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Free amino nitrogen improvement in sorghum grain brewing

By

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DECLARATION

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

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To my Creator,
my caring parents
Max & Magdalene Ng'andwe,
and my dearly beloved late brother
Sansa Emmanuel Ng'andwe

ABSTRACT

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Supervisor: Prof. J. R. N. Taylor

Free amino nitrogen (FAN) levels in sorghum grain brewing are characteristically low as a direct consequence of the very low levels of FAN in unmalted sorghum grain. During fermentation, FAN provides nutritional support to the yeast, enabling optimal yeast growth and efficient fermentation. Exogenous proteolytic enzymes are required to hydrolyse sorghum grain protein into FAN. Eight commercial proteolytic enzymes were studied in terms of their efficacy to produce FAN levels during mashing considered adequate (150 mg/L) to support optimal yeast functionality and their effect on extract levels.

FAN as determined by the ninhydrin assay, ranged between 25-72 mg/100 g sorghum grist (representing 0%-185% increase) when mashing was carried out at 55°C over 45 mins with the 8 different enzymes. The proteolytic enzymes varied greatly in terms of their efficacy, possibly as a result of having different optimal operating conditions. It is also suggested that the proteolytic enzymes differed in terms of their classification and exopeptidase/endopeptidase ratio.

In an attempt to further increase FAN levels, the effects of the reducing agent potassium metabisulphite (KMS) and different mashing temperatures (50°C, 55°C and 60°C) were also studied. Three enzymes (Flavourzyme, Neutrase, Papain) were selected for this part of the study. The addition of KMS caused a significant increase in the FAN in mashing systems that utilized Flavourzyme (22% when mashing at 55°C) and Neutrase (14% when mashing at 55°C), but not with Papain. It is suggested that KMS reduced the stabilizing disulphide bonds present in the sorghum kafirin, thus making the kafirin more

digestible. The KMS could have reduced the effectiveness of Papain because it is a sulphydryl protease which possesses stabilizing disulphide bonds which were reduced by the KMS. Flavourzyme and Papain were more active at 55°C, while Neutrase displayed maximum activity at 50°C.

The effect of mashing at a low temperature (40°C) over an extended time period (7 hr) on FAN was studied using Flavourzyme. Samples treated with Flavourzyme and KMS showed a five-fold increase in FAN, while those treated with Flavourzyme only showed a four-fold increase. It is proposed that the low temperature was able to suppress the rate at which disulphide bonds were formed in kafirin, therefore improving its digestibility. The lower temperature also retarded the enzyme denaturation rate, while the long time period allowed the enzyme more contact time with the substrate.

Microstructure analysis of sorghum protein bodies using transmission electron microscopy and of sorghum endosperm using confocal laser scanning microscopy revealed that the relative indigestibility of kafirin makes a significant contribution to the relatively low FAN levels. Although the action of the proteolytic enzyme and KMS on the sorghum endosperm protein matrix showed notable degradation, large fragments of undigested protein bodies were observed. This once again suggested that disulphide bond cross-linking played a major role in reducing the digestibility of sorghum protein.

The addition of Flavourzyme to a complete mashing cycle was also found to result in a significant increase (9%) in extract. The increase in extract is attributed to the increased availability of starch to amylase hydrolysis as a result of the degradation of the protein matrix which envelopes the starch granules.

The addition of KMS to the sorghum grain mashing system and the utilisation of a low temperature protein rest are effective methods of improving FAN in sorghum grain brewing.

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CHAPTER 1: INTRODUCTION

1.1 Statement of the problem

Sorghum (*Sorghum bicolor* (L.) Moench) is a cultivated tropical grass which originated in Africa about 3000 to 5000 years ago (Taylor and Belton, 2002). It is also widely grown in other parts of the world. Worldwide, sorghum is an important cereal crop to humankind as it is ranked fifth in terms of overall cereal production after wheat, rice, maize and barley (Taylor and Belton, 2002). Total world production was estimated at about 57.2 million tonnes, of which 31.2% was grown and harvested in Africa (FAO, 2004). Though this figure represents only 2.2% of the world's total cereal production, the importance of sorghum particularly in Africa cannot be underestimated. Sorghum accounts for approximately 15.2% of the total grain production in Africa.

In the more arid parts of Africa and other sub tropical areas, sorghum is grown mainly at small-scale level and is primarily used as a source of energy in the human diet as it is a starch rich food crop (Taylor and Belton, 2002). It is consumed in the form of various indigenous breads and porridges. It is also used in the production of traditional opaque beer, non alcoholic beverages in developing countries, and until more recently, production of lager-type clear beers.

Utilisation of sorghum in the production of clear beer has been documented in several countries like Mexico, Cuba, Nigeria, Israel and Cameroon (Agu and Palmer; 1998b; Goode and Arendt, 2003). The most successful developments in brewing clear beer with sorghum have been reported in Nigeria, where due to a 1988 government ban on imported cereals (barley inclusive), brewers were obliged to source alternative cereals such as maize and sorghum (Goode and Arendt, 2003). Despite the lifting of this ban, brewers continue using sorghum. It is reported that presently, most of the clear beer produced in Nigeria is brewed with at least some level of sorghum present (Taylor *et al.*, 2006). More recently, sorghum based clear beer has been produced on a commercial basis in Eastern and Southern Africa, and in the USA.

Sorghum has for a long time held immense potential as a suitable substitute for barley in lager beer production, particularly in developing countries where sustainable cultivation of barley cannot be undertaken. Sorghum is able to withstand what would be considered adverse climatic and soil conditions for barley which are experienced in many of the tropical semi-arid and arid areas (Agu and Palmer, 1998b). Utilisation of sorghum in lager beer production also has benefits at both the micro- and macroeconomic levels in developing countries. At microeconomic level, breweries are able to cut production costs through eliminating extended logistical and freight costs by using an input that is acquired locally (Mackintosh and Higgins, 2004). In many of these countries e.g. Uganda (Whittington, 2004) and Zambia (UNDP, 2005) governments provide tax incentives to breweries due to the use of locally produced inputs. This has enabled breweries to retail sorghum based lager beer at cheaper prices than conventional barley based lager beer, and in so doing, given them an opportunity to broaden their market by providing a product that is more accessible to lower income groups (UNDP, 2005). At the macroeconomic level, utilisation of a local ingredient like sorghum in brewing provides an opportunity for developing countries to save much needed foreign exchange and also provide a strong and sustainable market for a locally grown ingredient particularly for small scale farmers which greatly assists in poverty alleviation (Mackintosh and Higgins, 2004).

When sorghum is used as a brewing material, three approaches may be used. The first approach entails using the sorghum as an adjunct (Dale *et al.*, 1989; Bajomo and Young, 1992; Demuyakor *et al.*, 1994). In this approach, the sorghum is merely used as a source of starch to provide material that can be broken down into fermentable sugars. When this approach is used, malted barley or malted sorghum is used as a source of hydrolytic enzymes. The second approach involves using malted sorghum as opposed to malted barley (Olatunji *et al.*, 1993; Agu and Palmer, 1998b; Agu, 2005). Here, the malted sorghum provides both the hydrolytic enzymes and the starch. Exogenous enzymes (mainly microbial) may be added to supplement the indigenous enzymes that are present in the malt. The third approach involves the use of unmalted sorghum grain and

exogenous enzymes (Ugboaja *et al.*, 1991; Bajomo and Young, 1992; Goode *et al.*, 2003). In this approach it is absolutely essential that exogenous enzymes are employed because the enzyme activity in unmalted sorghum grain is low (Bajomo and Young, 1992).

Several reports (Aisien and Muts, 1987; Agu *et al.*, 1995; Agu and Palmer, 1998b; Goode *et al.*, 2003) have shown that when brewing with sorghum malt with or without exogenous enzymes and brewing with unmalted sorghum grain with exogenous enzymes, several challenges are encountered which are not present when brewing with barley malt. Problems associated with brewing with sorghum malt include development of insufficient amylase activity (leading to slow and incomplete saccharification), limited protein modification (leading to low levels of free amino nitrogen), poor wort and beer filtration, high malting costs and high malting losses (Agu *et al.*, 1995; Goode *et al.*, 2003). With the exception of the latter two problems, these are also encountered when brewing with unmalted sorghum.

Free amino nitrogen (FAN) is an essential component of yeast nutrition in brewing as it promotes proper yeast growth and fermentation efficiency (Goode *et al.*, 2003; Lekkas *et al.*, 2007). It also plays a role in the maintenance of foam stability (Power, 1993; Goode *et al.*, 2003). When brewing with sorghum grain, comparatively low levels of FAN are achieved as opposed to those produced when brewing with malted barley or malted sorghum. This is a direct result of the relatively poor protein digestibility (Taylor and Belton, 2002; Duodu *et al.*, 2003) of sorghum kafirin which constitutes up to 80% of the total protein in sorghum grain (Taylor and Belton, 2002). Apart from having an adverse effect on FAN levels, the poor protein digestibility of kafirin may also contribute to the comparatively low extract levels that are achieved when brewing with sorghum. The digestibility of starch in sorghum is partly dependant on the kafirin which exists in a network which envelopes the starch granules (Ezeogu *et al.*, 2005).

This project is aimed at improving FAN levels in wort by using commercial proteolytic enzymes and optimised mashing conditions. The project is also aimed at improving other sorghum wort properties that may be affected by limited protein hydrolysis.

1.2 Literature review

This review will examine the structure of sorghum grain with particular emphasis on the kafirin storage proteins and their digestibility. The proteolytic enzymes present in the sorghum grain will also be explored. An outline of the traditional clear beer brewing process will be given. Additionally, research focused on the production of sorghum lager with particular emphasis on use of unmalted grain will also be reviewed. A brief overview of proteolytic enzymes will also be given.

1.2.1 Sorghum grain structure

The sorghum kernel (Figure 1) is a naked caryopsis which is roughly spherical in shape and is typically 2-5 mm in length and 2-3 mm thick at the widest point (Taylor and Belton, 2002). It is composed of 3 major anatomical parts which are the pericarp, the germ and the endosperm.

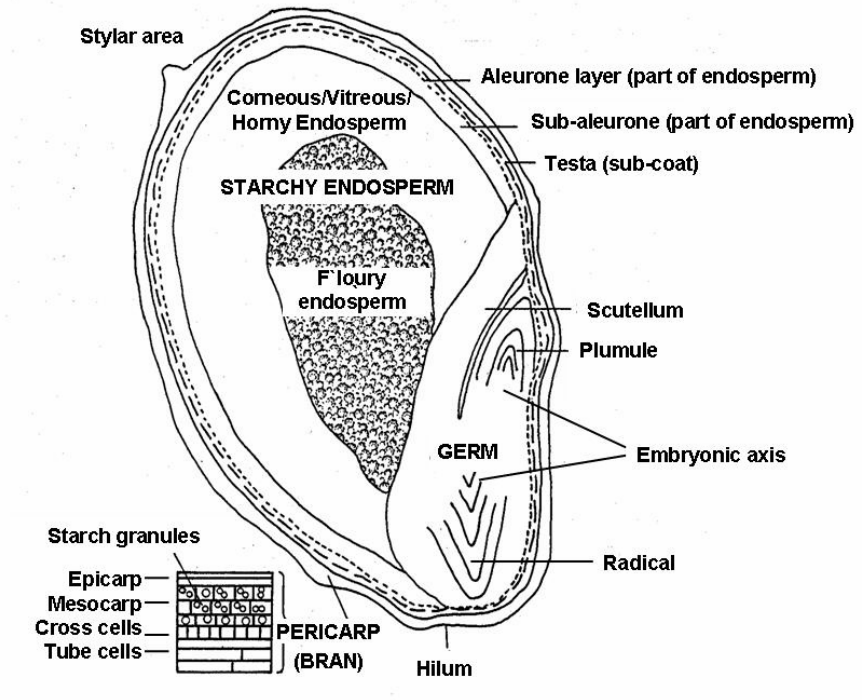


Figure 1: Diagrammatic section through a sorghum kernel (Taylor and Belton, 2002)

The pericarp consists of three different tissue types which are the epicarp, mesocarp and the endocarp (Hoseney *et al.*, 1974). The epicarp consists of two to three layers of rectangular cells which may be pigmented depending on genotype. The mesocarp cells are thin-walled and contain polygonal starch cells. The endocarp is composed of cross and tube cells. The testa is located directly below the endocarp and surrounds the endosperm. In some sorghum varieties the testa may be pigmented and this pigmentation is due to the presence of condensed tannins (Hulse *et al*., 1980).

The germ consists of a large scutellum (single cotyledon) and embryonic axis, a plumule and a primary root. It consists mainly of parenchymatous cells. It also contains a high concentration of oil (Hoseney, 1994).

The endosperm is the largest part of the sorghum kernel and consists of an aleurone layer, a horny (also known as corneous or vitreous) and a floury endosperm. The aleurone layer consists of a single layer of thick walled cells which contain large amounts of soluble vitamins, autolytic enzymes, fat and some protein bodies as well as a considerable amount of ash (Hoseney, 1994). The horny and floury endosperms are the kernels main store of starch and the two may be differentiated according to the relative proportion of protein and starch. The horny endosperm is a dense packed area where the protein is arranged in a matrix which envelopes the starch granules and other protein bodies; whereas the floury endosperm is a less densely packed area where the protein matrix is discontinuous (Rooney and Serna Saldivar, 1991).

1.2.2 Proteins of sorghum

Sorghum proteins may be classified on the basis of extraction and solubility as proposed by Osborne into prolamins, albumins, globulins, and glutelins (Shewry *et al.*, 1995). This review will focus primarily on the prolamin fraction.

1.2.2.1 Prolamins

The prolamins, which are exclusive to the grass family are the major protein fraction found in cereals (with the exception of oats and rice), accounting for approximately half of the total grain nitrogen (Shewry *et al.,* 1995). In sorghum, the prolamin protein present is called kafirin and is contained in distinct protein bodies (Taylor *et al.*, 1984b). The protein bodies are located mainly in the outer vitreous endosperm (Watterson *et al.*, 1993). Isolation of kafirin dates back as far as 1916 when Johns and Brewster (1916) were able to extract the protein using 70% ethanol. Studies by Shull *et al.* (1991) established extraordinary similarities between kafirin and zein based on electrophoretic mobility, alcohol solubility and immunological studies. Traditionally, prolamins were recognised as a group based on their solubility in alcohol/water mixtures (60-60% [v/v] ethanol or 50% [v/v] propan-1-ol) (Shewry, 2002). Kafirins are most efficiently extracted using 60% [v/v] tertiary butanol (2-methyl-2-propanol) which is a less polar solvent, as a result of their hydrophobic nature. A protein fraction which is insoluble in aqueous alcohol in its native state, but becomes soluble upon introduction of a reducing agent was discovered by Evans *et al.* (1987). This led to the expansion of the definition of prolamins to include components that are insoluble in aqueous alcohols in the native state but soluble in a reduced state (Shewry *et al.,* 1995).

The prolamins in sorghum have been divided into two classes based on their extraction method using a system devised by Landry and Moureaux (1970). Prolamin I, which is extracted by aqueous alcohol alone, is rich in monomeric α-prolamin with some oligomeric and polymeric components and prolamin II, which is extracted only in its reduced state using aqueous alcohol, is rich in β- and γ- prolamins (Taylor *et al.*, 1984c).

Kafirin accounts for up to 80% of the total protein in sorghum (Taylor and Belton, 2002). It has been found to contain high proportions of glutamine, proline, alanine, and leucine, and very low levels of lysine (Table I) (Taylor and Schüssler, 1986). The kafirin proteins also contain a high level of hydrophobic amino acids, and this has been said to account for the hydrophobicity of the proteins (Taylor and Belton, 2002). Based on its similarity

to zein (the prolamin of maize), kafirin has been separated into three species: α-, β- and γ- kafirin.

Table I: Amino acid content (mole % of amino acid) of the major protein fractions of sorghum (Taylor and Belton 2002)

Amino acid	α- Kafirin	β- Kafirin	γ- Kafirin
Asparagine	6.0	3.3[a]	0
Aspartic acid	0.4	[c]	0
Threonine	4.0	4.6	4.7
Serine	6.0	4.6	5.2
Glutamine	24.6	17.19[b]	11.9
Glutamic acid	0.4	[c]	1.0
Proline	7.7	9.7	23.3
Glycine	1.6	6.8	8.8
Alanine	14.9	13.4	5.7
Cysteine	0.4	4.9	7.8
Valine	4.4	5.2	6.2
Methionine	0.8	5.7	1.0
Isoleucine	5.6	2.3	2.6
Leucine	15.3	12.0	8.3
Tyrosine	2.8	3.0	2.1
Phenylanine	2.4	1.9	1.6
Histidine	1.2	0.9	7.8
Lysine	0.0	0.5	0.0
Arginine	0.8	2.7	2.1
Tryptophan	0.4	[c]	0.0

[a] Asparagine + Asparagine expressed as Aspartic acid

[b] Glutamine + Glutamic acid expressed as Glutamine

[c] Not available

1.2.2.1.1 Alpha-kafirin

The α-kafirins account for approximately 80% of the total kafirin content (Shewry, 2002). SDS-PAGE reveals two broad bands occurring at M_r 25 k and 23 k which represent α-kafirin (Figure 3) (El Nour *et al.*, 1998; Shull *et al.*, 1991). When analysed under reducing conditions, α-kafirin has been classed into two sub groups: α_1 (M_r 24-28 k) and α_2 (M_r 22 k) (Mazhar *et al.*, 1993). They contain a high content of glutamine as well as the hydrophobic amino acids phenylanine, leucine and isoleucine relative to β- and γ- kafirins (Table I). They also contain low amounts of lysine, cysteine and methionine. Immunocytochemical (Shull *et al.*, 1992) and digestibility studies (Rom *et al.*, 1992; Oria *et al.,* 1995a, b; Weaver *et al.*, 1998; Oria *et al.*, 2000) have revealed that α-kafirin is primarily located in the interior part of the spherical protein bodies (Figure 2).

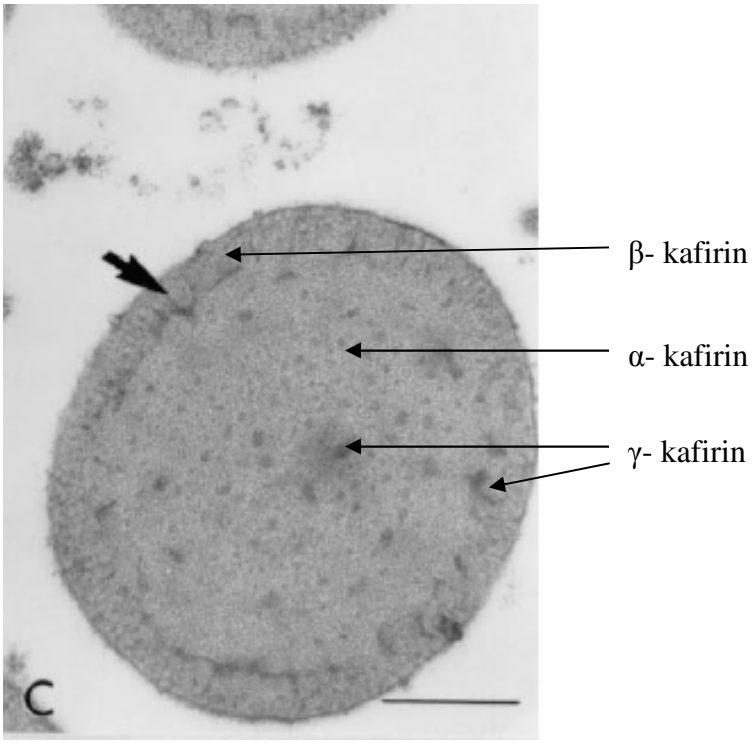


Figure 2: Micrograph of a sorghum protein body incubated with preimmune sera. The light staining interior region shows α-kafirin, the darker staining periphery region shows β-kafirin, and the dark staining inclusions throughout the protin body shows γ-kafirin (Oria *et al*., 2000).

1.2.2.1.2 Beta-kafirin

Beta-kafirin accounts for approximately 7-8% of the total kafirin (Shull *et al.*, 1992). On separation using SDS-PAGE, β-kafirin is accounted for by three distinct bands of approximate M_r 20, 18 and 16 (Figure 3). Compared to α-kafirin, it contains a relatively lower content of the hydrophobic amino acids phenylanine, leucine and isoleucine, but a significantly higher content of glycine (Table 1). It also contains a significantly higher content of the sulphur-containing amino acids methionine and cysteine; with 18 and 7 residues respectively present in the peptide chain (Shewry, 2002). The presence of the sulphur-containing amino acids in β-kafirin enables this kafirin fraction to participate in the formation of oligomers and polymers due to interchain disulphide bonding (Shewry, 2002). It is primarily located on the periphery of the protein body (Figure 2).

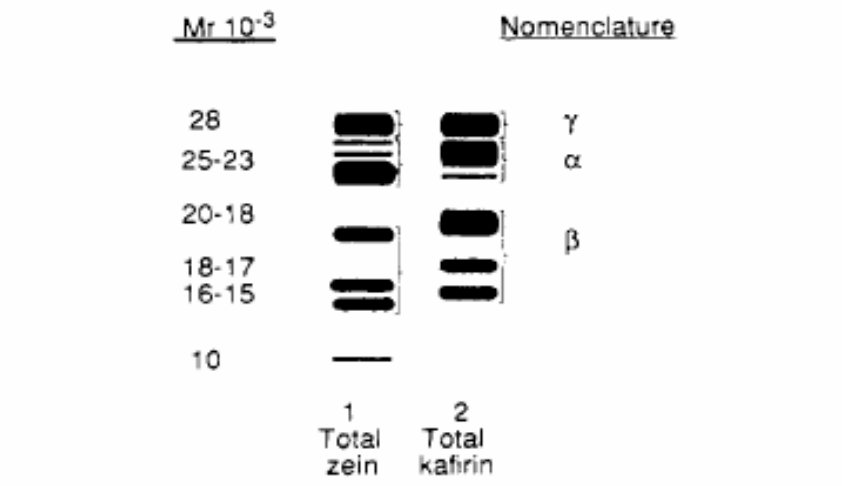


Figure 3: Diagramatic representation of total zein (lane 1) and total kafirin (lane 2) polypeptides extracted with 70% alcohol and 2- mercaptoethanol and separated by SDS-PAGE (Shull *et al.*, 1991).

1.2.2.1.3 Gamma-kafirin

Gamma-kafirin contributes approximately 9-12% to the total kafirin content (Shull *et al.*, 1992). In SDS-PAGE it displays the least molecular mobility of all the kafirin species and is characterised by a single band of approximately 27-28 k (Figure 3) (Taylor *et al.*, 1989). It contains a relatively higher content of the hydrophilic amino acids glycine and histidine (Table 1). It is also characteristically deficient in lysine and aspartic acid. The high presence of the sulphur-containing amino acid cysteine makes it possible for γ-kafirin to participate extensively in the formation of oligomers and polymers with itself and other prolamin fractions due to the formation of intermolecular disulphide bonds (Oria *et al.*, 1995a, b). It is located at the periphery (Figure 2) of the protein body and is also present in the interior as inclusion bodies (Shull *et al.*, 1992).

1.2.2.2 Other sorghum proteins

Apart from the prolamins, sorghum also contains the albumin, globulin and glutelin protein fractions.

The albumins and globulins are the salt soluble fraction of sorghum proteins and are the first to be extracted during sequential Osborne fractionation using 1.25 M NaCl (Daiber and Taylor 1982). Together these proteins make up approximately 23% of the total protein (Taylor and Belton, 2002). They are primarily located in the germ and include biologically important proteins such as enzymes, nucleoproteins, membrane proteins and glycoproteins (Wall and Paulis, 1978). This protein fraction is considered nutritionally significant because it is rich in the limiting amino acid lysine (Van Scoyoc *et al*., 1988).

The glutelins are classified as proteins that are soluble in dilute alkali or the residue that is left following the extraction of the albumins and globulins, and kafirin (Wilson, 1983). After the prolamins, this fraction represents the second largest individual protein fraction of whole sorghum grain (Taylor and Schüssler, 1986). They constitute a significant portion (24-29%) of the total endosperm protein. Glutelin proteins are high molecular

weight proteins found primarily within the endosperm and the pericarp. In the endosperm, they are located in the protein matrix where they surround the protein bodies (Taylor *et al*., 1984b) and exist as glutelin polymers linked by disulphide bonds (Wall and Paulis, 1978).

1.2.3 Sorghum protein digestibility

1.2.3.1 Effect of cooking

Several reports (Axtell *et al.,* 1981; Hamaker *et al*., 1986; Rom *et al*., 1992; Oria *et al*., 1995a, b; Arbab and El Tinay 1997; Duodu *et al*., 2002) have documented the relatively poor digestibility of sorghum grain protein. In all these studies, it has been found that cooking (in particular wet cooking) further reduces the digestibility of sorghum protein. Considering that wet cooking plays a vital role during the mashing stage of brewing, it is possible that the susceptibility of sorghum protein to hydrolysis when mashing with sorghum could be reduced.

The reduction in sorghum protein digestibility as a result of cooking has been attributed largely to the formation of enzyme resistant, disulphide bonded, cross-linked polymers particularly in the β- and γ-kafirin fractions, and possibly the glutelin matrix protein (Hamaker *et al*., 1987; Oria *et al*., 1995b; Weaver *et al*., 1998). Cross-linking in the mentioned protein fractions is owed to the relatively high content of cysteine.

Reducing agents have shown to partially alleviate the negative effects of cooking on sorghum protein digestibility (Hamaker *et al.*, 1987; Rom *et al.*, 1992; Oria *et al.*, 1995b; Arbab and El Tinay, 1997). Reducing agents such as 2-mercaptoehtanol (Hamaker *et al.*, 1987), sodium bisulphite (Rom *et al.*, 1992; Oria *et al.*, 1995b; Arbab and El Tinay, 1997) and ascorbic acid (Arbab and El Tinay, 1997) are said to prevent the formation of additional disulphide bonds during cooking. This in turn prevents the formation of enzyme resistant polymers.

1.2.3.2 Kafirin hydrophobicity

The kafirins are known to be hydrophobic in nature (Wall and Paulis, 1978) as a result of having an amino acid profile which is rich in the hydrophobic amino acids. Gamma-kafirin in particular shows a very high level of hydrophobicity (Duodu *et al.*, 2003). Since enzymes function in aqueous environments, their activity on kafirin will not be very effective because of the poor interaction between kafirin and water (Duodu *et al.*, 2003). The high presence of γ-kafirin on the periphery of the protein body further exacerbates this problem.

1.3.3.3 Other factors affecting sorghum protein digestibility

Duodu *et al.* (2003) have reviewed the factors that affect sorghum protein digestibility and have classified them under two classes: Exogenous factors and endogenous factors. In the previous section, some of the endogenous factors that may be relevant to consider when brewing with sorghum grain have been reviewed. This section will briefly look at what are termed exeogenous factors.

The exogenous factors that affect sorghum protein digestibility have been described by Duodu *et al*. (2003) as those that arise out of the interaction of sorghum proteins with non-protein grain components which include polyphenols, non-starch polysaccharides, starch and phytates. These interactions may result in interfering factors arising from the production of indigestible products as a result of chemical interaction between the protein and the non-protein grain components; or the non protein components forming a physical barrier which impedes access of proteases to the protein (Duodu *et al*., 2003).

1.3.3.3.1 Polyphenols

The interaction of sorghum protein with polyphenols (in particular tannins) has been studied extensively (Chibber *et al.,* 1980; Butler *et al*., 1984; Agudelo *et al.,*1997; Emmambux and Taylor, 2003). Studies have shown that the digestibility of tannin

sorghum varieties is lower than that of tannin-free varieties (Armstrong *et al*., 1974; Ebadi *et al*., 2005). Considering that low tannin content sorghum varieties are preferred for brewing (Mackintosh and Higgins, 2004), tannins would not be considered a major factor in this regard.

1.3.3.3.2 Non starch polysaccharides

Interactions between grain proteins and cell wall material are not unique to sorghum. Gram (1982) reported an association between protein and the pericarp or endosperm cell walls in barley. Similar associations in sorghum have also been reported. (Glennie, 1984; Bach Knudsen and Munck, 1985; Kavitha and Chandrashekar, 1993). It has been suggested that such association could lower protein digestibility by physical impediment of proteolytic enzymes to proteins; or the formation of enzyme resistant complexes (Duodu *et al*., 2003). Cell wall polysaccharides are known to be detrimental to brewing performance if they are not efficiently degraded (Bamforth, 1982; 1994).

1.3.3.3.3 Starch

Within the sorghum vitreous endosperm, starch granules are enveloped by a glutelin protein matrix embedded with protein bodies (Chandrashekar and Kirleis, 1988). As a result there is a very close association between the starch granules and the protein. Duodu *et al*., (2003) suggest that such a close association could result in starch (particularly when it is gelatinised) reducing the accessibility of proteolytic enzymes to the protein bodies resulting in reduced digestibility. In fact, treatment of sorghum endosperm flour with α-amylase has shown to improve protein digestibility (Duodu *et al*., 2002).

As a result of the close association between starch and sorghum within the vitreous endosperm, sorghum starch digestibility is also affected (Ezeogu *et al*., 2005). Treatment of sorghum endosperm flour with a proteolytic enzyme (pronase) has shown to significantly improve sorghum starch digestibility (Rooney and Pflugfelder, 1986). This is because the surface area of the starch granules exposed to amylase attack is increased

as a result of the rigid cover provided by the protein being degraded (Chandrashekar and Kirleis, 1988).

1.3.3.3.4 Phytate

Phytic acid has been implicated to play a role in protein digestibility. It is believed that physiologically unavailable complexes are formed as a result of phytic acid-protein interactions (Serraino *et al*., 1985). Knuckles *et al*. (1985) showed that phytate was able to significantly reduce the in vitro protein digestibility of casein and bovine-serum albumin. Addition of sorghum malt to unmalted sorghum flour has shown to decrease phytic acid levels with a concomitant increase in in-vitro protein digestibility (Elkhalil *et al*., 2001). When whole milled sorghum grain is mashed with commercial enzymes, it is likely that phytic acid may play a significant role in reducing the digestibility of sorghum proteins if processes that inhibit its activity such as malting are not conducted.

1.2.4 Proteolytic enzyme activity in sorghum

The two different classes of protease enzymes i.e. proteinases (endopeptidases) and peptidases (exopeptidases)[1] are located primarily in the sorghum endosperm and germ respectively (reviewed by Serna Saldivar and Rooney, 1995). Though it is generally accepted that unmalted cereal grains have little enzymatic activity, the work by Evans and Taylor (1990a) revealed that in resting (un-malted) sorghum grain, there was a considerable level of proteinase activity but this was accompanied by a low level of carboxypeptidase activity. After malting, proteinase activity increased between two to almost four-fold depending on the cultivar used. A significant increase (four to almost nine-fold depending on the cultivar) was observed in carboxypeptidase levels after malting (Evans and Taylor, 1990a, b).

Malted sorghum has been found to possess more endopeptidase activity than exopeptidase activity (Winspear *et al.*, 1984; Evans and Taylor, 1990a, b). The same

[1] See section 1.2.6 on proteases for definitions of the two classes.

could be said for other tropical cereal crops like maize and rice, while temperate cereal crops (e.g. wheat, barley, oats, wild oats, rye and triticale) have more exopeptidase activity than endopeptidase activity (Winspear *et al.*, 1984).

Different classes of proteolytic enzymes have been identified in malted sorghum grain (Ogbonna *et al.,* 2003; Ogbonna *et al.,* 2004; Ogbonna and Okolo, 2005). Using the intrinsic properties of the different proteases, it is possible to deduce what class of enzyme these fall under.

Ogbonna *et al.* (2003) classified the protease they isolated as a cysteine or sulphydryl protease based on its inhibitory properties. There was not sufficient evidence in this study to ascertain whether this protease was an endo- or exopeptidase. The protease characterized by Ogbonna *et al.* (2004) was also classified as a cysteine or sulphydryl protease. This protease was also classified as an endoprotease or proteinase. The protease isolated by Ogbonna and Okolo (2005) was classified as a metalloprotease based on the fact that it was inhibited by EDTA which is a metal chelator. This protease was found to have a similar molecular weight to a zinc containing endopeptidase from germinating buckwheat. The high pH optimum of this enzyme could also suggest that this particular protease was an endopeptidase.

1.2.5 The brewing process

The traditional brewing process entails various steps which all play a cardinal role in the quality of the end product. Essentially, the brewing process entails the following main steps: malting, wort production (mashing), fermentation and finishing (Hallgren, 1995), which are outlined in Table II. In this study, emphasis will be on the mashing process. Therefore, only the mashing process will be reviewed in detail.

The brewing process is primarily aimed at extracting the soluble components of the malt and the hops (Hallgren, 1995). Malting involves the steeping of grain in water and allowing the grain to germinate until the endosperm (starchy food store) has undergone

some degradation by enzymes produced by the germ (Hough *et al.,* 1971). The most important enzymes that are produced during the malting process are the amylases (α- and β- amylases) and proteases.

Table II: Main steps involved in the brewing process (adapted from Bamforth, 2000).

Stage	Events
1. Malting	Steeping- water added, interspersed by air rests, to raise moisture content of embryo and endosperm; up to 48 h at 14-18°C. Germination- controlled sprouting ('modification') of grain; typically 4-5 days at 16-20°C Kilning- heating of grain through increasing temperature regime (<24 hr at 50-220°C) for desired properties (enzyme survival, removal of moisture for stabilization, removal of 'raw' flavours, development of malty flavours and colour).
2. Milling and mashing	Extraction of milled malt at temperatures between 40 and 75°C. Temperature stands allowed at specific temperatures over a period of time to facilitate optimal enzymatic activity for hydrolysis of macromolecules (e.g. proteins, starch and non-starch polysaccharides).
3. Wort separation	Recovery of wort from spent grains.
4. Boiling and trub separation	1-2 hr at approx. 100°C before removal of insolubles (trub) and cooling.
5. Fermentation	Wort aerated and then pitched (inoculated) with yeast and fermented for 3-14 days at 6-25°C.
6. Finishing	Secondary fermentation process during which unacceptable 'immature' flavours are modified. In traditional lager brewing process this is carried out at -2°C over a stipulated period of time. Carbonation may also be done during this stage. Mature beer is then filtered, packaged and pasteurized.

The mashing stage is essentially a digestion process during which insoluble substances (primarily starch, non-starch polysaccharides and proteins) are hydrolysed into soluble substances which constitute the extract (Owuama, 1997; Briggs *et al.*, 2004). A wide variety of enzymes including amylases, proteases, peptidases and phosphorylases are involved in these hydrolytic reactions (Owuama, 1997). During mashing, crucial factors with a bearing on enzyme activity such as temperature, time, pH and wort concentration are regulated (Briggs *et al*., 2004).

There are two types of mashing procedures that are commonly used in brewing, namely infusion mashing and decoction mashing (Hough *et al.*, 1971; Beckerich and Denault, 1987). Traditionally, beer was produced using a single infusion mashing procedure which entails use of a single temperature (65-71˚C) throughout the process (Hough *et al.*, 1971). The single infusion mashing process is still used to produce stout and ale type beers. Modern brewery operations usually employ what is termed an upward step infusion or modified mashing procedure which involves using progressively rising temperatures with a series of rests at predetermined temperatures (Beckerich and Denault, 1987). This facilitates the action of the different enzymes (mainly proteolytic enzymes and amylases) at near optimum levels.

Decoction mashing involves removing a predetermined portion of the mash for boiling which is then later reintroduced into the cold mash to raise the temperature (Hough *et al.*, 1971, Briggs *et al.*, 2004). Decoction mashing also involves a series of rising temperature steps, which serve a similar purpose to those in the modified infusion mashing procedure.

The fermentation process entails conversion of the fermentable carbohydrates (glucose, fructose, sucrose, maltose and maltotriose) of the wort to ethanol, carbon dioxide and several other by products which have a bearing on the taste, aroma and other characteristics of the beer (Hough *et al.*, 1971). The yeast also utilizes the products of protein degradation (free amino nitrogen), which are essential for their growth and have a

huge influence on the flavour inducing substances produced as by products of yeast metabolism.

The finishing step entails storage, clarification and packaging of the beer (Beckerich and Denault, 1987). There are variations during this step depending on the type of beer being produced (e.g. lager, ale, stout). The finishing step may also include a secondary fermentation step which has a great influence on the flavour of the beer.

Clarification of beer is essential to prevent haze formation which is considered to be a defect in clear beer types (Hough *et al.*, 1971). This may be achieved by using proteolytic enzymes or adsorbant material (e.g. bentonite, polyvinyl pyrrolidone or nylon). In order to improve the microbiological safety as well as enhance the shelf life of the beer, pasteurization is also carried out during the finishing stage.

1.2.5.1 Sorghum brewing mashing regimes

Table III provides a summary of the methods used by different workers. Owuama (1997; 1999) reports that no significant differences exist in terms of FAN and reducing sugar content between worts produced using a three-step decoction method, and the upward infusion method when using sorghum malt. When using sorghum grain, it is likely that using a decoction method would result in a reduction in FAN and reducing sugars. This is because of the reduction in protein digestibility which is likely to occur when part of the mash is boiled. A reduction in protein digestibility is likely to be accompanied by a reduction in starch digestibility.

Table III: Sorghum mashing procedures used by different workers.

Source	Mashing procedure	Brewing material
Aisien and Muts (1987)	Infusion, decoction	100% sorghum malt
Dale *et al*. (1989)	Infusion	10%, 20%, 30%, 40%, 50% extruded sorghum
Dale *et al*. (1990)	Decoction	50%, 60%, 70%, 80% sorghum adjunct
Ugboaja *et al*. (1991)	Infusion	15%, 25% sorghum adjunct
Bajomo and Young (1992)	Infusion	80% sorghum adjunct
Taylor (1992)	Infusion	29% sorghum malt, 71% maize grits
Olatunji *et al*. (1993)	Infusion	100% sorghum malt
Bajomo and Young (1993)	Infusion	100% unmalted sorghum
Bajomo and Young (1994)	Infusion	100% unmalted sorghum
Demuyakor *et al*. (1994)	Decoction	66.7% sorghum malt, 33.3% barley malt
Agu *et al*. (1995)	Infusion	100% sorghum malt
Agu and Palmer (1998a)	Infusion	100% unmalted sorghum
Osorio-Morales *et al*. (2000)	Decoction	63.3% sorghum adjunct
Goode *et al*. (2002)	Infusion	20%, 40%, 60%, 80% sorghum adjunct
Goode *et al*. (2003)	Infusion	100% unmalted sorghum
Ogu *et al*. (2006)	Infusion	100% sorghum malt

1.2.5.2 Brewing with sorghum

As stated earlier, a number of problems are associated with using sorghum (malted and un-malted) as brewing material for clear European style beers. Despite these problems, commercialization of sorghum based clear beers has been successful in different geographical locations. The drawbacks associated with brewing with sorghum malt in clear beer production led some workers to propose that raw un-malted sorghum grain in conjunction with commercial enzymes be used as the best practical approach, since commercial enzymes are also used when brewing with malted sorghum (Dale *et al.*, 1989; Bajomo and Young, 1993).

Several reports have shown that when brewing with malted sorghum with or without incorporation of exogenous enzymes, higher levels of FAN and extract have been achieved in the wort (Dale *et al.*, 1990; Dufour *et al.*, 1992; Olatunji *et al.*, 1993; Agu and Palmer, 1998a; Owuama, 1999).

When brewing with sorghum grain much lower levels of free amino nitrogen are achieved when compared to brewing with malted barley or malted sorghum (Goode *et al.*, 2003). The low FAN levels are a direct consequence of very low levels of FAN in unmalted sorghum grain, which can be attributed to several factors associated with poor protein digestibility.

It has been generally agreed that at least 120 mg of FAN per litre is required to support proper yeast growth during brewing, though with the high gravity brewing processes employed in most modern breweries, recommended levels are at about 150 mg/L (Beckerich and Denault, 1987). Interestingly, Bajomo and Young (1994) were able to successfully ferment wort with a FAN level of only 51 mg/L. The wort was able to attenuate fully and yeast development was not negatively affected as the authors were able to use the same yeast crop over five consecutive fermentations. This however is not the case in industry where only two to three consecutive fermentations are achieved using the same yeast crop (Mr. I. Kennedy, Brewing Consultant Novozymes, 2006: Personal

communication). Although low overall FAN levels have been achieved when brewing with sorghum, it has been reported that qualitatively, the FAN present in sorghum wort is superior to that in malted barley wort (Taylor, 1983; Bajomo and Young, 1993, Bajomo and Young, 1994). Work by Bajomo and Young (1994) showed that when malted barley wort of a similar specific gravity to that of sorghum grain wort had its FAN diluted, full attenuation was not achieved. This is because the FAN produced in sorghum contains a higher proportion of the group A amino acids (i.e. aspartate, threonine, serine, glutamate, asparagine, glutamine, cysteine, lysine and arginine) compared to group D amino acids (i.e. proline). Group A amino acids are assimilated immediately by yeast while the group D imino acid which accounts for almost a third of total FAN in malted barley wort is only slightly absorbed under normal anaerobic fermentation conditions (Jones and Pierce, 1964).

In wort produced from 100% malted barley, FAN values lie in the range of 200 to 250 mg/L (Goode *et al.*, 2003). In malted sorghum, FAN values reported by different workers range between 90mg/L and 358mg/L (Taylor and Boyd, 1986; Olatunji *et al.,* 1993; Demuyakor *et al.,* 1994; Ogu *et al*. 2006). The wide range of FAN values reported by these workers is a result of differences in sorghum varieties used, varying malting conditions, and varying mashing conditions. Different workers have also reported different values of FAN in wort produced from un-malted sorghum grain and commercial enzymes. Similar to the work done with malted sorghum, these differences have been attributed to varietal effects and varying mashing conditions (Agu and Palmer, 1998a). Goode *et al.* (2003) investigated the effect of different levels of a commercial protease enzyme (Bioprotease) on the levels of FAN (Table IV). It can be observed that the relationship between increase in FAN and levels of enzyme addition was not directly proportional. When economic considerations are borne in mind, it can be observed that bringing the FAN levels to a level within those of the recommended range for yeast nutrition would not be feasible because of the high amount of enzymes used.

Table IV: Free amino nitrogen levels of worts derived from mashes containing increasing levels of Bioprotease (Goode *et al.*, 2003)

Bioprotease (U/g of grist)	FAN (mg/L)[a]
0	38 (1.5)
50	61 (0.5)
100	75 (0.8)
200	87 (4.3)
1000	103 (4.2)

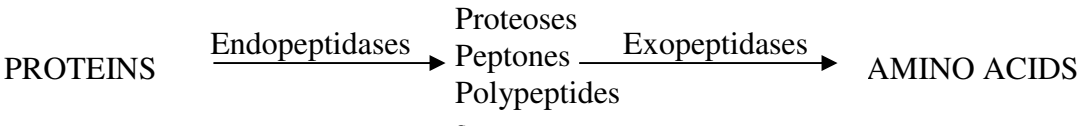
[a]Figures in brackets indicate standard deviations

Exogenous proteolytic enzymes have also been shown to have a positive effect on extract levels in sorghum wort. Bajomo and Young (1994) were able to increase extract by approx. 7% when a proteolytic enzyme (Proteinase 200L) was used in addition to amylases. Similarly, Goode *et al.* (2002) were able to increase extract levels from 70% to 76% (dry weight basis) when a proteolytic enzyme (Bioprotease) was used. The degradation of sorghum protein (in particular endosperm protein) is essential if acceptable extract levels are to be achieved because of the role that the proteins play in starch digestibility. Poor saccharification in sorghum grain could be attributed to several different factors. In the vitreous endosperm region of the sorghum grain, the starch granules are embedded within a protein matrix (rich in kafirin). As earlier described, there is a high degree of cross-linking in the kafirins that are present in sorghum grain and this has been regarded by Duodu *et al.* (2003) as the major factor affecting sorghum protein digestibility. The relative indigestibility of the protein surrounding the starch granule makes it difficult for the amylases to act efficiently on the starch granule. Apart from protein-protein cross linking, protein-starch interactions, as stated have also been identified as a factor affecting starch digestibility in sorghum (Duodu *et al.*, 2003).

1.2.6 Proteolytic enzymes

Proteolytic enzymes find extensive use in a wide range of commercial applications and are considered to be the most significant enzyme group traded globally (Rao *et al.* 1998; Haki and Rakshit, 2003). Proteolytic enzymes are hydrolytic enzymes that catalyse the cleavage of peptide bonds in proteins leading to their total hydrolysis (Rao *et al.* 1998).

Apart from being classified according to their source (animal, plant or microbial) Proteolytic enzymes may be classified broadly under two groups: endopeptidases also known as proteinases, and exopeptidases (Underkofler *et al.,* 1958; Adler-Nissen, 1993). Underkofler *et al.* (1958) explain the action of the two classes of proteases as follows:



In order for complete or near complete protein hydrolysis to occur, both types of proteases need to be present in a system (Stauffer, 1987). The two classes of enzymes are analogous to α- and β-amylase in that they act synergistically. Commercially available proteases normally comprise a mixture of the two types (Underkofler *et al.,* 1958)

1.2.6.1 Endopeptidases

Endopeptidases preferentially cleave polypeptide chains within the chain at any susceptible point away from the N and C termini (Rao *et al.,* 1998). The presence of the free amino or carboxyl group has been said to have a negative effect on the activity of endopeptidases.

Endopeptidases are subdivided into four major classes based on their catalytic mechanism. These are (i) serine proteases, (ii) aspartic proteases (iii) cysteine proteases, and (iv) metalloproteases (Adler-Nissen, 1993).

1.2.6.1.1 Serine proteases

Serine proteases are characterised by the presence of a serine group in their active site (Rao *et al.* 1998). They have been sub divided into twenty families based on their structural similarities, and these twenty families have been further sub divided into about six clans with common ancestors. Trypsin and chymotrypsin are typical examples of serine proteases of animal origin (Adler-Nissen, 1993). Serine proteases do not have a cleft in their surface, which facilitates extensive interaction with peptide chains in other proteases (Stauffer, 1987). The major interaction of serine proteases is with the side chains of amino acids contributing the carboxyl group to the bond being hydrolysed.

Serine proteases are generally active at neutral and alkaline pH (optimum between pH 7 and 11) and have a very broad range of substrate specificities (Rao *et al.,* 1998). Alcalase (subtilisin Carlsberg) is a typical example of a serine protease belonging to the group subtilisins (Adler-Nissen, 1993; Haki and Rakshit, 2003). It is produced by *Bacillus licheniformis* and has traditionally found application in the detergent industry owing to its broad pH activity profile which exhibits a maximum at pH 10 (Adler-Nissen, 1993). It is also regarded as moderately thermostable with an application range between 55 and 70°C, with optimum activity being reported at 60°C (Adler-Nissen, 1993; Rao *et al.,* 1998). Subtilisin Carlsberg is said to have an active site conformation similar to that of tryspin and chymotrypsin with a triad being formed by serine (Ser221), histidine (His64) and aspartic acid (Asp32) (Rao *et al.,* 1998).

Based on their substrate preference, serine proteases may also be classed into three groups: (i) trypsin-like, which cleave after positively charged residues; (ii) chymotrypsin-like, which cleave after large hydrophobic residues, and (iii) elastase-like, which cleave after small hydrophobic residues (Rao *et al.,* 1998).

1.2.6.1.2 Aspartic proteases

Aspartic proteases are more commonly known as acidic proteases. As the name suggests, these proteases depend on aspartic acid residues at their active site for their catalytic activity (Rao *et al.,* 1998). They are divided into three families which are: pepsin (A1), retropepsin (A2) and proteases from pararetroviruses (A3). Proteases from the A1 and A2 families are highly related, while those of A3 only show a low level of relatedness to the former two. The best two studied aspartic proteases are mammalian pepsin and penicillopepsin from *Penicillium janthinellum* (Stauffer, 1987). Microbial aspartic proteases may be subdivided into two groups, (i) pepsin like enzymes produced by *Aspergillus*, *Penicillium*, *Rhizopus*, and *Neurospora* and (ii) rennin like enzymes produced by *Endothia* and *Mucor* spp. (Rao *et al.,* 1998).

The optimum pH level for aspartic proteases which are from the pepsin like group is around pH 3 (*Aspergillus* spp. pH 4.5) (Adler-Nissen, 1993), although this has been reported to vary depending on the substrate and ionic strength of the medium (Stauffer, 1987). Microbial aspartic proteases have been reported to exhibit specificity against aromatic or bulky (i.e. phenylanine, tyrosine and tryptophan) residues on both sides of the peptide bond (Rao *et al.*, 1998).

1.2.6.1.3 Cysteine proteases

Cysteine proteases are also known as thiol or sulphydryl proteases (Stauffer, 1987). The catalytic activity of this group of proteases is owed to the presence of a dyad consisting of cysteine and histidine (Rao., *et al.*, 1998). About twenty different families of cysteine proteases have been identified; and the order of cysteine and histidine (i.e. either Cys-His or His-Cys) has been used as the basis for differentiation. They have also been grouped into four broad classes based on their side chain specificity: (i) papain like, (ii) trypsin like with preference for cleavage at the arginine residue, (iii) specific to glutamic acid, and (iv) others. Generally, cysteine proteases are said to be active only in the presence of reducing agents such as HCN or cysteine and are said to have a neutral pH optima

(Stauffer, 1987). However, some proteases e.g. lysosomal proteases are active at acidic pH (Rao, *et al.*, 1998).

1.2.6.1.4 Metalloproteases

Metalloproteases are proteolytic enzymes that contain an essential metal atom which is usually zinc for their catalytic activity (Muntz, 1996). Initially, they were termed neutral proteases due to their fairly sharp pH optimum (7-8). However, the term metalloproteases is perhaps more suitable as it defines this class of enzymes purely on a mechanistic basis. This therefore accomodates enzymes that possess similar biochemical features and follow a hydrolysis pattern similar to the classical neutral proteases but have more acidic or alkaline optima (Stauffer, 1987). They have been described as the most diverse group of proteolytic enzymes, with about thirty recognised families (Rao *et al.*, 1998). These families have been grouped into different clans based on the nature of the amino acid that completes the metal-binding site. Metalloproteases have further been divided into four groups based on the specificity of their action: (i) neutral, (ii) alkaline, (iii) *Myxobacter* I, and (iv) *Myxobacter* II. The neutral proteases have been found to show specificity for hydrophobic amino acids; alkaline metalloproteases possess a very broad specificity; *Myxobacter* I metalloproteases are specific for small amino acid residues on either side of the cleavage bond; and *Myxobacter* II proteases are specific for lysine residues on the amino side of the peptide bond.

The metalloprotease produced by *Bacillus amyloliquifaciens* is commercially known as Neutrase (Adler-Nissen, 1993). It has a pH optimum range between 6 and 8 and has low temperature stability (optimum at 50°C) (Adler-Nissen, 1993). Neutrase has been reported to find commercial application in the brewing industry to aid in increasing soluble nitrogen due to its resistance to serine protease inhibitors present in barley.

1.2.6.2 Exopeptidases

Exopeptidases are said to hydrolyse only near the terminals of proteins. This mechanism enables them to cleave off either single amino acids, or very short peptides (di- and tripeptides). Exopeptidases may be classified into two classes (aminopeptidases and carboxypeptidases) based on their site of action (i.e. N or C terminus) (Table V).

1.2.6.2.1 Aminopeptidases

Aminopeptidases hydrolyse bonds at the free N termini of polypeptide chains to liberate amino acids, dipeptides or tripeptides (Rao *et al*., 1998). They occur in a wide variety of microbial species including bacteria and fungi and are generally intracellular enzymes. The substrate specificities of the enzymes sourced from bacteria and fungi are distinctly different in that the organisms can be differentiated on the basis of the profiles of their hydrolysis products (Cerny, 1998). Most aminopeptidases are from the metalloprotease group (Zeffren and Hall, 1973)

Table V: Classification of exopeptidases (Rao *et al.*, 1998)

Class	Group	Mode of Action[a]
Aminopeptidase		
	Dipeptyl peptidase	●↓○-○-○-○-○---
	Tripeptyl peptidase	●-●↓○-○-○-○---
Carboxypeptidase		---○-○-○-○-○↓●
	Serine type protease	
	Metalloprotease	
	Cysteine type protease	
	Peptidyl dipeptidase	---○-○-○-○↓●-●
	Dipeptidases	●↓●

[a]○ represents amino acid residues in the polypepide chain; ● represents terminal amino acids; ↓ represent sites of action of the enzymes

1.2.6.2.2 Carboxypeptidases

The carboxypeptidases hydrolyse bonds at the C termini of polypeptide chains to yield a single amino acid (dipeptidases) or dipeptide (peptidyl dipeptidase) (Rao *et al.*, 1998). Carboxypeptidases may be divided into three major groups, serine, metallo- and cysteine carboxypeptidases. This subdivision is based on the nature of the amino acid residues at their active sites (similar to endopeptidases).

Many commercial enzyme preparations particularly of fungal origin are reported to contain appreciable amounts of carboxypeptidases (Adler-Nissen, 1993). The most studied carboxypeptidases are metallocarboxidases A and B, which are produced mainly by mammals (Zeffren and Hall, 1973; Firsht, 1985). Serine carboxypeptidases have been reportedly isolated from *Penicillium* spp., *Saccharomyces* spp., and *Aspergillus* spp. These have been reported as having very similar substrate specificities but different pH optima, stability and molecular weight (Rao *et al.*, 1998). According to the authors, metallocarboxypeptidases have been isolated from *Saccharomyces* spp., and *Pseudomonas* spp., which require zinc and cobalt for their activity.

1.2.7 Conclusions

Though un-malted sorghum grain has been used successfully in both laboratory and commercial scale production of clear beer, challenges are still faced which render the process not as efficient as it ought to be. These challenges emanate from both the physical and chemical composition of sorghum grain. Exogenous proteolytic enzymes are capable of improving the efficiency of brewing with un-malted sorghum grain. Unfortunately, in order to improve the technical specifications of un-malted sorghum grain wort, proteolytic enzymes have been used at high dosage levels. Identifying proteases that would effectively degrade sorghum endosperm protein at low dosage levels, as well as identifying optimal conditions in which these (proteolytic enzymes) operate would greatly improve the efficiency of brewing with un-malted sorghum grain.

1.3 Objectives

To determine the effect of different industrial proteolytic enzymes and different mashing conditions on the free amino nitrogen (FAN) levels of wort from un-malted sorghum grain.

To determine the effect of proteolytic enzymes on the sorghum grain endosperm protein matrix.

To determine the effect of proteolytic enzymes on the wort extract of un-malted sorghum grain.

1.4. Hypotheses

Exogenous proteases will cause an increase in the levels of FAN due to their hydrolytic action on the protein matrix and protein bodies, which will release more free amino acids by 'breaking up' stabilizing bonds in the protein matrix.

Exogenous proteases will cause significant physical damage to the microstructure of the sorghum grain endosperm matrix protein because of their hydrolytic activity on.

Exogenous proteases will cause an increase in the levels of wort extract as a result of the breakdown of the endosperm protein matrix enveloping the starch granules. This will lead to greater hydration of the starch granules during gelatinization which will be more susceptible to amylosis and thus produce higher levels of fermentable sugars.

CHAPTER 2: RESEARCH

This section is written in the style of a scientific paper essentially as required for the Journal of Cereal Chemistry. Figure 3 shows a flow diagram of the experimental design used in this study

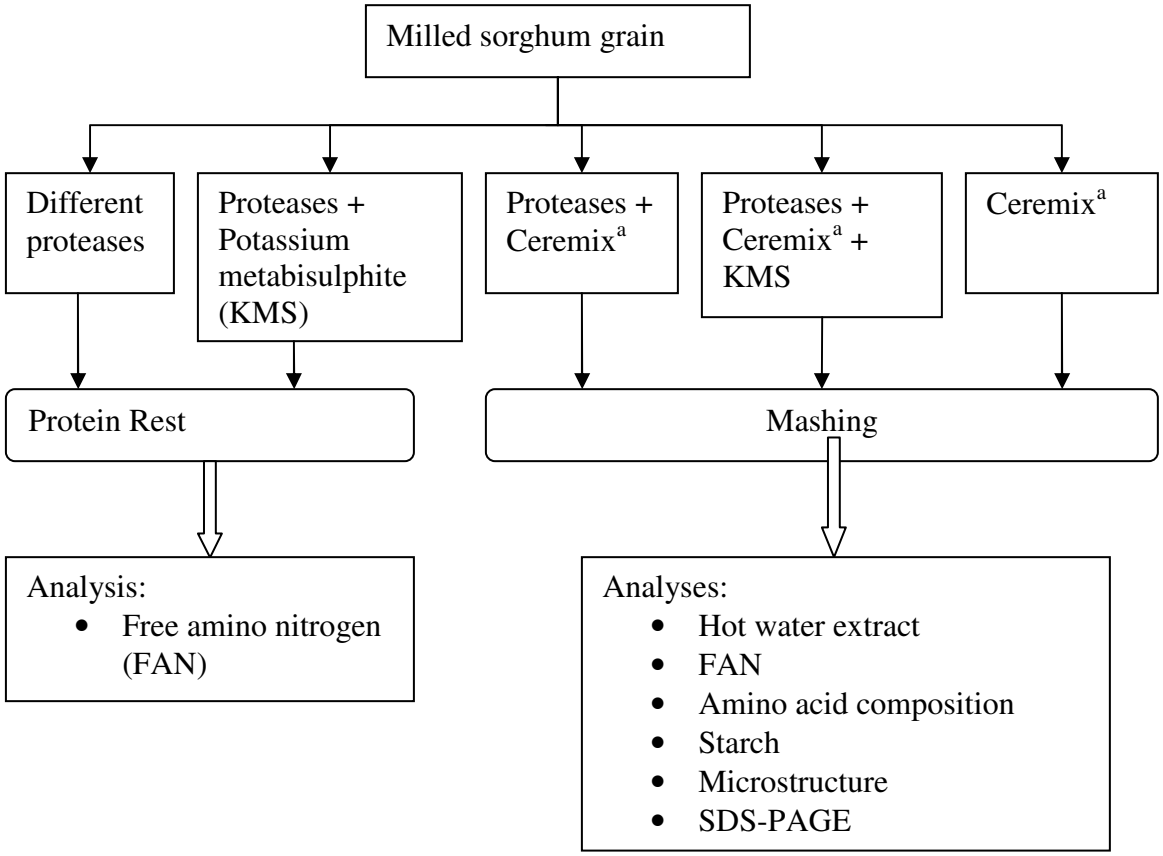


Figure 4: Experimental design for the study of free amino nitrogen improvement in sorghum grain brewing.

[a]Ceremix is composed of a mixture of a variety of necessary enzymes: α- amylase, β-glucanase, and protease.

2.1. Effect of proteolytic enzymes and mashing conditions on sorghum endosperm proteins and wort properties

ABSTRACT

The effects of commercial proteolytic enzymes and mashing conditions on sorghum proteins was investigated with regard to FAN production, effect on protein microstructure and other wort properties. The 8 enzymes studied produced varied increases in FAN (0% – 185%) when mashing was conducted at 55°C over 45 minutes. The addition of KMS to mashing systems where Neutrase and Flavourzyme were employed resulted in a further increase in FAN. A low-temperature, long time mashing system (40°C for 7 hr) increased FAN levels substantially (250%) when mashing with Flavourzyme and even more (350%) when KMS was introduced to the mashing system. The addition of KMS also resulted in more extensive physical damage to the sorghum endosperm protein matrix. Flavourzyme significantly increased (8%) the hot water extract of sorghum wort, but the addition of KMS did not cause any further increase. The relatively low digestibility of sorghum endosperm proteins (particularly kafirins) played a major role in the low FAN levels that are experienced when mashing with unmalted sorghum grain. Undigested sorghum grain endosperm protein had a negative effect on starch hydrolysis.

2.1.1 Introduction

In the past, sorghum has primarily found commercial application in the production of traditional opaque-beer and other non alcoholic beverages in developing countries (Murty and Kumar, 1995). Though research into the use of sorghum as an alternative for malted barley in clear beer production has been reported as far back as 1943 (Agu and Palmer, 1998b), the importance of sorghum in clear beer production was only realized in 1988 when a government ban on imported cereals was imposed in Nigeria (Hallgren, 1995). This brought about widespread use of sorghum in the production of lager beer both as an adjunct and as the principle brewing grain. More recently, utilisation of sorghum in commercial clear beer production has been reported in Central Africa (Mackintosh and Higgins, 2004), Southern and Eastern Africa, as well as the USA (Taylor *et al*., 2006).

Most of the research conducted on sorghum clear beer brewing has been focused on the use of malted sorghum as a partial (Dhamija and Singh, 1978; Demuyakor *et al*., 1994; Osorio-Morales *et* al., 2000) or total replacement for malted barley (Aisien and Muts, 1987; Ugboaja *et al.*, 1991; Bajomo and Young, 1992; Dufour *et al., 1992*; Olatunji *et al.*, 1993; Agu *et al.*, 1995; Agu, 2002; Agu, 2005; Ogu *et al*, 2006). In these studies, the identified problems that have been associated with brewing with malted sorghum are similar. Among the major problems cited are high malting costs for sorghum, generally high malting losses, insufficient amylase activity (low β-amylase activity leading to slow and incomplete saccharification), limited protein modification (which results in low free amino nitrogen (FAN) levels) and high gelatinization temperature of sorghum starch. In order to address these problems, researchers have proposed employing exogenous microbial enzymes to augment the efficacy of the indigenous malt enzymes (Agu *et al.*, 1995; Ogu *et al.*, 2006).

An alternative to brewing with malted sorghum is the use of un-malted sorghum. In this particular case it is essential to use a mixture of exogenous enzymes because there are no endogenous enzymes (with the exception of a small amount of protease) (Adams and Novellie, 1975; Evans and Taylor, 1990b) present in contrast to when sorghum malt is

used (Delcour *et al.*, 1989; Dale *et al.,* 1989, Bajomo and Young, 1993). It has been argued that using this approach is a more logical and cost effective method of brewing as opposed to using malted sorghum because the high malting costs and losses are not encountered and the used of exogenous enzymes is more justified in this case (Dale *et al.,* 1989, Bajomo and Young, 1993). However, when using un-malted sorghum, the problem of low FAN levels is even more profound. Several works have shown that the levels of FAN in unmalted sorghum grain wort mashed with commercial enzymes are well below those obtained with sorghum and barley malt (Ugboaja *et al*., 1991; Bajomo and Young, 1993; Bajomo and Young, 1994; Agu and Palmer, 1998a; Goode *et al*., 2002; Goode *et al*., 2003). In order to achieve a level of at least 120 mg/L (Beckerich and Denault, 1987) of FAN which is considered acceptable for optimal yeast growth and fermentation efficiency (Lekkas *et al.*, 2005), unacceptably high levels of proteolytic enzymes have been employed (Goode *et al.*, 2003). The low levels of FAN attained when using such exogenous proteolytic enzymes could be attributed primarily to the factors that contribute to the low digestibility (Duodu *et al.*, 2003) of the kafirin fraction of the protein which accounts for up to 80% of the total protein in sorghum grain (Taylor and Belton, 2002).

Apart from having an adverse effect on FAN levels, low protein digestibility also has an effect on sorghum starch digestibility (Ezeogu *et al.*, 2005). This is because in the vitreous region of the endosperm, starch granules are enveloped within a protein matrix. This is said to prevent full starch gelatinisation (Chandrashekar and Kirleis, 1988) and also limit access of amylase enzymes to the starch granule. The result of this is lower than expected extract levels.

The objective of this research was to study the effect of various commercial exogenous proteolytic enzymes and brewing conditions on sorghum grain protein hydrolysis and FAN production during mashing.

2.1.2 Materials and methods

2.1.2.1 Materials

Grain

White, tanplant, tannin free *Sorghum bicolor* (L.) Moench 'NK 8828' harvested in 2005 was used for all experiments. Whole grain sorghum was ground using a hammer mill (Falling Number AB, Huddinge, Sweden) fitted with a 1.6 mm screen. Samples were stored in polythene zip lock type bags at ≈5°C until needed for analysis.

Enzymes

The commercial enzymes Flavourzyme, Neutrase 1.5 MG, Papain, NS26001, NS26009, NS26068, NS26023 and Ceremix were kindly provided by Novozymes SA (Pty) Ltd (Benmore, Johannesburg, South Africa). Bioprotease P Conc. was kindly donated by Kerry Bioscience (Johannesburg, South Africa). Table VI shows the information that was provided about the enzymes by the suppliers.

Table VI: Description of the commercial enzyme types used in the study.

Commercial Enzyme	Source	Description	pH Optima	Temp. Optima	Activity	Form
Flavourzyme 500 MG	*Aspergillus oryzae*	Aminopeptidase	NP	NP	500 LAPU/g [1]	Granular
Neutrase 1.5 MG	*Bacillus amyloliquefaciens*	Neutral protease	5.5-7.5	45-55	1.5 AU /g[2]	Granular
Papain	Papaya		4.0-5.0	55-60	6100NFu/ mg[3]	Liquid
NS26001	NP	Exo-peptidase, endoprotease	NP	NP	NP	Granular
NS26009	NP	Exo- and endo-proteases; acid, neutral and alkaline	NP	NP	NP	Liquid
NS26068	NP	1.Protease 2.α-amylase 3. glucanase 4. xylanase	NP	NP	NP	Granular
NS26023	NP	Acid protease	NP	NP	NP	Liquid
Bioprotease P Conc.	*Aspergillus oryzae*	Protease	4.0-7.0	55	400,000 HUT u/g[4]	Granular
Ceremix Plus MG	*Bacillus stearothermophilus, Humicola spp, Bacillus spp.*	1.α-amylase 2.Neutral protease 3.β-glucanase 4.Pentosanase, xylanase 5.Cellulase			115 KNU_B/g[5]	Granular

[1]LAPU = Leucine amino peptidase units; [2]AU = Anson units; [3]NFU = NF papain units; [4]HUT = Haemoglobin units on a tyrosine basis ;[5]KNU = Kilo Novo α-amylase units; NP = Not provided

2.1.2.2 Small Scale Mashing

Mashing regime A.

Milled grain (10 g) was mixed with tap water (15 g) pre-heated to 55°C contained in conical flasks, and maintained at this temperature in a shaking water bath. Calcium chloride (200 ppm with respect to flour) was added to each sample. Proteolytic enzymes were dosed in at a rate of 1% (w/w) of the milled grain. Mashing was carried out for 45 min at 55°C. In some cases, samples were treated with potassium metabisulphite (KMS) (0.1 % (w/w) of milled grain) at the beginning of the mashing period. In one experiment, the temperature was varied to include mashing temperatures at 50°C and 60°C.

Mashing regime B.

Milled grain samples were treated as in Mashing regime A. Mashing was carried out for 7 hr at 40°C.

Complete modified mashing regime.

Mashing was conducted in stirred stainless-steel beakers using a BRF mashing bath (Brewing Research Foundation, Nutfield, UK). Milled sorghum (100 g) was weighed into the beakers and mixed with tap water (150 mL) to give a grist/liquor ratio of 1:1.5. Calcium chloride (200 ppm with respect to flour) was added to each sample. KMS (0.1 % (w/w) of flour) was added to some treatments. The mashing regime used is shown in Figure 5. After the mashing period, the total weight of the mash was made up to 250 g with tap water to replace any evaporative losses. The mash was then centrifuged at 14,020 g and vacuum filtered through filter paper (Whatman Grade 1: 11 µm pore size) to

obtain a clear sweet wort for analysis. Both the wort and the spent grain were stored at 0°C for not more than 24 hr before analysis.

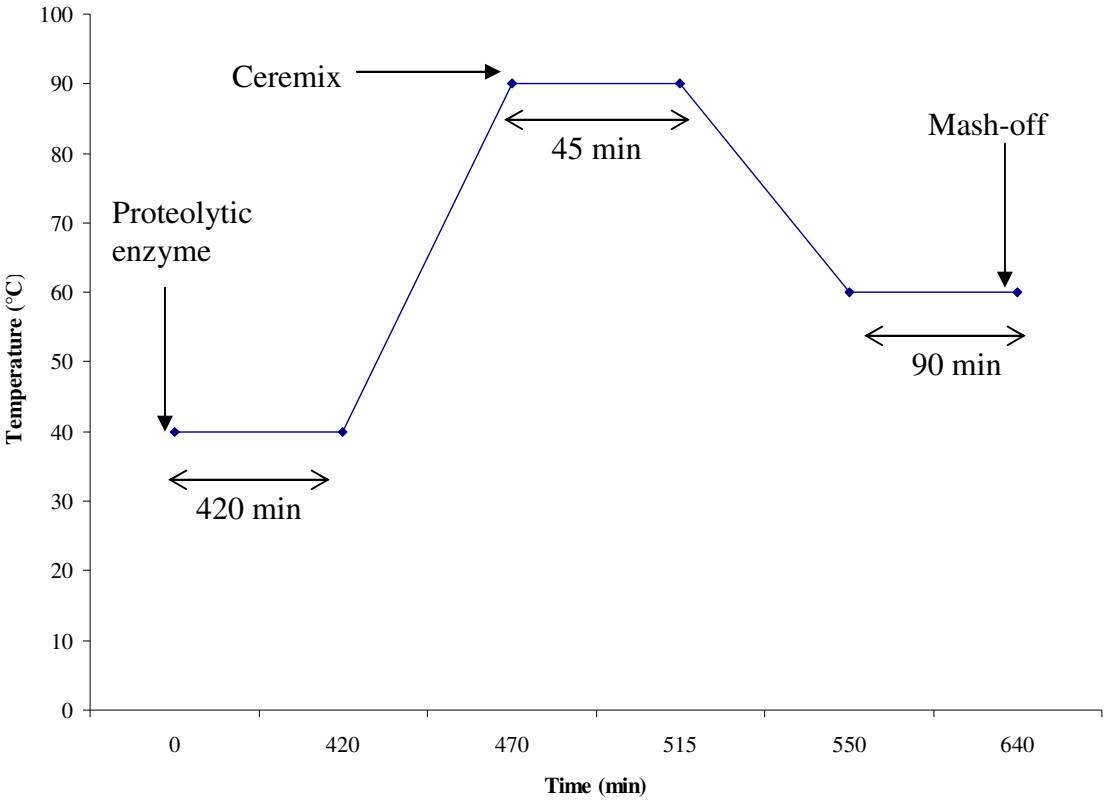


Figure 5: Complete modified mashing regime used in the Study.

Sample preparation of samples for Transmission Electron Microscopy

Milled sorghum grain (100 g) was defatted using hexane (300 ml). The grist was mixed and stirred with fresh hexane 3 times at 10 min intervals. After each ten minute interval the supernatant was discarded. After defatting, the grist was air-dried in a fume cupboard overnight. The samples were then mashed using the complete modified mashing regime. After mashing, samples were wet milled using a Retsch ZM 200 wet mill (Haan, Germany) fitted with a 1.0 mm screen at 10,000 rpm and then later with a 250 µm screen at 10,000 rpm. The samples were then passed through a 150 µm sieve. The material that passed through the sieve was centrifuged using a swing out rotor centrifuge at 2000 g for 2 min. The supernatant was discarded and the cream/brown protein rich layer was

carefully scraped off using a spatula. The protein rich material was then resuspended in a sodium azide solution (0.0004% w/v) and stored at ≈ 5°C.

Preparation of samples for CLSM

Sorghum grist (100g) was mashed using mashing regime B followed by a cooking (90°C for 45 min) step. Samples were taken at the beginning of the 7 hr period and after cooking and then stored in a sodium azide solution (0.0004% w/v). Samples were kept at ≈ 5°C until ready for analysis.

2.1.2.3 Analyses

2.1.2.3.1 FAN

One mL samples were taken at the beginning and at the end of the mashing period (mashing regime A and B). The samples were then diluted to 500 mL in volumetric flasks. Ten mL samples were removed from the initial diluted samples and centrifuged (Hettich Zentrifugen Rotanta 460R, Tuttlingen, Germany) at 1,900 g for 10 minutes. FAN was then determined using the ninhydrin assay as prescribed by the European Brewery Convention Method 8.8.1 (European Brewery Convention, 1987).

2.1.2.3.2 Extract

The specific gravity of the wort was measured using the American Society of Brewing Chemists approved method WORT-2 (American Society for Brewing Chemists, 1976) using a Reischauer pycnometer. The refractive index and °Brix of the wort was obtained using a refractometer. Wort extract (Plato, °P) was calculated from the specific gravity using the following formula (DeClerck, 1957).

$$Extract \quad (°P) = (-463.37) + (668.72 \ \ SG) - (205.35 \ \ SG^2)$$

in which SG = specific gravity of the wort sample.

2.1.2.3.3 Amino acid analysis

Wort samples for amino acid analysis were freeze dried. The PICO.TAG-Method (Bidlingmeyer *et al.*, 1984) was used for amino acid anlysis. This method is based on the principle of reversed phase chromatography with pre-column derivatisation. The apparatus used was a Waters high performance liquid chromatography model 440 unit (Waters, Milford, USA) with a Waters 717 plus Autosampler unit (Waters, Milford, USA) attached. The column used in the method was a PICO.TAG column for hydrolysate amino acid analysis 150 x 3.9 mm internal diameter.

2.1.2.3.4 Starch

Both the wort and spent grain were qualitatively analysed for starch using the iodine starch test.

2.1.2.3.5 Transmission Electron Microscopy (TEM)

One mL of the suspended protein rich material obtained during the preparatory stage was mixed with 1 mL double distilled water and centrifuged at 10,000 g for 30 sec. The pellet was then fixed in a fixative (1 mL 2.5% glutaraldehyde + 5 mL 0.075M sodium: potassium (1:1) phosphate buffer + 4 mL double distilled water) for 30 min. The sample was then centrifuged at 7200 g for 30 sec. The pellet was rinsed 3 times using the 0.075M Na:K (1:1) phosphate buffer with centrifuging following each rinse. After the final rinse, the pellet was suspended in osmium tetroxide and left to stand for 1 hr. This was followed by another rinsing step as described. The sample was then dehydrated in a series (50 %, 70%, 90% ethanol for 15 min then 100% ethanol for 15 min repeated 3 times) with a centrifuging step following each rinse. The dehydrated pellet was then infiltrated in quetol epoxy resin and polymerized in an oven at 60°C for 24 hr. The sample was then sectioned and stained with toludine blue for TEM.

2.1.2.3.6 Confocal Laser Scanning Microscopy (CLSM)

One mL double distilled water was added to about 1 mL suspended spent grain. A drop of the sample was placed on a glass microscope slide which was then covered with a cover-slip. Samples were then viewed under a CLSM (ZEISS LSM 510, Zeiss SMT, Jena Germany). The microscope system was equipped with a Plan-neofluar 40×/1.3 Oil DIC objective lens. Excitation was at 405 nm for the sorghum proteins. Fluorescing proteins were detected after passing through a 420 long pass filter, with a pinhole set at 55 µm. 3-D images were obtained by collecting ≥25 laser generated optical planes. Digital images were processed using Zeiss LSM image browser (Zeiss SMT, Jena, Germany)

2.1.2.3.7 SDS-PAGE

An XCell SureLock Mini-Cell electrophoresis unit (Invitrogen Life Technologies, Celsbad CA USA) was used to conduct SDS-PAGE under both reducing and non reducing conditions. Protein samples were prepared as described for TEM. The pre-prepared gel consisted of 12 % and 3.9 % (v/v) acrylamide respectively. Premixed molecular weight markers (Roche Diagnostics, Basel, Switzerland) and consisted of the following premixed proteins: phosphorylase B (M_r = 97.4 k); bovine serum albumin (M_r = 66.2 k); aldolase (M_r = 39.2 k); triose phosphate isomerase (M_r = 26.6 k); trypsin inhibitor (M_r = 21.5 k); lysozyme (M_r = 14.4 k). The elctrophoresis system was set at 13 mA and 120 V (constant) for 4 hr. The gel was stained overnight with 1% Coomasie blue, destained and scanned using a flat bed scanner. A similar method to that used by Byaruhanga *et al.* (2007) was used in this study.

2.1.2.3.8 Statistical analysis

Single factor analysis of variance was used to determine the effect of the different proteolytic enzymes on FAN. Multifactor analysis of variance was used to determine the effect of temperature, KMS and proteolytic enzymes on FAN. Means of at least two replicate experiments were separated using the least significant test ($p < 0.05$).

2.1.3 Results and discussion

Table VII: Effect of different exogenous proteolytic enzymes on free amino nitrogen (FAN) levels when sorghum grain meal was mashed at 55°C[1] for 45 min.

Enzyme treatment	FAN (mg/100 g sorghum)[2]	% increase in FAN
No enzyme	25.3[a] (3.4)	
Bioprotease P Conc.	72.1[e] (0.8)	185.0
Flavourzyme	52.2[d] (3.0)	106.3
Neutrase	32.3[b] (1.3)	27.7
Papain	50.2[cd] (0.4)	98.4
NS 26001	47.6[c] (2.0)	88.1
NS 26009	49.8[cd] (0.8)	96.8
NS 26068	30.7[b] (0.5)	21.3
NS 26023	24.6[a] (0.4)	-2.8

[1] All results are mean of two replicate experiments (n = 2). Figures in parentheses indicate standard deviations

[2] Values with different letter superscripts in the column are significantly different ($p \leq 0.05$) from each other.

Table VII shows the amount of FAN produced by 8 different enzymes used to mash whole sorghum grain at 55°C. All the enzymes with the exception of NS 26023 brought about a significant increase in FAN levels. Bioprotease P Conc gave the highest degree of proteolysis which resulted in an almost 3-fold increase in FAN. This could be attributed to the high concentration of active protease present in the Bioprotease P Conc preparation (Table VI). Flavourzyme, Papain and NS 26009 were the second most effective enzymes producing a 2-fold increase in FAN.

In modern day brewery operations which often employ high gravity brewing methods, it is generally believed that 150 mg/L of FAN is necessary to support proper yeast function (Beckerich and Denault, 1987). None of the enzymes used in this study were able to generate even half the amount that is considered acceptable even when using a supposedly very high dosage level. This is in agreement with findings of other workers (Bajomo and Young, 1992; Bajomo and Young, 1993; Agu and Palmer, 1998a; Goode *et al.*, 2002; Goode *et al.*, 2003) whose findings all suggested that very high levels of proteolytic enzymes were required to produce sufficiently high FAN values when mashing with unmalted sorghum.

It was expected that the different proteolytic enzymes used in this study would produce varying levels of FAN (which is an indication of varying levels of proteolysis). These findings are in agreement with those reported in similar studies where the efficacies of different proteolytic enzymes have been directly or indirectly studied in brewing with sorghum grain (Bajomo and Young, 1992; Bajomo and Young 1993; Goode *et al.*, 2003). There are several possible explanations for this occurrence. The mashing was carried out at 55°C for all the enzymes and no pH adjustments were made. It has been shown that proteolytic enzymes in general function optimally within well defined temperature and pH ranges both of which are very specific to different enzymes (Kaur *et al.,* 2001; Poza *et al.*, 2001; Pekkarinen *et al.*, 2002; Kim and Lei, 2005). In particular, proteolytic enzymes from the family of acid proteases generally have a low pH optimum range (Whitaker, 1972).

Proteolytic enzymes can be subdivided into two major groups, i.e. exopeptidases (carboxypeptidase) and endopeptidases (proteinases) depending on their site of action (Rao *et al.*, 1998). Exopeptidase activity is limited specifically to the peptide or ester linkage connecting an appropriate carboxy- or amino-terminal residue to the remainder of the substrate molecule, whereas endopeptidase activity occurs at appropriate linkages regardless of their positions with respect to the ends of the peptide chain (Zeffren and Hall, 1973). It would therefore be safe to presume that exopeptidases are more responsible for the release of free amino acids than endopeptidases which produce more

peptides in addition to lower levels of amino acids. The varying levels of FAN produced by the different enzymes suggest that the ratio of exopeptidase to endopeptidase in the different enzymes also could differ, resulting in some of the proteolytic enzymes producing more amino acids than others.

Proteolytic enzymes are also further subdivided into four prominent groups (serine, aspartic, cysteine and metallo-proteases) based on the functional group present at the active site (Rao *et al.*, 1998). The active sites of proteolytic enzymes cleave at specific bonds within the peptide chain (Keil, 1992). For instance, Papain has been reported to cleave the Insulin B chain at bonds between asparagine and glutamine; glutamic acid and alanine; leucine and valine; cysteine and glycine; phenylanine and tyrosine; and tyrosine and threonine (Keil, 1992). It is therefore imperative that the presence of appropriate bonds between amino acids at which specific enzymes cleave occur close to the terminal ends of the peptide chain if FAN is to be released.

The catalytic activity of some enzymes is dependant on the presence of non-proteinaceous compounds such as metal ions or organic material (Zeffren and Hall, 1973). For instance, enzymes from the metalloprotease family depend on the presence of specific bound divalent cations for their catalytic activity (Rao *et al*., 1998). In this particular study, calcium chloride was added during mashing. Though calcium chloride was not added for the sole purpose of enhancing proteolytic activity, it could have been beneficial to some of the enzyme preparations by acting as a cofactor, but it could also have been detrimental to some because the presence of some cations in the environment in which certain enzymes operate have a negative effect on enzyme activity (Ogbonna *et* al., 2003; Ogbonna *et al.*, 2004; Ogbonna and Okolo, 2005).

Kafirin has been reported to contribute significantly to the poor digestibility of sorghum proteins when compared to other cereal storage proteins (Hamaker *et al.*, 1987; Oria *et al.*, 1995a, b; Duodu *et al*. 2003) primarily because of its hydrophobic nature (Taylor and Belton, 2002) and the presence of disulphide bonds (Oria, 1995a, b). Evidence suggests that the digestibility of kafirin is further reduced when it undergoes wet cooking (Rom *et*

al., 1992; Hamaker *et al.*, 1986; Oria *et al.*, 1995a, b; Arbab and El Tinay, 1997). This occurs because disulphide bonds are formed within the protein structure which reduces the accessibility of proteolytic enzymes to susceptible bonds. Enzyme resistant polymers are also formed between the already highly folded β- and γ-kafirins (Hamaker *et al.*, 1986; Rom *et al.*, 1992; Oria *et al.*, 1995b; Duodu *et al.*, 2002). It has been reported that the formation of polymeric kafirin increases as cooking temperatures are increased (Ezeogu *et al.*, 2005). Although the temperature at which the mashing was carried out in this study is relatively low (55°C), a certain degree of cross linking may have occurred even at this temperature thus limiting the access of the enzymes to susceptible bonds in the proteins. As enzymes generally operate in aqueous environments, the hydrophobic nature of kafirin may also have been a contributing factor to the low FAN values achieved.

Table VIII shows the effect of mashing temperature and KMS on proteolysis. Neutrase showed its highest level of proteolysis when mashing was carried out at 50°C where a 2-fold increase in FAN was achieved, while Flavourzyme and Papain showed to be most effective when mashing was carried out at 55°C with both treatments resulting in a 2-fold increase in FAN. The addition of KMS did not cause any significant increase in FAN when papain was used, regardless of the mashing temperature. When using Flavourzyme and Neutrase, KMS addition resulted in a significant additional increase in FAN irrespective of the mashing temperature used. The addition of KMS to mashing systems where Neutrase and Flavourzyme were employed resulted in significant increase in FAN (Table III). On average, the addition of KMS brought about an increase of 13% and 15% in FAN in mashes containing Neutrase and Flavourzyme resepectively. When mashing at 55°C with Flavourzyme, addition of KMS resulted in a 22% increase in FAN. It is likely that the activity of Papain was negatively affected by KMS because it is classified as a thiol/ sulphydryl protease (Firsht, 1985). As the structure of papain is stabilized by 3 disulphide bonds (Whitaker, 1972), it is possible that the KMS could have reduced the stabilising disulphide bonds present in papain.

Other studies have shown that reducing agents including 2-mercaptoehtanol (Hamaker *et al.*, 1987), sodium bisulphite (Rom *et al.*, 1992; Oria *et al.*, 1995b; Arbab and El Tinay, 1997) and ascorbic acid (Arbab and El Tinay, 1997) have been able to enhance the digestibility of sorghum protein. Reducing agents are able to reduce inter- and intra-molecular disulphide bonds that are formed in kafirin during the cooking process. This facilitates the unfolding of the protein and renders bonds that would otherwise be inaccessible to the enzymes more available. The increased availability of susceptible peptide bonds in the kafirin fractions (particularly β- and γ-) should result in more FAN being produced. In addition to that, higher degradation of the β- and γ-kafirin fractions which are located at the periphery of the protein body should result in better access of enzymes to the more digestible α- kafirin which will also result in higher FAN production.

Table VIII: Effect of mashing temperature (50°C, 55°C and 60°C) and potassium metabisulphite (KMS) on free amino nitrogen (FAN) levels in sorghum grain mashed with three different enzymes[1].

Enzyme	Temperature (°C)	KMS	FAN (mg/100 g sorghum grist)[2]
Neutrase	50	Absent	46.3^{c} (0.2)
	50	Present	51.3^{d} (1.5)
	55	Absent	32.3^{a} (1.3)
	55	Present	36.8^{b} (0.4)
	60	Absent	33.2^{a} (0.9)
	60	Present	37.6^{b} (1.3)
Papain	50	Absent	41.8^{a} (0.8)
	50	Present	41.8^{a} (0.7)
	55	Absent	50.2^{b} (0.4)
	55	Present	54.6^{b} (0.4)
	60	Absent	35.3^{a} (7.0)
	60	Present	39.7^{a} (4.1)
Flavourzyme	50	Absent	47.9^{a} (0.0)
	50	Present	54.2^{b} (0.6)
	55	Absent	52.2^{ab} (3.0)
	55	Present	63.6^{c} (0.1)
	60	Absent	49.7^{ab} (2.5)
	60	Present	55.2^{b} (4.5)

[1] All results are mean of two replicate experiments (n = 2). Figures in parentheses indicate standard deviations

[2] Values with different letter superscripts in a block are significantly different ($p \leq 0.05$).

Table IX: Effect of a combination of 3 different enzymes (Neutrase, papain and Flavourzyme) and potassium metabisulphite on free amino nitrogen levels when mashing with sorghum grain[1]

Enzyme combination	Mashing Temperature (˚C)	Final FAN (mg/100 g sorghum grist)[2]
Flavourzyme, neutrase and papain without 100 mg KMS	55	46.7[a] (0.7)
Flavourzyme, neutrase and papain with 100 mg KMS	55	56.3[b] (1.4)

[1] All results are mean of two replicate experiments (n = 2). Figures in parentheses indicate standard deviations

[2] Values with different letter superscripts in the column are significantly different ($p \leq 0.05$).

Table IX shows the effect of using a combination of 3 enzymes in conjunction with KMS on proteolysis levels in grain. There was no synergistic effect observed when the 3 enzymes were used in combination. However as observed previously, a significant ($p \leq 0.05$) increase was observed when KMS was introduced. It was hoped that by using the enzymes in combination, a synergistic effect could be achieved with a resultant increase in FAN. This was thought possible because the presumed specificity of the different enzymes would have been used to cleave the proteins at different bonds. This, however, was not the case as the levels of FAN obtained when using the enzymes in combination at the chosen temperature were very similar to those obtained when Papain was used on its own. It is highly likely that in addition to having hydrolytic action on the sorghum protein, papain could have had a similar effect on the two other enzymes (Neutrase and Flavourzyme). This is possible because enzymes are proteins as well and would therefore also be susceptible to hydrolysis by other enzymes especially if they do not naturally operate within the same enzyme system.

Mashing at 55°C and even with the addition of KMS did not produce FAN levels considered sufficiently high enough for brewing purposes. It was hypothesised that increasing the enzyme hydrolysis time with the substrate while simultaneously retarding the rate of enzyme denaturation would have a positive effect on increasing FAN. It was thus decided to explore using a low temperature-long time mashing period. It was also decided to use Flavourzyme in this part of the study because it had proved to be the most effective of the enzymes in terms of overall FAN production in the preceding study (Table VIII). Figure 6 shows the effect of using a low (40°C) temperature over a long period (7 hr) of mashing in the presence of KMS. Using the proteolytic enzyme in conjunction with KMS resulted in an almost 5-fold increase in FAN reaching a maximum of 93 mg/100 g sorghum grist after the 7 hr mashing period. Using the enzyme on its own caused a 4-fold increase in FAN with a maximum of 78 mg/100 g sorghum grist after the 7 hr mashing period. Mashing without the proteolytic enzyme and KMS only caused a 2-fold increase in FAN.

Even in the absence of proteolytic enzyme, the level of FAN produced over a 7 hr period doubled. This can be attributed to the endogenous proteases that have been reported to be present in un-malted sorghum grain (Evans and Taylor, 1990a, b). In their study these authors established that in un-malted sorghum, the level of endopeptidase is considerable but this is accompanied by a very low level of carboxypeptidase activity. Therefore the low level of FAN produced in the absence of exogenous proteolytic enzymes could be because endopeptidases produce more peptides than they do free amino acids.

The large increases in FAN observed when mashing over a 7 hour period could indicate that the enzyme was given more time to access susceptible bonds within the protein. Though the temperature was reduced to 40°C from 55°C which would have resulted in a reduction in the reaction rate of the enzyme, the longer time period permitted more than compensated for the lower reaction rate by reducing the rate at which the enzyme was denatured. It was observed that after 45 min of mashing (the time used in the previous mashing experiments) almost 60% and 70% of the total FAN produced when mashing was done at 55°C with and without KMS respectively was already produced.

Apart from reducing the reaction rate of the enzyme, reducing the mashing temperature to 40°C could possibly have also reduced the rate at which formation of disulphide bonds occured. Work done by Ezeogu *et al*. (2005) suggests that disulphide bond formation in kafirin is enhanced with increasing temperatures. Therefore, when mashing at 40°C, it is probable that less polymerization of the kafirin sub-units occurred. This could have enabled the enzyme to have more access to susceptible bonds within the protein chains, which allowed more FAN being produced.

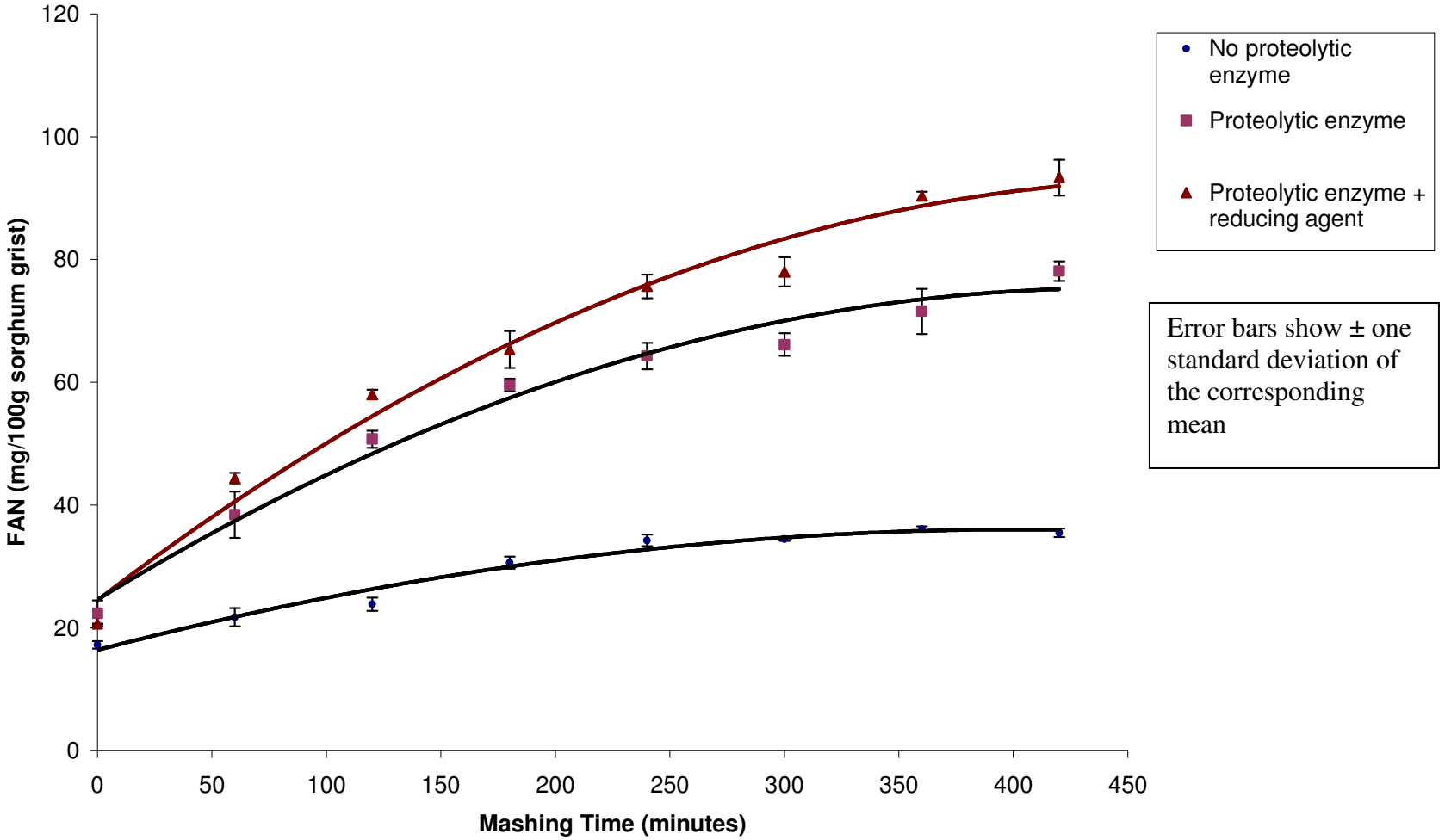


Figure 6: Effect of a long time low temperature mashing period and reducing agent (KMS) on free amino nitrogen levels in sorghum mashed with Flavourzyme

Table X: Effect of proteolytic enzyme (Flavourzyme) and reducing agent (KMS) on hot water extract, free amino nitrogen levels and starch presence in sorghum grain wort and spent grain.

						Iodine test	
Treatment	**Specific gravity (g/cm^3)[2]**	**Extract (°Plato) [2]**	**R.I.[2,3]**	**°Brix [2]**	**FAN (mg/100 g)[2]**	**Wort**	**Spent grain**
Ceremix	1.1120 (0.0033)^a	26.3 (0.5)^a	1.375 (0.000)^a	26.5 (0.0)^a	43.7 (1.3)^a	Positive	Positive
Ceremix + Flavourzyme	1.1215 (0.0035)^b	28.3 (0.7)^b	1.379 (0.001)^b	28.4 (0.2)^b	84.6 (5.1)^b	Positive	Positive
Ceremix + Flavourzyme KMS +	1.1234 (0.0026)^b	28.7 (0.7)^b	1.379 (0.001)^b	28.9 (0.2)^b	96.7 (7.7)^c	Negative	Positive

[1] All results are means of three replicate experiments (n = 3). Figures in parentheses indicate standard deviations.

[2] Values with different letter superscripts in each column are significantly different ($p \leq 0.05$).

[3] R.I. = Refractive index.

Table X shows that a significant increase in extract (as measured by specific gravity and refractive index) was observed when Flavourzyme was introduced into the mashing system. Similarly, a significant increases in FAN also occured. However, in all parameters measured with the exception of FAN, the addition of KMS did not bring about any further significant increases.

Considering that the contribution of soluble sugars to wort HWE is very high (Briggs *et al*. 2004); extract thus provides a good indication of the degree of starch degradation. The four parameters measured (specific gravity, °Plato, refractive index and °Brix) gave a similar result and will therefore be discussed collectively using the term extract. The presence of amylolytic enzymes in Ceremix was the main contributor to the extract levels because the amylase enzymes were able to hydrolyse the sorghum grain starch. The increase in FAN observed when Flavourzyme was introduced was probably because the Flavourzyme was able to partially degrade the endosperm protein matrix in which the starch granules are embedded within the vitreous endosperm (Rooney and Serna Saldivar, 1991). This occurrence could have had two positive effects which ultimately led to the increase in extract. Firstly, the hydrolysis of the intimately bound matrix protein on the surface of the starch granule provided a larger surface area on which the amylase could act to produce more reducing sugars. Secondly, more extensive breakdown (Figure 8) of the sorghum endosperm matrix protein could have allowed the starch granules to take up more water and therefore expand more during gelatinization. This would make them more susceptible to amylase attack. Work by Chandrashekar and Desikachar (1981) supports this theory. Their studies showed that the addition of papain to sorghum semolina greatly increased the water absorption during cooking.

As stated, a further increase in extract level with the addition of KMS was not achieved. Though the increase of 12.5% in FAN as a result of addition of KMS represents a significant increase, this was not manifested in the extract. This could suggest that the additional protein matrix degradation was not extensive enough to ‘free’ a corresponding high amount of starch granules for hydrolysis.

Table. XI: Amino acid (g/100 g protein) spectrum of worts separated from sorghum mashed with commercial enzymes Ceremix and Flavourzyme plus KMS at 40°C for 7 hr.

Group[1]	Amino acid	Enzyme Treatment		
		Ceremix	Ceremix + Flavourzyme	Ceremix + Flavourzyme + KMS
A	Aspartic acid	8.0	9.0	7.3
	Glutamic acid	11.7	15.0	15.6
	Serine	4.5	5.4	4.9
	Arginine	6.5	7.8	7.1
	Threonine	4.2	4.6	4.1
	Lysine	5.3	6.2	5.2
	Cysteine	0.0	0.0	0.2
	Sub-total A	40.2 (52%)	48.0 (49%)	44.4 (47%)
B	Histidine	1.9	2.7	3.1
	Valine	4.8	6.5	6.2
	Methionine	0.0	1.6	1.3
	Isoleucine	2.9	4.2	4.1
	Leucine	5.4	7.9	7.6
	Sub-total B	15.0 (19%)	22.9 (23%)	22.3 (24%)
C	Glycine	5.3	6.0	5.7
	Alanine	5.8	8.3	7.2
	Tyrosine	2.5	3.6	3.6
	Phenylanine	2.9	3.9	3.7
	Sub-total C	16.5 (22%)	21.8 (22%)	20.2 (22%)
D	Proline	5.7 (7%)	6.1 (6%)	7.3 (8%)

[1]Classification of amino acids based on their rate of uptake by yeast during fermentation (Jones and Pierce, 1964)

Table XI shows the amino acid composition of sorghum wort under the given enzyme treatments. Overall, in all the treatements, there is a high concentration of the group A amino acids. The addition of Flavourzyme and KMS did not cause any substantial change to the distribution of the respective classes of amino acids.

Overall all three treatments produced wort with a similar ratio of 5:2:2:1 (group A:B:C:D amino acids). Amino acids have been grouped based on their assimilation patterns by yeast (Jones and Pierce, 1964). The group A amino acids (glutamic acid, aspartic acid, asparagine, glutamine, serine, threonine, lysine and arginine) are absorbed the fastest. The group B amino acids (valine, methionine, leucine, isoleucine, histidine) have an intermediate absorption rate. The group C amino acids (glycine, phenylalanine, tyrosine, tryptophan, alanine) are absorbed slowly. The only amino acid in group D, proline, is almost not absorbed at all. The findings of this work are in agreement with the findings of Taylor and Boyd (1986) where the same ratio of the respective amino acid groups was observed, though in their study, sorghum malt was used without addition of any exogenous enzymes. Similar to the findings in this study, Taylor and Boyd (1986) also found that aspartic acid and glutamic acid were the most abundant. When compared to raw untreated sorghum whole flour (Taylor and Schüssler, 1986) where the amino acid group ratio was 4:3:2:1, it can be seen that the proteolytic activity increased the levels of group A amino acids in the wort.

These results also suggest that the proteases that are present in Ceremix could cleave the protein at similar positions to those present in Flavourzyme, resulting in amino acid compositions that are very similar.

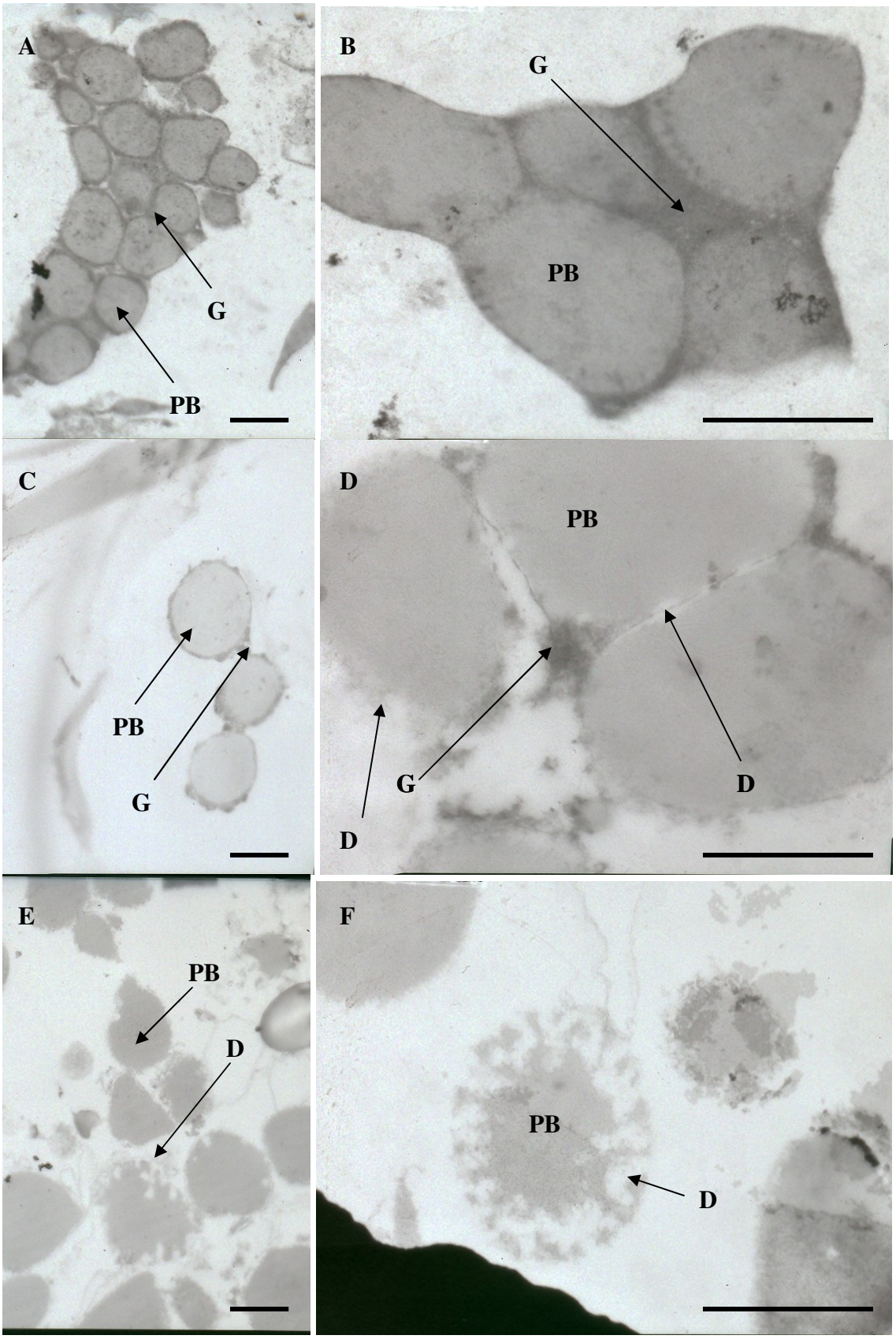

A
G
PB
B
G
PB
C
PB
G
D
PB
G
D
D
E
PB
D
F
PB
D

Figure 7: Transmission electron microscopy of sorghum protein bodies isolated from sorghum grain mashed at 40°C for 7 hr: (A) and (B) control (no Flavourzyme or KMS). (C) and (D) mashed with Flavourzyme only; (E) and (F) mashed with Flavourzyme and KMS. PB, protein body; G, glutelin matrix protein; D, degradation. Scale bar = 1 μm

The effect of Flavourzyme and KMS on the microstructure of the sorghum endosperm protein matrix was studied using TEM (Fig 7). Extensive damage to the glutelin matrix protein which surrounds the protein bodies was observed when the proteolytic enzyme was used (Figs. 6 C and D). There was also evidence of protein body degradation primarily at the periphery of the protein bodies when the proteolytic enzyme was used. The addition of KMS (Figs. 6 E and F) brought about a complete breakdown of the glutelin matrix. More extensive degradation of the protein bodies was also observed when KMS was added.

When mashing with Flavourzyme only, very partial hydrolytic activity on the protein bodies was observed. The addition of KMS was able to augment the activity of the protease by reducing the disulphide bonds that are formed primarily at the periphery of the protein bodies involving β- and γ-kafirin (Oria, 1995b). The reduction of the disulphide bonds opened up the protein structure making it more digestible. The increased digestibility of the periphery regions of the protein body allowed the enzymes to have better access to the more digestible α- kafirin which is located throughout the protein body (Oria *et al,* 1995a). The pattern of protein degradation is very similar to that observed by Taylor *et al.* (1985b). They studied the degradation of sorghum protein bodies during germination and found that protein body degradation began on the margins (periphery) of the protein bodies and progressed inwards.

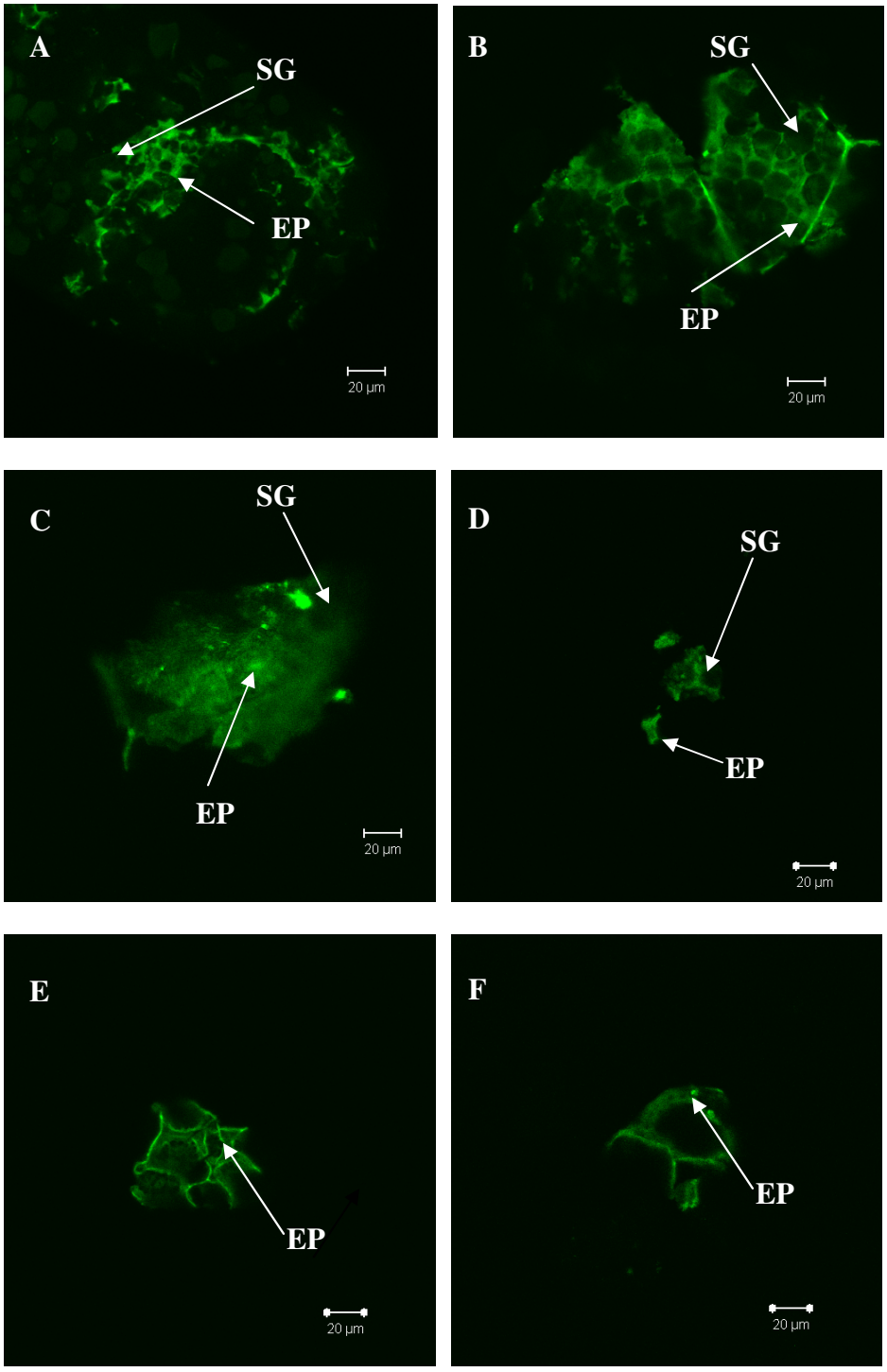


Figure 8: Confocal laser scanning microscopy of sorghum grain mashed at 40°C for 7 hr: (A) Control (no enzyme or KMS) uncooked; (B) Control cooked; (C) mashed with Flavourzyme and uncooked; (D) mashed with Flavourzyme and cooked; (E) mashed with

Flavourzyme and KMS and uncooked; (F) mashed with Flavourzyme and KMS and cooked. EP, endosperm protein matrix; SG, starch granule.

CLSM showed that after cooking the sorghum grist, there was an increase in the size of the starch granules (Fig 8 B). The increase in the size of the starch granules is an indication of the gelatinisation process taking place as a result of cooking. It has been postulated that endosperm proteins may play a role in limiting starch gelatinisation because they provide a rigid envelope around the starch granules (Chandrashekar and Kirleis, 1988). The envelope provided by the protein has been said to impede uptake of water by the starch granule, which prevents granules from swelling. Introduction of Flavourzyme did not alter the size of the covered starch granules. However, the endosperm particle size was greatly reduced in size (Fig 8 D). This is possibly an indication of more extensive starch gelatinisation which was made possible by the partial degradation of protein matrix. The partial degradation of the protein matrix caused weakening in its structure and this allowed the starch granules to take up more water and also expand more. The expanding starch granules were then able to rupture the weakened surrounding protein matrix into smaller fragments. Addition of KMS did not result in any apparent increase in the size of the starch granules (Fig 8 F).

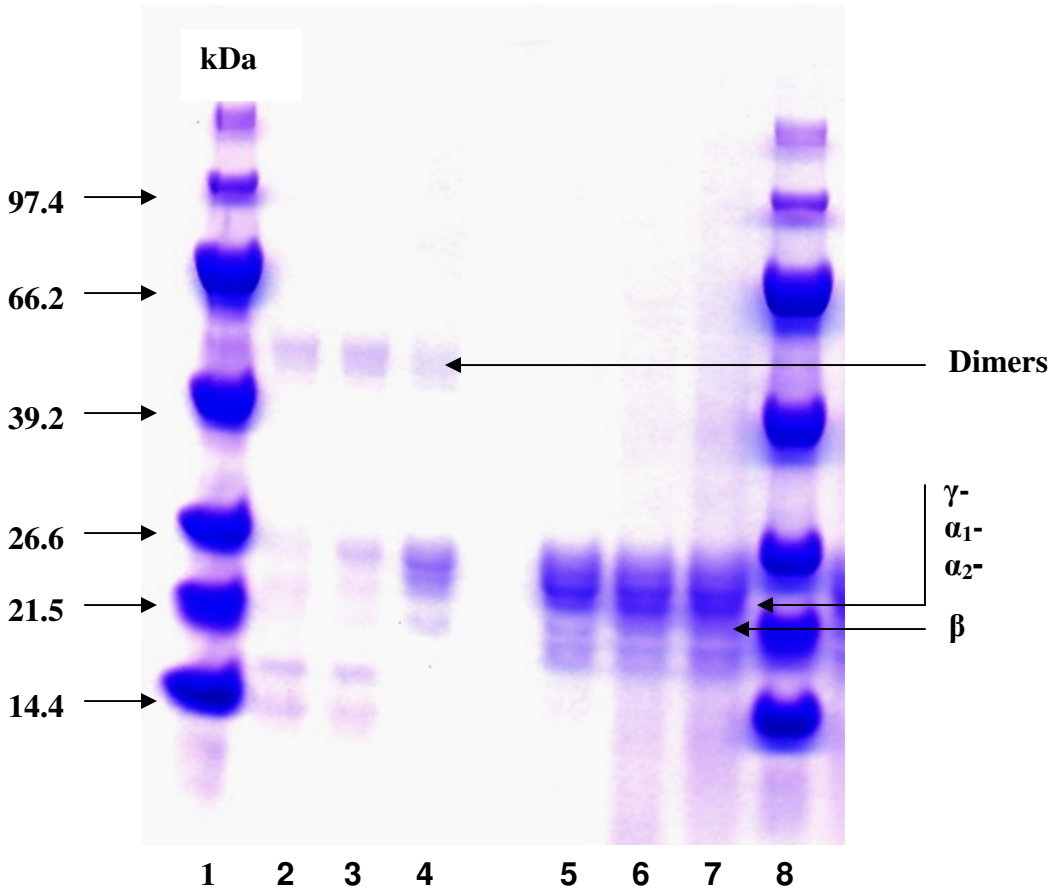


Figure 9: SDS-PAGE of non solubilised protein of sorghum after mashing at 40°C over 7 hr. Tracks 1 and 8, molecular weight standards; Tracks 2 to 4 under non-reducing conditions, where Track 2, control (no Flavourzyme and no KMS); Track 3, mashed with Flavourzyme; Track 4, mashed with Flavourzyme and KMS; Tracks 5 to 7 under reducing conditions, where Track 5, mashed with Flavourzyme and KMS; Track 6, mashed with Flavourzyme; Track 7, control (no Flavourzyme and no KMS).

With SDS-PAGE under reducing conditions (tracks 5, 6 and 7) (Fig 9), the intensity of bands representing the kafirin monomeric sub-units was much greater than the corresponding tracks under non-reducing conditions. The increased intensity was a result of the reduction of disulphide bonds which link the respective monomeric kafirin sub-units. Under non-reducing conditions (tracks 2, 3 and 4), disulphide linked dimers (M_r 40,000-50,000) were observed in all treatments. The intensity of the bands representing dimers in tracks 2 and 3 was greater than that in track 4 because the sample in track 4 was treated with KMS. The KMS present in this sample reduced the bonds in some of the dimeric and polymeric kafirin. As a result, the intensity of the bands representing monomeric kafirin sub-units in the sample treated with KMS (Track 4) were much higher than the corresponding bands in tracks 2 and 3.

In tracks 2, 3, 6 and 7, bands $\leq M_r$ 14,400 were observed. It is suggested that these bands could represent disulphide bond linked peptides. These did not occur in tracks 4 and 5 because these tracks represented samples that were treated with KMS. Although the control sample (tracks 2 and 7) was not treated with any proteolytic enzyme, it is possible that the endogenous endopeptidases that have been reported in unmalted sorghum grain (Evans and Taylor, 1990) could have cleaved bonds within the kafirin to yield cross-linked peptides.

2.1.4 Conclusions

Efficacy of different proteolytic enzymes on sorghum protein hydrolysis to produce FAN varies because of their varied optimal conditions and type. Their efficacy is also greatly affected by the characteristic poor digestibility of sorghum protein. The addition of KMS into the mashing system significantly improves the rate of sorghum protein hydrolysis because of the reduction of inter- and intra molecular stabilizing disulphide bonds. Allowing Flavourzyme to act over a longer period of time at a lower temperature was the best way of enhancing FAN production. The longer time period allows the enzyme more contact time with the substrate whilst the lower mashing temperature probably retards the denaturation of the enzyme as well as formation of additional disulphide bonds in the

substrate protein. The addition of Flavourzyme gives a substantial increase in extract because the protein enveloping the starch granules within the vitreous endosperm is significantly degraded. This seems to be because there is an increase in the surface area of starch granules exposed to amylase attack.

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CHAPTER 3: DISCUSSION

In this chapter, the principles of the major experimental methods used in this study will be explored namely, the choice of sorghum variety, the mashing regime used, free amino nitrogen (FAN) determination (ninydrin assay), microstructure analysis (using transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM), SDS-PAGE and amino acid analysis (PICO-TAG method). The problems encountered in the experimental work will also be discussed. Furthermore, the strengths and weaknesses of these methods will be discussed. This discussion will then go on to address the efficacy of the different proteolytic enzymes investigated with respect to sorghum protein degradation, the effect of the mashing conditions employed in this study on sorghum protein degradation and the effect of optimized mashing conditions on wort properties. Finally, the practicality of employing the optimized mashing conditions, which have been found in this study to result in improved wort FAN will also be discussed.

3.1 Methodological Considerations

For this study, a known white tannin free sorghum cultivar NK 8828 was selected. In lager style beer production using sorghum, it generally known that use of "red" sorghums with high levels of tannin and other polyphenols is undesirable (Mackintosh and Higgins, 2004) because tannins are able to irreversibly bind to kafirins, thus reducing digestibility and causing haze in beer (Emmambux and Taylor, 2003). In several brewing studies involving both un-malted and malted sorghum grain, white sorghum has been used (Dale *et al.*, 1989; Dufour *et al.*, 1992; Bajomo and Young, 1993, 1994; Olatunji *et al.*, 1993; Demuyakor *et al.*, 1994; Osorio-Morales *et al.,* 2000; Goode *et al.,* 2003). This said, in most of these studies, workers had not specified whether the sorghum they have used was tannin free or not.

Interestingly, in one study, Goode and Arendt (2003) used a red sorghum to produce a lager beer. It is, however, important to note that in this particular study, un-malted sorghum was only used at a 50% level of substitution for malted barley. The tannin levels as well as levels of other polyphenols (phenolic acids and flavonoids) of the red sorghum used were not stated. In a comparative study of white and red sorghum cultivars, Dufour *et al*. (1992) observed that generally the white sorghum varieties, which had lower polyphenol content, recorded a higher level of both α- and β-amylase after malting. This was partly attributed to the presence of polyphenols, which have been found to inactivate malt amylases (Daiber, 1975). A similar effect would possibly occur when industrial amylase enzymes are used when brewing with un-malted sorghum grain and this would result in poor saccharification. It is, however, also important to note that some white sorghum varieties may not perform optimally during brewing (Dufour *et al*, 1992). This is because the presence of other inhibitors such as phytic acid (Serraino *et al.*, 1985) could also possibly interfere with enzyme activity particularly where necessary steps are not taken to reduce levels of phytic acid.

In this study, hammer milled grist was mixed with mash-in liquor (tap water) to give an initial liquor to grist ratio of 1.5:1. This mixture was then mashed in a laboratory-scale BRF type mashing bath using an upward 3- step temperature-programmed infusion mash system with stands at 40°C for 420 min, 90°C for 45 min, and 60°C for 90 min (total mash cycle of 570 minutes). Different authors have used different liquor/grist ratios for different brewing materials (Table VII) and the ratio used in this study is lower than those used in other studies. Brewing with such concentrated mashes (liquor/grist <2.0:1) is a typical example of what is termed high gravity brewing (Briggs *et al.*, 2004). The liquor/grist ratio chosen in this study represents a rather extreme example of high gravity brewing. Based on information provided by Novozymes (Personal communication), the liquor/grist used in this study is used in commercial practice in Central and East Africa. The choice of temperature programmed infusion mash system in this study was to facilitate general as well as specific recommended optimal temperatures for enzyme function (i.e. proteolytic enzymes and amylolytic enzymes). The temperature at which the protein rest was conducted in this study falls within the range (40°C-60°C) used by other

workers (Taylor and Boyd, 1986; Dale *et al.*, 1989; Olatunji *et al.*, 1993; Demuyakor *et al.*, 1994; Agu and Palmer, 1998a; Osorio-Morales *et al.*, 2000; Goode *et al.*, 2002; Goode and Arendt, 2003; Goode *et al.*, 2003). The wide range in temperatures used is an indication of the varied optimal temperatures of different proteolytic enzymes from different sources i.e sorghum malt, plant and microbial sources. Initially, the protein rest was carried out at 55°C over a period of 45 min. During subsequent studies, the temperature for the protein rest was reduced to 40°C and conducted over a period of 7 hr. This was done in order to allow more protein degradation as a result of allowing the enzyme more contact time with the substrate at a lower temperature. The lower temperature also retarded enzyme denaturation. It was also thought that conducting a protein rest at a lower temperature would retard development of protein cross-linking (Ezeogu *et al.*, 2005), which would result in increased FAN as a result of more digestible protein being available.

Table XII: Liqour/Grist ratios and mashing systems used by various authors

Liqour/Grist	Mashing method	Main brewing material	Reference
2.0:1	Infusion	Malt, up to 50% extruded sorghum, barley and wheat.	Dale *et al* (1989)
3.0:1	Infusion	100% raw sorghum	Bajomo and Young (1993, 1994)
4.3:1	Infusion, decoction	80% raw sorghum, 20% malted barley or sorghum malt	Bajomo and Young (1992)
4.0:1	Infusion	100% sorghum malt	Olatunji *et al* (1993)
3.5:1	Decoction	66% sorghum malt, 34% malted barley	Demuyakor *et al* (1994)
2.0:1	Double extraction	90% raw sorghum,10% malted barley	Osorio-Morales *et al* (2000)
2.5:1	Infusion	50% raw sorghum, 50% malted barley	Goode and Arendt (2003)
3.0:1	Infusion	100% raw sorghum	Goode *et al* (2003)
2.2-2.4:1	Infusion	Malted barley, rice or maize	Briggs *et al* (2004)
3.5-5.0:1	Decoction	Malted barley	Briggs *et al* (2004)

Following the protein rest, a cooking step was conducted at 90°C for 45 min in order to gelatinize the sorghum starch. The temperature at which cooking was carried out, is considerably higher than temperatures that are employed in conventional brewing with barley malt where infusion mashing systems are used. Typical cooking temperatures seldom exceed 70°C when brewing with barley, with the optimal temperature range for obtaining maximum levels of fermentable sugars occurring between 60°C and 63°C (Briggs *et al.*, 2004). In order for complete and efficient (rapid) saccharification to take place, the starch has to first be gelatinized. For sorghum, this occurs at a much higher temperature (68-78˚C) (Taylor and Belton, 2002) than for barley (60-62°C) (Briggs *et al.*, 2004). Therefore, conducting the amylase rest at 90°C ensures that gelatinisation of the

sorghum starch is as near to completion as possible. It is important to note the temperature stability of enzymes (amylases) being used during mashing. If the temperature optimum of the amylase enzymes being employed is below the gelatinization temperature, there is a high risk of obtaining wort with incomplete saccharification. In this study, the amylase used was Termamyl. Termamyl is a heat stable endo-amylase which randomly hydrolyses 1,4-alpha-glucosidic linkages in amylose and amylopectin. It shows maximum activity at 90°C with an optimum pH between 6.5 and 7.5 in the presence of 30-60 ppm CaCl. Therefore, at a cooking temperature of 90°C, the enzyme was still highly thermal stable and was still able to function optimally. The addition of calcium chloride in the mash also helped improve the thermal stability of the amylase (Taylor and Daiber, 1988).

The ninhydrin assay is the official method of FAN determination stipulated by the European Brewery Convention. The ninhydrin method is a colorimetric protein estimation technique based on the total amino acid content of protein hydrolysate (Ahmed, 2005). This method is dependant on the reaction between the free α-amino acids and the yellow/amber coloured ninhydrin (2,2-dihydroxy-1,3-indanedione) solution, which forms a purple-coloured product (Sun Wang, 2007). In this reaction (Figure 10), an oxidative deamination reaction occurs during which two hydrogen atoms are removed from the α-amino acid, yielding an α-imino acid. At the same time, the ninhydrin is reduced and it loses an oxygen atom with the formation of a water molecule. The NH group in the α-imino acid is hydrolysed resulting in the formation of an α-keto-acid with the production of an ammonia molecule. The α-keto-acid undergoes further decarboxylation under heated conditions to form an aldehyde which has one less carbon atom than the original amino acid with the formation of a carbon dioxide molecule. These reactions yield the reduced ninhydrin and ammonia that are involved in the production of the purple coloured compound.

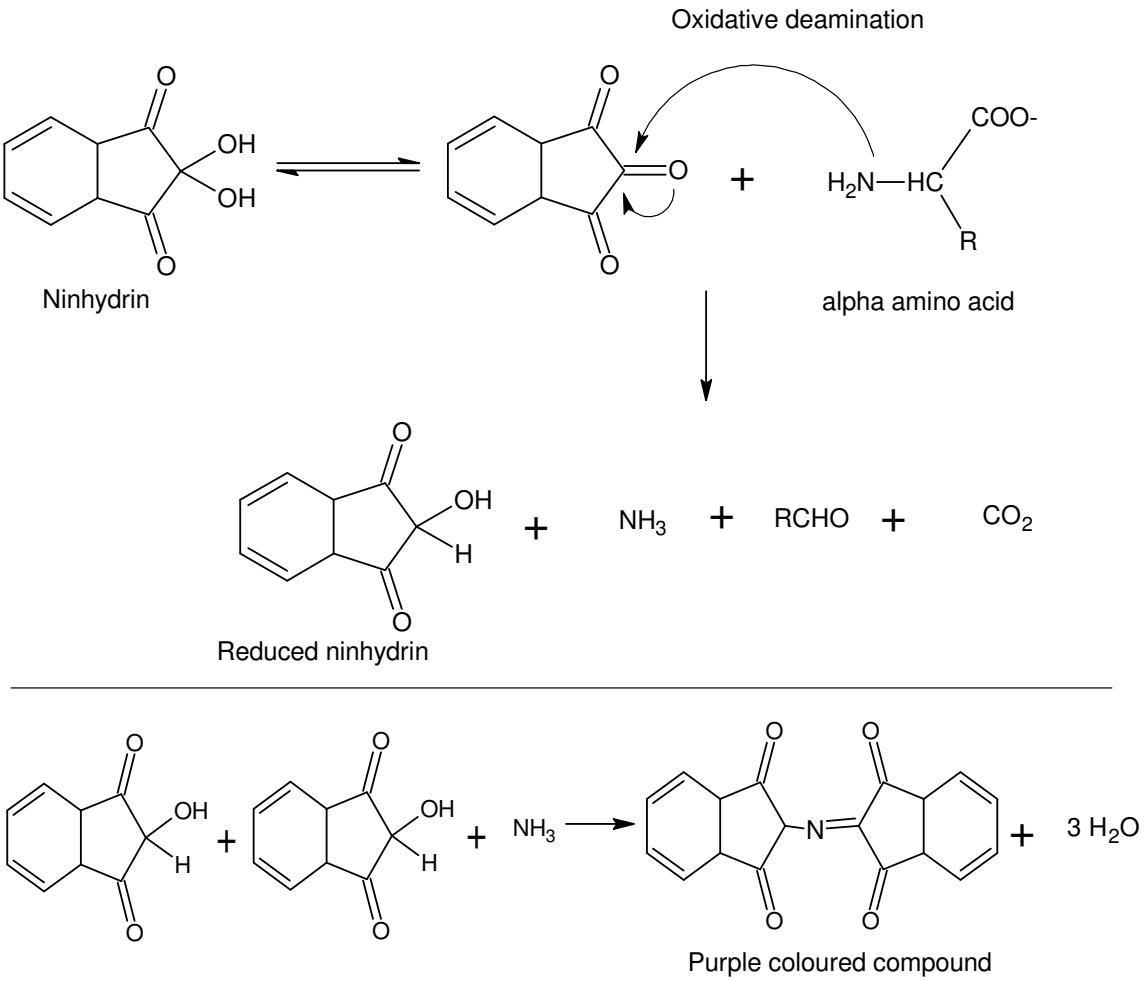

Figure 10: Reaction series of the ninhydrin assay (adapted from Ahmed, 2005).

The ninhydrin assay is a highly effective tool for measuring FAN. Even though ninhydrin reacts with the free α-amino group which is contained in all amino acids, peptides and proteins, the decarboxylation reaction which is an essential part of the reaction only occurs for free amino acids and not for peptides and proteins (Sun Wang, 2007). The ninhydrin assay is also a fairly sensitive method as it has a sensitivity range of 0.1–1 mg/mL (Ahmed, 2005).

One limitation of the ninhydrin assay is that it does not react with tertiary or aromatic amines (Sun Wang, 2007). This would result in the underestimation of the total amino acid composition of a sample that contains high levels of amino acids that possess aromatic groups. Another limitation of this method is that it is unable to detect other

important forms of FAN *viz*, ammonium ions and short chain peptides (dipeptides and tripeptides) (Lekkas *et al*, 2007). Essentially, the ninhydrin method does not give an entirely accurate estimation of 'true' FAN but gives a good indication of the presence of amino acids in wort. The inclusion of assays to quantify the levels of ammonia and short chain peptides in the wort could have given a more accurate reflection of the 'true' FAN present the wort. In work done by Agu (2002), a comparison was made between sorghum, maize and barley as brewing adjuncts. In this study, the ninhydrin method as well as the 2,4,6-trinitrobenzene sulphonic acid (TNBS) assay was used to quantify FAN. Wort produced with the sorghum adjuncts recorded the lowest level of FAN when the ninhydrin assay was used. However, when the TNBS assay was used, wort containing sorghum as an adjunct recorded the highest level of FAN. The TNBS method quantifies the presence of short peptides in addition to free amino acids present in wort (Agu and Palmer, 1998a). Agu (2002) in this study was able to establish that when mashing with sorghum, a high level of short peptides is produced. The ninhydrin assay therefore underestimates the true value of FAN to a certain extent. Notwithstanding, the limitations of the ninhydrin assay, it is the official approved method of the European Brewery Convention. The work by Taylor *et al*. (1985a) provides evidence to support the ninhydrin assay as a good indicator of yeast fermentation performance.

Microstructure analysis using transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM) was used to observe the physical effect of treatments on sorghum protein and starch. TEM is an imaging technique that employs electrons as opposed to light which is used in conventional light microscopy for image creation (Wikepedia, 2007). The electrons are focused onto the sample and are able to provide far greater image resolution than is possible with light microscopy (Wikepedia, 2007). Images in TEM are formed by a beam of electrons that is transmitted through a specimen and the image is magnified and directed to appear either on a fluorescent screen or a layer of photographic film (Wikepedia, 2007). Structural details of a sample in TEM are enhanced by the use of compounds of heavy metals such as osmium, lead or uranium. These compounds selectively deposit electron dense atoms in the sample and the dense

nuclei of their atoms scatter the electrons out of the optical path. The electrons that remain in the beam are then detected (Wikepedia, 2007).

The main advantage of TEM is the high image resolution achieved. This enabled very clear observation of structural degradation of the sorghum endosperm protein matrix (protein bodies and glutelin) in this study. Preparation of samples for TEM is very laborious; hence, the risk of structural changes emanating from sample preparation is relatively high. The potential of sample damage by the electron beam is also high particularly in the case of biological materials (Wikipedia, 2007) such as sorghum protein. There is also a high possibility of obtaining images that are not truly representative of the sample under observation because the field of view is etremely small.

Confocal laser scanning microscopy (CLSM) is a technique that uses light for image creation as does light microscopy. In CLSM, the source of light used is laser. In CLSM, contrary to conventional light microscopy where object-to-image transformation takes place simultaneously and parallel for all object points, the specimen in CLSM is irradiated (fluoresced) in a point-wise fashion, i.e. serially, and the physical interaction between the laser light and the specimen detail irradiated is measured point by point as well (Wilhelm *et al.*, 2003). Information about the entire specimen is obtained by moving the specimen relative to the laser beam (scanning). During the scanning process, individual 'slices' are obtained and with the aid of computer software, these 'slices' may be reconstructed to give a 3-D view of the specimen. CLSM enables one to observe the structure of typically 'thick' samples via fluorescence. Sample preparation in this technique is minimal; therefore interference to the native structure of samples being observed is also kept minimal.

In this study, the auto-fluorescence property of protein was used. In native sorghum endosperm, a 'chicken-mesh wire' like structure was observed, where the protein fluoresced and the starch did not. This showed the typical arrangement of starch granules and endosperm protein matrix (i.e starch granules enveloped by the sorghum endosperm

matrix protein) within the vitreous endosperm of the sorghum grain. It was hypothesized that when gelatinised, an expansion of the starch granules would be observed. In samples that were treated with the proteolytic enzyme and KMS, more extensive expansion of starch granules could have been observed because the break-down of the endosperm matrix protein would not have as an effective a restrictive effect on starch granule expansion. It was thought that with these observations, the degree to which starch granules expanded (underwent gelatinization) could be quantified (physically measured as the diameter across starch granules) and compared for the different treatments.

Choi *et al*. (2008) used CLSM to study the effect of cooking and sodium bisulphite on in-vitro digestibility of waxy sorghum flour. In their study, they used a double-labelling technique to monitor both the protein and starch in sorghum. With this technique, they were able to fluoresc both the starch and protein using the periodic acid Schiff reaction and fluorescamine dye respectively. Using the double labeling technique could have enabled better observation of the extent of starch granule disruption in this study.

Physical measurement of the diameter across the starch granules could have provided valuable information with regard to the extent of expansion of the starch granules. However, using CLSM as a technique to achieve this may be questionable. It is important to consider that the size of sorghum starch granules is not constant within a single grain and may range between 2 µm and 30 µm (Taylor and Belton, 2002). Another important consideration is the starch and protein matrix within the endosperm exist in a 3-D structure. If they were arranged in a 2-D structure and the starch granules were of equal size, it would be possible to 'slice' the sample using CLSM at exactly the same level and measure the diameter. The random distribution of the starch granules within the protein matrix in 3-D would make it almost impossible to accurately measure the diameter of granules. This is because granules could be sliced at different positions in the y-dimension.

Notwithstanding the limitations discussed above, CLSM was able to provide very valuable information regarding the effect of the proteolytic enzyme and KMS on starch

granule gelatinization. Interpretation of the results observed made more use of the size of the structures in the samples observed. In both the uncooked and cooked untreated samples, large sized structures showing the characteristic protein matrix enveloped starch granules were observed. Treatment with the proteolytic enzyme and KMS revealed smaller structures that were not similar in appearance to the untreated samples. It was considered that the smaller structures were a result of the action of proteolytic enzyme and KMS weakening the protein matrix. As a result, more extensive starch granule expansion occurred to the extent that the native structure of some of the starch granules became totally disrupted resulting in fragmentation of the protein matrix.

SDS-PAGE is an electrophoretic protein analysis method. In this method, the test sample is treated with sodium dodecyl sulphate (SDS), which is an anionic detergent (Ahmed, 2005). The SDS molecules are able to mask the surface charge of the native proteins and create a net negative charge caused by the sulphate groups on the SDS molecule (Figure 11).As a result, the charge/size ratio is equal for all proteins contained in a heterogeneous mixture and separation can be achieved on the basis of molecular size.

The separation of polypeptides is achieved in a porous media (polyacrylamide gel) in which an electric field exists. The porosity of the polyacrylamide gel results in a molecular sieving effect where low molecular polypeptides travel faster in the gel, and those of high molecular weight travel slower. The molecular weights of proteins contained within the test sample are estimated by running appropriate standard proteins of known molecular weights on the same gel.

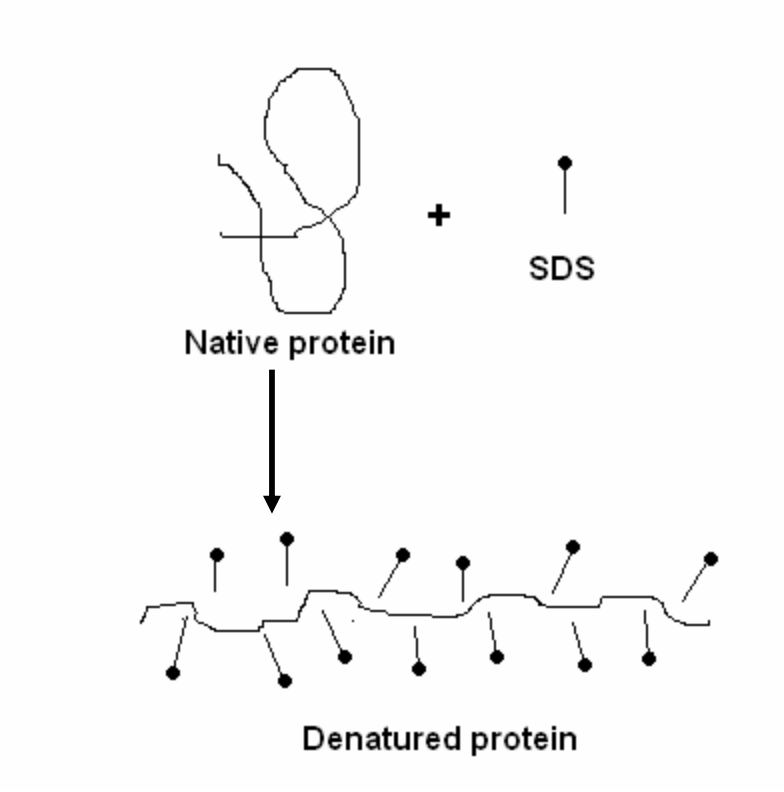

Figure 11: Model showing protein denaturation with sodium dodecyl sulphate and the creation of a highly negative charged molecule (adapted from Ahmed, 2005).

SDS-PAGE is the most commonly used method to separate and identify heterogeneous polypeptides of prolamins. Many workers have employed this method in the study of sorghum prolamins (Daiber and Taylor, 1982; Taylor, 1983; Taylor and Schüssler, 1984a; Taylor *et al*., 1984b; Taylor *et al.,* 1984c; Taylor et al., 1985b; Taylor and Schüssler, 1986; Taylor and Evans, 1989; Evans *et* al., 1987; Watterson *et al*., 1993; Subramanian *et al*., 1995; Bean *et al*., 2006). It has also been used to determine prolamins in beer (Linko *et al*., 1989; Ellis *et al*., 1990; Sheehan and Skerrit, 1997; Kanerva *et al.*, 2005). Considering that up to 80% of the protein in sorghum is accounted for by kafirin, using SDS-PAGE in this study provided a powerful tool to determine the presence of kafirin and its effect on the FAN. This provided further information on the efficacy of the proteolytic enzyme and mashing conditions used in the study. However, SDS-PAGE was not able to quantify the respective kafirin fractions present. Quantification of the

respective kafirin sub units could have provided more information to ascertain whether cross-linking of kafirin (particularly β- and γ-kafirin on the periphery of the protein bodies) (Hamaker *et al*. 1987; Oria *et al*. 1995b) did make a significant contribution to the FAN levels reported by limiting access of the enzyme to the more digestible α-kafirin which is present in the greatest quantity (Belton and Taylor, 2002). The inclusion of a method known as densitometric scanning could have provided quantitative information on the electrophoresis.

The amino acid composition of FAN in sorghum wort could be considered equally as important as the quantity of FAN present. As stated in the literature review, the findings of a study by Bajomo and Young (1994) could possibly suggest that the overall quality of FAN (i.e. amino acid composition) may be more vital than the quantity. In their study, they were able to successfully re-pitch a yeast crop over five consecutive fermentations without any deterioration in the physiological condition of the yeast. With this considered, it was thought that determining the amino acid spectrum of the wort was essential. The amino acid analysis was also able to quantify the total amount of the different classes (Table. XIII) of amino acids that are considered important in brewing. Amino acids are classified in groups A to D on the basis of the rate at which they are assimilated by yeast during fermentation (Jones and Pierce, 1964).

Although the FAN level produced in sorghum grain is low, there is evidence to suggest that the overall quality of the FAN (i.e. amino acid composition) is relatively better than that of barley (Taylor and Boyd, 1986; Bajomo and Young 1993, 1994). The results in this study show that the proportion of class A amino acids (Jones and Pierce, 1964) is higher in sorghum than in malted barley. This concurs with the findings of Taylor and Boyd (1986) and Bajomo and Young (1993, 1994). In barley, proline which is classified under group D (little or no absorption by yeast) in Jones and Pierce's (1964) classification system, contributes 22.4 to 31% of the total amino acid composition (Miflin and Shewry, 1978 according to Taylor and Boyd, 1986). Proline metabolism requires oxygen and a functioning electron chain (Wang and Brandriss, 1987). The anaerobic nature of the fermentation process thus makes it impossible for proline to be assimilated

by yeast. In this study, it was established that after mashing, sorghum wort only contained between 5.7 and 7.3% proline/ 100 g protein. Bajomo and Young (1993) and more recently Lekkas *et al*. (2007) have also shown how important a role the ammonia and oligopeptide (di- and tripeptides) components of FAN play in yeast fermentation. It has already been suggested that these parameters should also have been determined in this study. A comparative study should have also been done to determine if the amino acid composition in the wort mashed with a 420 minute protein rest at 40°C was better than that of the wort mashed with a 45 minute protein rest at 55°C.

Table XIII: Classification of wort amino acids according to Jones and Pierce (1964) based on assimilation by yeast (adapted from Lekkas *et al*, 2007).

Group A (Fast absorption)	Group B (Intermediate absorption)	Group C (Slow absorption)	Group D (Little or no absorption)
Glutamic acid	Valine	Glycine	Proline
Aspartic acid	Methionine	Phenylalanine	
Asparagine	Leucine	Tyrosine	
Glutamine	Isoleucine	Tryptophan	
Serine	Histidine	Alanine	
Threonine		Ammonia	
Lysine			
Arginine			

The PICO.TAG method (Bidlingmeyer *et al.*, 1984) was used to determine the amino acids present in the wort. This method is based on the principle of reversed phase chromatography with pre-column derivatisation and subsequent hydrolysis. One of the biggest drawbacks with this method is the variability in the intrinsic liability of different amino acids to undergo hydrolysis (West *et al.*, 1996). Cystine and tryptophan are susceptible to total destruction, while glutamine and asparagine undergo deamidation, which results in their transformation to glutamic acid and aspartic acid respectively (West *et al*., 1996). Therefore, the content of these four amino acids is reported as glutamine

plus glutamic acid, and asparagine plus aspartic acid. If the total kafirin protein fraction were to be considered on its own, the reporting of the four (glutamine, glutamic acid, asparagines and aspartic acid) amino acids as they were reported i.e. glutamine plus glutamic acid, and asparagine plus aspartic acid may not have a major impact on the value of results obtained. Aspartic acid and glutamic acid contribute a very small quantity compared to asparagine and glutamine (See Table I). However, the contribution of these amino acids in the albumin and glutelin protein fractions is not known because in literature, they are also reported as glutamine plus glutamic acid, and asparagine plus aspartic acid. However, considering that the stated amino acids are all classified under group A, this does not make a substantial difference in respect of FAN.

3.2. Efficacy of proteolytic enzymes on sorghum protein degradation

The mashing process, which arguably constitutes the most vital part of brewing, is essentially a digestion (hydrolysis) process. A myriad of enzymes is required to bring the grain components into solution. When malted grain is used (e.g. barley or sorghum), the enzymes that are required during the mashing process generally develop during malting. Generally when barley malt is used, it is not absolutely vital to incorporate exogenous enzymes during mashing (Briggs *et al.,* 2004). When mashing with malted sorghum (particularly on a commercial basis), exogenous enzymes are often employed to augment malt enzyme activity. One of the reasons cited for this is the low levels of β- amylase present in sorghum malt (Dufour *et al.*, 1992; Taylor and Belton, 2002) compared to malted barley. This point amongst others has been used by some scientists (Dale *et al.,* 1990; Bajomo and Young, 1992; Agu *et al.*, 1995; Hallgren, 1995; Agu and Palmer, 1998a; Goode *et al.*, 2002) to justify the use of unmalted sorghum grain with exogenous enzymes as a more efficient approach to brewing with sorghum.

As stated in the literature review, mashing with unmalted sorghum and exogenous enzymes at laboratory scale has been characterized by the use of excessively high levels of proteolytic enzymes in order to produce FAN levels (100-140 mg/L) sufficient to support efficient fermentation (Goode *et al.,* 2003). In most cases, despite the high

proteolytic enzyme dosages being used, levels of FAN produced were still considerably lower than the standard for brewing (Goode *et al.* 2003). A very high enzyme dosage was used in this study (1 g enzyme/ 100 g of grist). When one considers that sorghum typically contains approximately 11.3% protein (Serna-Saldivar and Rooney, 1995) it is apparent that the dosage rate is essentially 1 g enzyme/ 11 g protein, provided that the commercial proteolytic enzyme preparations consisted entirely of proteolytic enzymes. Even with such a high dosage, none of the enzymes used in this study were able to produce the lower limit (i.e. 100 mg/L) of recommended FAN levels when using the prescribed mashing regime (i.e. protein rest for 45 min). It was only after exposure to an extended protein rest (420 min) and the inclusion of KMS did one of the enzymes (Flavourzyme) produce a sufficiently high level of FAN. The results presented in this study are in agreement with the findings of Albini *et al.* (1987), Bajomo and Young (1993) and Goode *et al.* (2003), which also showed that unacceptably high levels of commercial proteolytic enzymes were required to produce wort with FAN levels deemed adequate to support effective yeast activity.

Three major factors need to be considered when evaluating the efficacy of the proteolytic enzymes used in this study. These factors are: i). Properties of the enzymes; ii). Mashing environment; iii). The substrate.

The intrinsic properties of the enzymes being used have a very large bearing on their effectiveness to hydrolyse sorghum proteins. A very important consideration in this regard is the suitability of the enzyme for a particular application. At laboratory scale, adjusting mashing conditions to suit enzyme functionality is relatively easy. At commercial level, making adjustments to normal processing conditions has greater implications (cost and final product quality), thus enzymes are selected to suit existing processes. Radial diffusion tests (Albini *et al*, 1987) have revealed that commercial enzyme preparations are rarely a single enzyme. They often consist of several different enzyme activities. In some instances, it could be possible that the activity for which a particular enzyme preparation is marketed merely constitutes a side activity and may therefore exist in a low concentration of the total enzyme preparation. This may work for

some substrates (i.e. protein of other cereals which may be easier to digest) but not necessarily on others. Application of proteolytic enzymes that have proved suitable for use in some processes where proteolysis forms an integral part, may not work for others. In this particular case, enzymes such as Neutrase which have been developed for the baking industry have not performed optimally with regard to sorghum protein hydrolysis. Another very important consideration discussed earlier is the exopeptidase/endopeptidase ratio in the proteolytic enzymes. A high ratio which would mean a higher presence of exopeptidase enzymes is essential if optimal release of free amino acids is to be achieved. A thorough understanding (specificity, pH and temperature optima, co-factors etc.) of proteolytic enzymes to be applied in sorghum brewing is necessary if extensive sorghum protein degradation is to be achieved.

3.2. Effect of mashing conditions on sorghum protein degradation and wort properties.

As stated earlier, making adjustments to mashing conditions to suit particular enzymes to sorghum brewing has implications. At commercial level, enzymes should be developed to suit mashing conditions. The major considerations that have to be made regarding the mashing environment are the pH and the temperature of the mashing system; the presence of co-factors and inhibitors in the mashing system; and the time the enzyme is exposed to the mashing system. With this knowledge, suitable enzymes may be identified that may work optimally in sorghum mashing systems

A thorough understanding of the substrate the enzyme is required to act on is very important. Studies have been conducted to determine the primary structure (i.e. amino acid sequence) of the various kafirin fractions. The primary structure of α-, β- and γ kafirin have been deduced based on their similarity to the corresponding prolamin fractions in zein (DeRose *et al.*, 1989; De Barros *et al.*, 1991; Shull *et al.*, 1991). This knowledge would greatly contribute to the isolation and subsequent development of commercial enzymes suited to sorghum brewing. Extensive studies on factors affecting the digestibility of sorghum proteins have been done and have been reviewed by Duodu

et al., (2003). This knowledge also provides a very valuable tool into understanding why there has been very little success in producing sorghum worts with appreciable levels of FAN.

The reaction rate of enzyme catalysed reactions is directly proportional to the concentration of the substrate (Whitaker, 1972). The TEM and SDS-PAGE results in this study suggest there was a substantial amount of undigested protein present after the protein rest. If the enzyme present in the mashing system was still active further degradation of the remaining protein would have happened. The SDS-PAGE results revealed that formation of enzyme resistant, cross-linked polymers probably occurred. In the treatment with added KMS, the presence of the polymeric kafirin (dimers) was reduced, as shown by the low intensity of the band (≈ 45 k) representing dimers. This was accompanied by an increase in the intensity of the bands representing the monomeric α- and γ-kafirins. This suggests that with this particular treatment, there was more available substrate that was not broken down probably because the enzyme was inactivated.

After employing a prescribed mashing regime which had a protein rest conducted at 55°C for 45 minutes, it was observed that the most successful enzymes used in this study produced just over half the recommended minimum FAN levels. Three enzymes were selected for further work. Flavourzyme and Papain were selected based on their relatively good performance, and Neutrase was selected based on the reported success of neutral proteases in similar studies (Dale *et al*., 1990; Agu and Palmer, 1998a). Although Bioprotease P Conc recorded the highest level of FAN, it was not selected for further work because its highly concentrated form would have rendered it economically unviable.

The three selected enzymes were used in mashing regimes where the temperature of the 45 minute protein rest was varied (i.e. 50°C, 55°C and 60°C). With the exception of Neutrase, the other enzymes performed best at 55°C, whereas Neutrase performed best at 50°C. The introduction of KMS into the mashing system resulted in an increase in FAN at all mashing temperatures for both Neutrase and Flavourzyme treated samples. This

could be attributed to the reduction of disulphide bonds in the protein structure because of the presence of KMS (Hamaker *et al.*, 1986). This presumably opens up the structure of the protein and allows the proteolytic enzymes access to bonds that were not available before. In the sample treated with Papain, no significant increase in FAN was observed. It is highly likely that the activity of Papain was negatively affected by KMS because it is classified as a thiol/ sulphydryl protease (Fersht, 1985). The structure of papain is stabilized by 3 disulphide bonds (Whitaker, 1972). As stated, it is possible that the KMS could have reduced the stabilising disulphide bonds present in papain.

A long protein rest at a lower temperature increased FAN levels substantially. Even in the control which contained no added exogenous proteolytic enzyme, a 77% increase in FAN was observed. This increase in FAN was because of the presence of endogenous endopeptidase which has been reported in unmalted sorghum grain (Evans and Taylor, 1990b). Though this figure (77%) may seem substantial, when one considers that the initial FAN content in sorghum grain is about 20 mg/ 100 g grist, an increase to a final value of 35.5 mg/ 100 g grist (77% increase) was not very substantial in brewing terms. Upon addition of the proteolytic enzyme, a final FAN value of 78.1 mg/ 100 g grist was reached when using the 420 minute protein rest. This represented an increase of almost 290%. When compared to the initial 45 minute protein rest at 55°C where a final FAN value of 52.2 mg/ 100 g grist was achieved, it is evident that allowing the enzyme to act over a longer period at a lower temperature was more effective. The addition of KMS further increased the FAN content to 93.4 mg / 100 g grist, representing a total increase of 375%. This provided some evidence to support the notion that protein cross-linking contributed to the poor digestibility of sorghum protein and subsequent low FAN levels.

Apart from FAN, other wort properties that were studied were the hot water extract (HWE) and starch presence. Addition of the proteolytic enzyme (Flavourzyme) during the protein rest resulted in a 2°P significant increase in HWE. Overall this increment represented an 8% increase in wort solids. Considering that a considerable portion of the HWE is composed of sugars (Briggs *et al.,* 2004), it would be safe to presume that a considerable portion of HWE is a result of starch hydrolysis. The degradation of the

protein matrix which envelopes the starch granules had a positive effect on HWE. The protective barrier which the protein matrix forms around the starch granules loses its integrity thus allowing amylases easier access to the starch granules. The degradation of the protein matrix could have also promoted more extensive pasting. Pasting is the phenomenon which follows gelatinization and involves starch granule swelling, the leaching out of molecular components from the granule and eventually the total disruption of the starch granule (Atwell *et al*., 1988). The disrupted starch granules became more prone to hydrolysis by amylases and thus increased the HWE level. The CLSM results suggest that the integrity of the protein matrix could have been damaged and permitted more extensive starch granule swelling eventually leading to total disruption. The findings of a number of studies on sorghum starch digestibility (Rooney and Pflugfelder, 1986; Chandrashekar and Kirleis, 1988; Ezeogu *et al.*, 2005) have suggested that the protein matrix enveloping the starch granules has a limiting effect on starch hydrolysis. This limiting effect is further enhanced by wet cooking methods which further limit the hydrolysis of the enveloping protein because of enhanced disulphide bond formation.

The addition of KMS did not significantly further increase the extract levels. Considering that KMS facilitated further breakdown of the protein, one would have expected a further increase in HWE to occur. The TEM results could provide a possible explanation as to why a further increase in HWE was not observed. There was still a high presence of only partially degraded protein bodies in the samples that were treated with the proteolytic enzyme and KMS. The KMS treatment could have had a more profound effect on individual protein bodies but did not necessarily have a very big overall effect on the protein envelope surrounding the starch granules.

The worts produced from the Ceremix, and the Ceremix plus Flavourzyme treated samples did test positive for starch. It was surprising to see that the sample treated with Ceremix plus Flavourzyme plus KMS tested negative for starch even though the addition of KMS did not provide any further significant increase in HWE. Using a quantitative starch test could have provided a better comparison as opposed to using the iodine starch

test which is qualitative. The iodine starch test is a highly subjective test and it is possible that a subtle colour change to indicate the presence of starch could not have been noticed.

A comparative study of the adapted mashing conditions and the prescribed mashing conditions should be done to determine whether there were any significant improvements in wort properties. Further work to determine the fermentability of the wort, as well as the properties of beer produced using the adapted mashing conditions should also be conducted.

Addition of KMS and adoption of a long protein rest at a low temperature has been shown to improve the FAN level of sorghum grain wort. It cannot be said for sure whether there was an improvement in the other wort properties determined in this study as there is no basis for comparison. It is also difficult to make a direct comparison with work done by other workers with reference to HWE. The brewing gravities used in other studies and that used in this study differ significantly (a very high gravity was used in this study).

3.3 Practicality of employing optimized mashing conditions commercially.

Before recommending the optimized mashing conditions used in this study for commercial use, a number of cost benefit considerations need to be made. KMS is routinely used as an aid to food processing. It is routinely used in grain wet milling operations as a reducing agent and is therefore approved for use in foods. The cost of KMS (at a dosage rate of 0.1% (w/w) sorghum) is also relatively cheap and would therefore not contribute significantly to brewing costs. Based on a retail price of US$ 870.00 per 50 kg (www.spectrumchemical.com), it would cost US$ 17.40 worth of KMS per ton of sorghum in order to improve FAN in sorghum grain brewing. Therefore, KMS provides a suitable and economic solution to improving FAN in the sorghum grain brewing process. However, one would need to consider the effect that the KMS could have on the sensory properties of the final beer. In this study, it was observed that the addition of KMS to the mashing system produced wort tainted with a typical sulphur dioxide smell. Nevertheless, it should be considered that in a complete brewing cycle,

there is a wort boiling step involved (Briggs *et al*., 2004) during which the KMS smell could be eliminated.

Using a long protein rest at a lower temperature may necessitate capital investment in the form of additional infrastructure. This would probably be in the form of steeping tanks in which the low temperature long time protein rest would be carried out. In order to maintain the temperature of the protein rest at 40°C, these steeping tanks should ideally be jacketed vessels. The energy required to raise the temperature to 40°C may be obtained from 'recycled' steam that has been used in other heating processes such as wort boiling, provided it is not used in any other processes. The additional energy that will be required to pump the mash from the steeping tanks to the mash tun has to be factored in as well.

In brewhouse operations, the number of brews that can be carried out in one day is a very important factor (Briggs *et al*., 2004). If a brewhouse were to operate on a 24 hr basis using the mashing regime that was prescribed for this study, it would theoretically be able to run 6 brews per day with each brew lasting 215 min. The optimized mashing procedure used in this study lasted 640 min. The maximum number of brews that could theoretically be produced in a 24 hr period is only 2. If a brewery were to produce the an equal amount of output using the 640 min mashing regime as when using a 215 min mashing regime using the same brewing gravity, the capacity of the brewhouse would have to be increased 3-fold. This would entail a substantial capital investment which may not necessarily provide a cost-benefit to the brewer.

Though the long time low temperature protein rest was able to increase the level of FAN to an almost acceptable level in brewing terms, the long time period may be a concern when this method is employed at a commercial level. One also needs to consider that the enzyme dosage rate used was also extremely high and possibly uneconomical.

CHAPTER 4: CONCLUSIONS AND RECOMMENDATIONS

The exogenous proteolytic enzymes investigated in this study are not effective enough to raise FAN levels in sorghum wort to recommended brewing levels. There are two likely reasons for this. Disulphide cross-linking which occurs in sorghum protein (primarily kafirin) reduces the digestibility of the sorghum proteins by forming enzyme resistant protein polymers. KMS is able to alleviate to some extent the negative effects of disulphide cross-linking on FAN. It is also highly likely that the proteolytic enzyme preparations studied were primarily composed of endopeptidases. Endopeptidase activity preferentially occurs in the inner regions of the polypeptide chain away from the N and C termini. It is thus likely that other products of protein degradation (i.e. peptides) were formed in greater amounts than free amino acids, as suggested by the extensive physical damage to the endosperm matrix protein observed by TEM and CLSM. KMS can be used to improve FAN in sorghum wort because it is able to reduce inter- and intra- molecular disulphide bonds in proteins. This opens up the protein structure more and provides more peptide bonds at which proteolytic enzymes are able to cleave.

Employing a low temperature protein rest over an extended period is an effective way of increasing FAN. The low mashing temperature retards the rate at which the enzyme denatures. It could also possibly reduce the rate at which disulphide bonds are formed in the protein.

The increase in HWE levels when Flavourzyme was used in the mashing system suggest that sorghum starch degradation is affected by the sorghum endosperm protein matrix. It has been postulated that the endosperm protein which envelopes the sorghum starch granules provides a protective coating around the starch granules which prevents amylases from accessing the starch granules. Therefore, the increase in endosperm protein hydrolysis resulted in a concomitant increase in starch hydrolysis, as evidenced by the increase in HWE.

A more thorough understanding of commercial proteases with regard to their classification, specifity and optimal environmental conditions is required if they are to be used for the purpose of improving FAN levels in sorghum brewing. The exopeptidase/endopeptidase ratio in proteolytic enzymes intended for use in sorghum brewing should be high if a high quantity of free amino acids (FAN) is desired in sorghum wort.

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