African Journal of Biotechnology Vol. 10(27), pp. 5355-5369, 15 June, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB09.1594
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

The effects of calcium regulation of endosperm reserve protein mobilization of the Nigeria sorghum cultivars, ICSV 400 and KSV 8 during malting

Bartholomew N Okolo[1]*, Anene N Moneke[1], Lewis I. Ezeogu[1] and Francis S. Ire[2]

[1]Brewing Science Laboratory, Department of Microbiology, University of Nigeria, Nsukka, Nigeria.
[2]Department of Microbiology, University of Port Harcourt, Rivers State, Nigeria.

Accepted 28 April, 2011

The effects of steep liquor calcium ion on sorghum endosperm reserve protein mobilization were evaluated using two improved Nigeria sorghum cultivars (ICSV 400 and KSV 8). The key protein modification factors evaluated were free amino nitrogen (FAN), total non protein nitrogen (TNPN) and soluble protein of cold water extract (CWS-P). Ca^{2+} treatment highly significantly ($P < 0.001$) repressed FAN development in both sorghum cultivars ICSV 400 and KSV 8. TNPN accumulation significantly enhanced in ICSV 400 by Ca^{2+} treatment in contrast to KSV8 malts which showed 23 to 69% repression. Similarly, Ca^{2+} treatment was effective in stimulating peptide accumulation in ICSV 400 at all levels of treatment indicating that the enhancement of TNPN accumulation in this cultivar was derived mainly from the stimulation of peptide accumulation. KSV 8 in contrast showed highly significant repression of peptide accumulation. Protein solubilisation, soluble protein accumulation and cold water soluble protein modification in both cultivars were all highly significantly repressed by Ca^{2+} treatment; although, ICSV 400 appeared to be better modified. Carboxypeptidase development was stimulated significantly by Ca^{2+} treatment in both cultivars. Existence of multiple high points in carboxypeptidase activity suggests heterogeneity of this enzyme in sorghum while Ca^{2+} treatment caused reduced proteinase development in ICSV 400, the enzyme activity was enhance in KSV 8 albeit marginally.

Key words: Sorghum malt, steep water Ca^{2+} treatment, modification, free amino nitrogen, total non protein nitrogen, carboxypeptidase.

INTRODUCTION

Sorghum (*Sorghum bicolor* (L) Moench) is a cultivated tropical grass which originated in Africa about 3000 to 5000 years ago (Taylor and Belton, 2002). Sorghum like other cereal grains such as barley, maize, rice and wheat belong to the grass family – the Gramineae and it is widely grown in other parts of the countries. Different cultivars are found in different regions depending on the climate. It is an important cereal crop to humankind ranking fifth in terms of overall cereal production after wheat, rice, maize and barley (FAO, 2004; Taylor and Belton, 2002). Among other applications, sorghum is used in the production of traditional opaque beer, non-alcoholic beverages in developing countries and until recently, production of lager-type clear bear (Taylor and Dewar, 2001; Palmer, 1989). Barley is traditionally the cereal chosen for malting in order to develop enzymes (Kuntz and Bamforth, 2007). In Nigeria, where attempts to cultivate barley have met with little success, the high cost of importing barley malt, in conjunction with the rising demand for European-type lager, has forced the use of local cereals particularly sorghum, as a malting and brewing grain. Thus, sorghum has successfully replaced barley in Nigeria as the primary source of extract for brewing (Okolo and Ezeogu, 1996). Ca^{2+}, Mg^{2+} and $^{K+}$, play important roles in the regulation of endosperm mobilization during cereal grain germination. Among these ions, calcium ion has been associated with important physiological functions during germination of

*Corresponding author. E-mail: annymoneke@yahoo.com. Tel: +234 803 3357 734.

Abbreviations: FAN, TNPN etc

cereals (Morrall et al., 1986; Stewart et al., 1988). The mobilization of endosperm reserve proteins during malting is critical to efficient grain structure modification and malt quality development (Bathgate and Palmer, 1974; Glennie et al., 1983; Jones, 1969; Okolo and Ezeogu, 1995a; Okolo and Ezeogu, 1996; Slack et al., 1979; Taylor, 1983). Numerous earlier workers have recognized manipulation of steep treatment as an effective strategy in modulation of cereal storage protein modification (Axcell et al., 1983; Brookes et al., 1976; Chrispeels and Varner, 1967; Jones and Jacobsen, 1983; Macleod, 1978; Ogbonna et al., 2003; Palmer, 1989; Pollock, 1962; Varner and Mense, 1972). These workers demonstrated that cations particularly calcium ion exerted a strong regulatory influence on barley endosperm protein mobilization during malting. Although, the site and molecular mechanism of this Ca^{2+} effect is still not resolved (Beck and Ziegler, 1989; Fincher, 1989), it is believed to participate in gibberellic acid induced stimulation of synthesis and release of barley endosperm protein hydrolyzing enzymes and indeed other hydrolases (Bush et al., 1986; Chrispeels and Varner, 1967; Jones and Jacobsen, 1983; Varner and Mense, 1972).

However, with the successful replacement of barley with sorghum in brewing processes, it becomes necessary to evaluate the influence of calcium ion in regulating sorghum endosperm protein mobilization during malting. Ezeogu and Okolo (1995b) and Okolo et al. (2010) reported the repression of carbohydrate modification when sorghum steeps were subjected to calcium treatment of sorghum steeps was shown to repress carbohydrate modification. Previous studies (Okolo and Ezeogu, 1996) revealed that two improved Nigerian sorghum cultivars ICSV 400 and KSV 8 possess high malting qualities which compared well with barley. Therefore, in this study, the two improved Nigerian sorghum cultivars ICSV 400 and KSV 8 with good malting quality were used to assess the role of calcium in regulation of endosperm protein mobilization in sorghum.

MATERIALS AND METHODS

Grain samples

Grains from two improved Nigeria sorghum cultivars ICSV 400 and KSV 8 were obtained from the National Seeds Service, Zaria, Nigeria and used in this study. All the grains had good germinative energies and were not water sensitive (Ezeogu and Okolo, 1994).

Sorghum malting

Grains were malted as described previously (Ezeogu and Okolo, 1995b) using double deionized water steeps as controls. Steeping was for a total of 53 h at 30°C with warm water (40°C) final steep lasting 6 h. Varying concentrations of $CaCl_2$ in the range of 100 to 500 ppm were added to the steep water to verify the effect of Ca^{2+} on protein modification in the grains. Grains were processed at the end of steeping, germinating for 4 days and processed for analyses as described previously (Ezeogu and Okolo, 1995a).

Analyses

Cold water extract and cold water soluble protein

Cold water extract (CWE) of the sorghum malts was determined according to Holmes (1991) modification of the recommended methods of analysis # 25 (1986). Soluble protein of cold water extract (CWS-protein) was measured using a modification of Coomassie brilliant blue method of Lewis et al. (1979) as described by Holmes (1991) using bovine serum albumen as standard. CWS-protein values were expressed as mg CWS-protein percent dry malt.

Free α-amino nitrogen

Free α-amino nitrogen (FAN) of the sorghum malts was determined using the methods of Taylor and Boyd (1986). Samples of malt (1.0 g) were extracted with 40 ml of 5% trichloroacetic acid at 30°C for 1 h. After centrifugation for 25 min at 4000 g, 1 ml of the clear supernatant was diluted to 25 ml in distilled water and FAN was measured using the EBC (1975) ninhydrin method.

Total non-protein nitrogen

The supernatant from FAN determination was used for total non-protein nitrogen (TNPN) determination. Nitrogen in the extract was measured using Kjeldahl digestion procedure as modified by Hach et al. (1987). The extract 2.5 ml was dispensed into 100 ml clean volumetric flask and digested using 3 ml concentrated H_2SO_4 with 12 ml of 50% H_2O_2 added via a capillary flow funnel attached to the fractionating head of the digesting vigreux column. The digest was made up to 100 ml with deionized water on cooling and the nitrogen determined spectrophotometrically as ammonia using Nessler's reagent.

Carboxypeptidase and proteinase assays

The assay procedure for these enzymes has been described elsewhere (Okolo and Ezeogu, 1995; Ogbonna et al., 2003).

Statistical analysis

All analyses were performed in triplicate. The influence of steep water Ca^{2+} treatment on the grain endosperm storage protein modification indicators examined were resolved by two-way analyses of variance and the Kruskal-Wallis test (Cohen, 1988). Correlation analyses were also performed to determine how the protein modification indices varied in relation to the level of Ca^{2+} treatment. Means that differed significantly were shown by the t-test and least significant difference (LSD) tests.

RESULTS AND DISCUSSION

The effects of steep liquor $CaCl_2$ concentration and cultivar on free alpha amino nitrogen (FAN) development in the two Nigerian sorghum cultivars ICSV 400 and KSV 8 were evaluated. The results expressed as mg % dry malt are presented in Table 1 where it can be deduced that the total FAN varied highly significantly with Ca^{2+} treatment in an inverse manner for both cultivars. Highest FAN was obtained for both cultivars with the double

Table 1. Effects of steep liquor $cacl_2$ level on fan of malts from two improved Nigeria sorghum cultivars ICSV 400 and KSV 8 (means, n = 3).

Cultivar	Malt FAN level (mg % dry malt) by $CaCl_2$ level (ppm)					
	0	100	200	300	400	500
ICSV 400	193.5	133.73	108.12	79.70	34.17	36.98
KSV 8	181.8	85.38	59.78	73.95	54.09	31.29

deionized steep water control malts. For ICSV 400, addition of $CaCl_2$ to steep liquor across the range of 100 to 500 ppm examined caused a progressive repression, in the range of 31 to 82%, in malt FAN development. A similar trend was observed in KSV 8 except that at 300 ppm Ca^{2+} treatment, malt FAN appreciated significantly ($P < 0.001$) compared with the value recorded at 200 ppm Ca^{2+} treatment. Further increases thereafter caused significant repression of FAN accumulation. Although, FAN values obtained for grains treated with Ca^{2+} were significantly lower than control values for both cultivars, ICSV 400 in general, exhibited higher FAN than KSV 8.

The net malt FAN has been described as the balance of rate of catabolic processes which degrade grain storage proteins to amino acids and the rate of anabolic processes at which these degradation products are removed to the new tissues for plant structure and enzyme synthesis (Morrall et al., 1986; Shutov and Vaintraub, 1987; Taylor, 1983; Taylor and Boyd, 1986). It is therefore conceivable and even very probable that the observed repression in FAN accumulation as a result of Ca^{2+} treatment might be due to possible inhibition of the capacity of the malts to release FAN. This would presumably arise from reduced ability to synthesize adequate amounts and/or complements of the appropriate enzymes necessary for protein degradation or reduction in ability to activate inactive enzyme forms. Similar views have been proposed by some other workers for barley (Jones and Jacobsen, 1983). It is also probable that the low FAN accumulation would result from highly significantly improved malt FAN utilization due to considerable increases in anabolic activities operating within the growing seedling. However, considering that for both cultivars, Ca^{2+} treatment caused significant repression of metabolic activities of the grains as indicated by highly significantly repressed kernel growth and enormous reductions in malting loss (Ezeogu and Okolo, 1995b), inhibition of proteolytic enzyme synthesis/activity would be a more plausible explanation for low FAN accumulation due to Ca^{2+} treatment. Jones (1969) had earlier proposed the reduction in yeast assimilable malt FAN as a mediator in the reduction of kernel growth in cereal seedlings after treatments that repress rooting during barley malting. The highly significantly ($P < 0.001$) higher FAN obtained for ICSV 400 malts compared with KSV 8 under all levels of Ca^{2+} treatment suggest superior FAN development in ICSV 400 malts. This observation agrees quite well with our earlier findings (Ezeogu and Okolo, 1995a; Okolo and Ezeogu, 1995a, b) and that of (Ogbonna et al., 2003) that ICSV 400 exhibited better malting qualities than most other sorghum cultivars reported so far. Analyses of variance data confirm that cultivar and Ca^{2+} treatment as well as their pairwise interactions are highly significant factors in FAN development in these grains. Furthermore, Kruskall-Wallis tests indicated the existence of highly significant linear relationships between malt FAN and steep Ca^{2+} treatment. Correlation analyses established very significant inverse relationships between the two factors for ICSV 400 at $r = - 0.95$ and $r = - 0.84$ for KSV 8 (Figure 1).

Calcium ion has been associated with important physiological functions during cereal germination (Jones and Jacobsen, 1983; Stewart et al., 1988). The effects of steep liquor $CaCl_2$ concentration on malt total non-protein nitrogen (TNPN) was therefore, investigated for sorghum cultivar ICSV 400 and KSV 8. As illustrated in Table 1, Ca^{2+} treatment and cultivar, plus their pairwise interactions highly significantly ($P < 0.001$) influenced the pattern of sorghum malt TNPN development. For ICSV 400, Ca^{2+} treatment appeared to significantly ($P < 0.001$) stimulates TNPN accumulation in the malts at all levels of treatment. Highest TNPN was recorded for grains exposed to 100 ppm steep water Ca^{2+}. KSV 8 in contrast exhibited a different trend of TNPN development in response to Ca^{2+} treatment. TNPN obtained for malts derived from double deionized water steep controls, at 725 mg % dry malt was considerably higher than TNPN values obtained for KSV 8 malts subjected to Ca^{2+} treatment. This indicates significant repression of between 23 and 69% of TNPN development in KSV 8 malts due to Ca^{2+} treatment. KSV 8 malts exhibited significantly higher TNPN accumulation compared with the corresponding ICSV 400 malts at 200, 300 and 400 ppm levels of treatment. These differences in the pattern of malt TNPN of the sorghum cultivars are further confirmation of cultivar-dependent differences in sorghum grain physiology. Although, Kruskall-Wallis test established a linear relationship between TNPN and Ca^{2+} treatment, there was poor correlation between the two factors for both cultivar at $r = - 0.51$ for KSV 8 (Figure 2).

TNPN consists mainly of products of storage protein hydrolysis specifically amino acids and small peptides (Hii and Herwig, 1982; Koehler and Ho, 1990; Rastogi and Oaks, 1986; Taylor, 1983). In fact, like malt FAN, the net TNPN is the balance of rates of TNPN release by

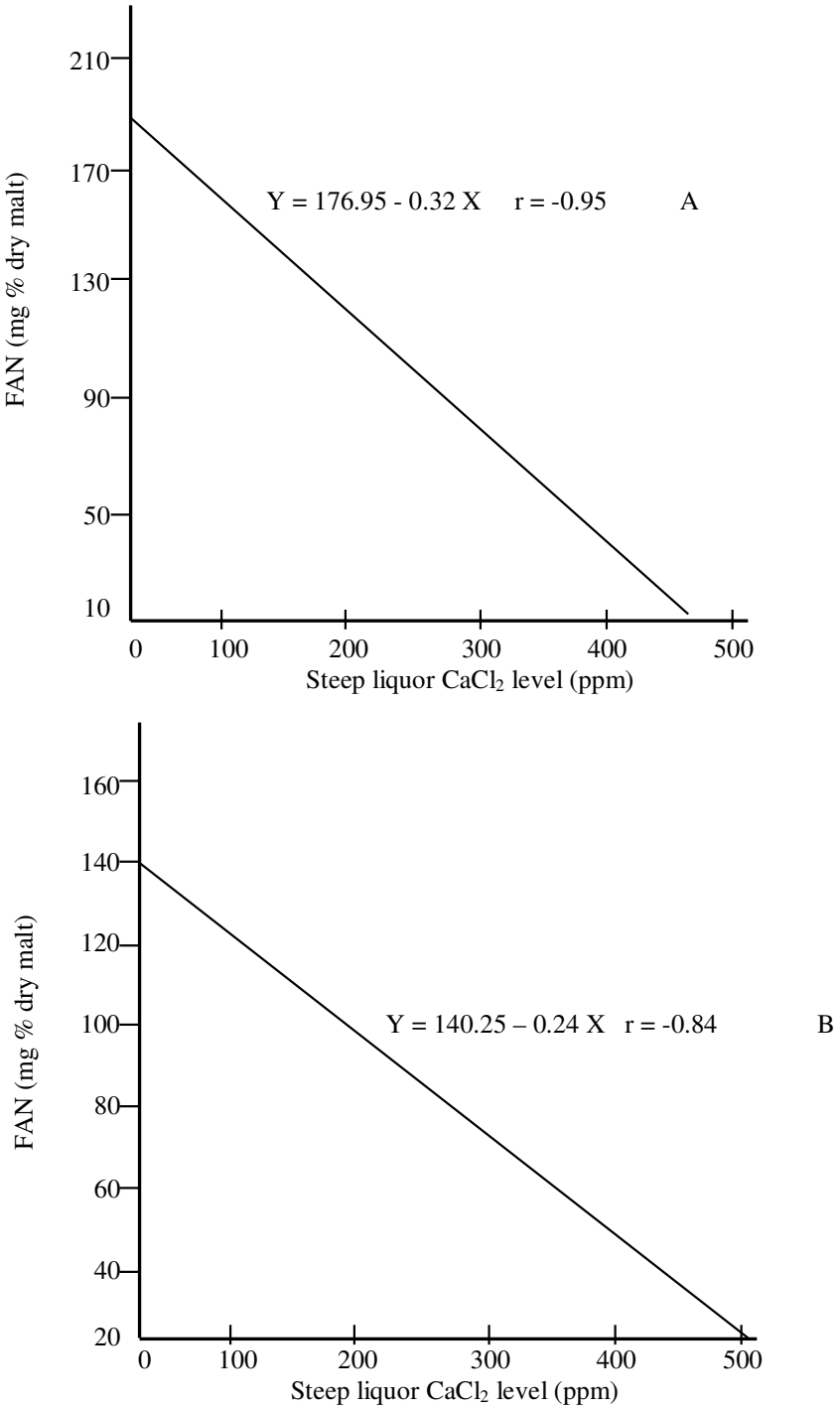

Figure 1. Correlation of steep water $CaCl_2$ concentration with malt FAN for sorghum cultivars (A) ICSV400 and (B) KSV 8.

protein degradation processes and its removal to the growing tissues of the germinating seedling. The differences in TNPN accumulation of the grains might therefore, be attributed to major differences in the physiological responses of these sorghum cultivars induced by Ca^{2+} treatment which may be cultivar dependent. Palmer (1993) linked grain maltability to physiological or metabolic status of the grain. It is therefore probable that steeping sorghum grains in $CaCl_2$ containing liquor elicited significant differential alterations in their metabolism; such that may include qualitative as well as quantitative differences in the development of

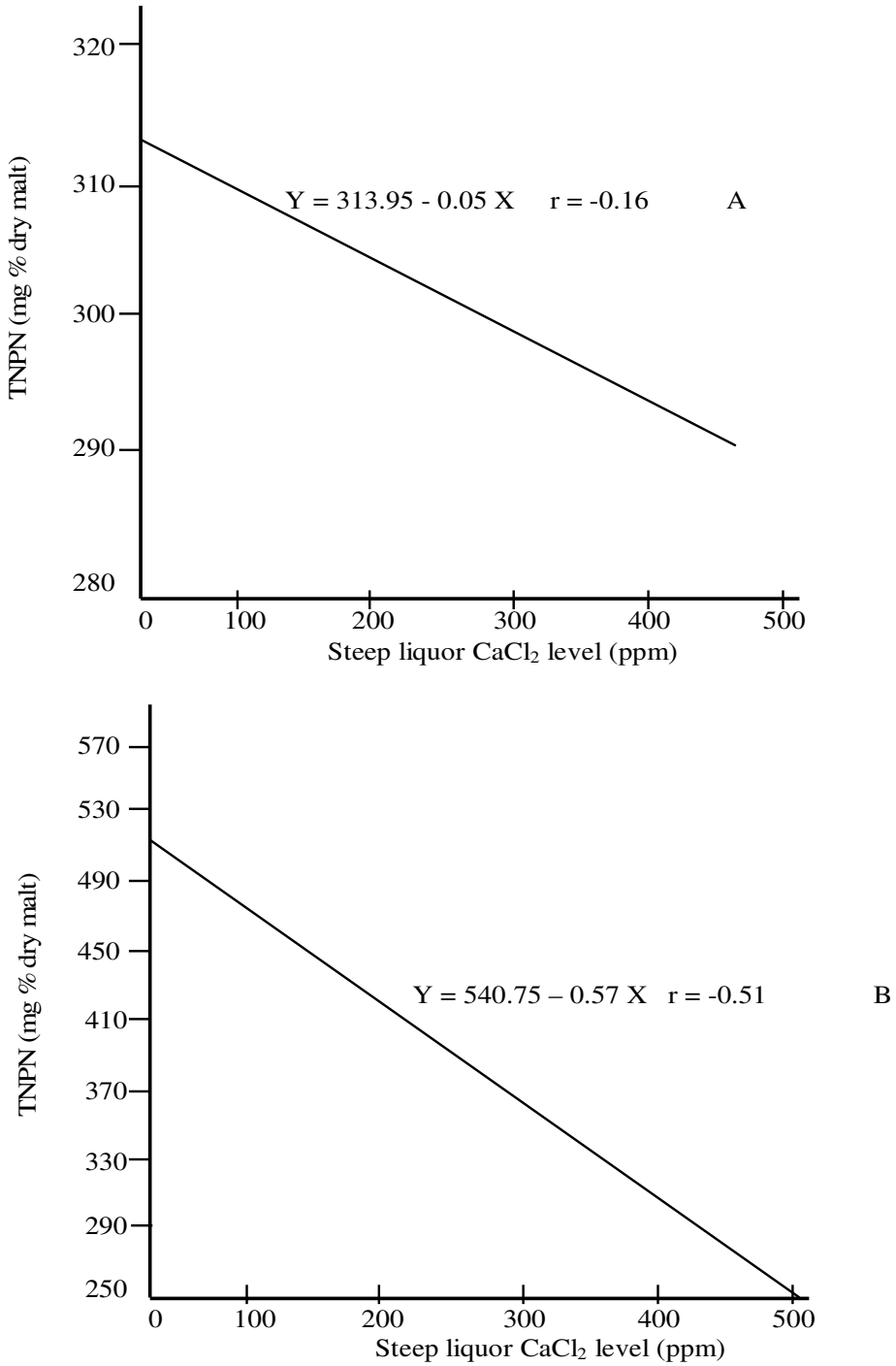

Figure 2. Correlation of steep liquor $CaCl_2$ concentration with malt TNPN for sorghum cultivars (A) ICSV400 and (B) KSV 8.

certain important enzymes of protein degradation and efficiency of TNPN translocation across the scutellum to the growing tissues of the young seedling. Since Ca^{2+} treatment caused repression of kernel growth and invariably reduced metabolic rate in sorghum (Ezeogu and Okolo, 1995b), enhancement of TNPN accumulation in ICSV 400 over control values would presumably indicate reduced TNPN utilization as well as possibly improved TNPN releasing activities. In KSV 8, however, where Ca^{2+} treatment highly significantly ($P < 0.001$) repressed TNPN development, a significant reduction in the activity of the TNPN releasing processes would seem to play significantly more important role in determining malt TNPN accumulation.

Table 2. Effects of steep $CaCl_2$ level on the total non- protein nitrogen (TNPN) level of malts from two improved Nigeria sorghum cultivars ICSV 400 and KSV 8 (means, n = 3).

Cultivar	Malt TNPN level (mg %) by $CaCl_2$ level (ppm)					
	0	100	200	300	400	500
ICSV 400	260.0	390.0	270.8	335.8	271.9	281.7
KSV 8	725.0	168.9	453.1	498.4	325.0	226.6

Table 3. Effects of steep $CaCl_2$ level on the ratio FAN/TNPN of malts from two improved Nigeria sorghum cultivars ICSV 400 and KSV 8 (means, n = 3).

Cultivar	Ratio FAN/TNPN level (%) by $CaCl_2$ level (ppm)					
	0	100	200	300	400	500
ICSV 400	74.4	34.3	39.9	23.7	12.6	13.1
KSV 8	25.1	50.5	15.12	14.8	16.6	13.8

Since TNPN comprises FAN and TCA-soluble peptides, the response of the ratio malt FAN/TNPN to Ca^{2+} treatment was evaluated. As shown for malt FAN and TNPN, the ratio FAN/TNPN of the malts varied highly significantly with cultivar, steep Ca^{2+} treatment and their pairwise interactions (Table 3). For ICSV 400, significantly higher FAN/TNPN ratio was obtained for control malts compared with malts derived from Ca^{2+} treated steeps. Ca^{2+} treatment of KSV 8 malts similarly appeared to generally repress FAN/TNPN ratio except in the case of 100 ppm Ca^{2+} treated malts, where FAN/TNPN ratio was about double the control value.

Both partial and final products of protein hydrolysis during grain germination are known to be trans-located to the growing regions of the young seedlings for the synthesis of plant structural materials and enzymes (Koehler and Ho, 1988; Mitsuhashi and Oaks, 1994; Shutov and Vaintraub, 1986). Thus, alongside amino acids, partial products of protein degradation such as peptides of up to 5 amino acids are also believed to be trans-located across the scutellum into the growing embryo region of the plants during grain outgrowth 17, 29, for a more efficient anabolic protein turnover. The FAN/TNPN ratio of the malts would therefore represent the net balance of rates at which these two major components of malt TNPN are generated and utilized during grain germination. Since the rates at which these two processes are prosecuted depend greatly on the biochemical/ physiological state of the germinating seed, it invariably follows that any factor which induces even the slightest alteration in grain metabolism might affect the FAN/TNPN ratio. This view is supported by the data presented in Table 3, which seemed to strongly suggest a highly significant ($P < 0.001$) variation in malt FAN to TNPN ratio with Ca^{2+} treatment which may be cultivar-dependent. This may reflect variety-dependent differences in both proteolytic activities and the nature of peptide translocation systems of these two cultivars. Our data also reveal that the assimilable peptide level may be higher in ICSV 400 than in KSV 8. This observation might provide further explanation for the significantly higher average malt FAN/TNPN ratios for ICSV 400 compared with KSV 8 malts. Results of Kruskall-Wallis tests indicated that malt FAN/TNPN ratio varied highly significantly ($P < 0.001$) in a linear manner with Ca^{2+} treatment. A strong inverse correlation was obtained between FAN/TNPN ratio and Ca^{2+} treatment for ICSV 400 ($r = -0.90$), while correlation coefficient (r) for KSV 8 was quite low at -0.60 (Figure 3) indicating the wide variety dependent differences in the abilities of grains from these cultivars to accumulate products of seed storage protein hydrolysis during germination.

The effects of steep $CaCl_2$ concentration on the level of none FAN total non-protein nitrogen that is, small peptides accumulation was evaluated. Since TNPN comprises only amino acids and small peptides, the difference between TNPN and FAN would represent the level of small peptides. As illustrated in Table 4, small pepetide accumulation was significantly influenced by Ca^{2+} treatment and cultivar plus their pairwise interactions. For ICSV 400, peptide accumulation was highly significantly at $P = 0.001$ enhanced by Ca^{2+} treatment at all levels of treatment. This observation reveals therefore that enhancement of TNPN in this cultivar (Table 2) apparently resulted from stimulation of peptide accumu-lation only rather than both components of TNPN as shown in Table 1, FAN generally repressed was highly significant at all levels of Ca^{2+} treatment. Conversely, KSV 8 showed highly significantly repressed small peptide accumulation across all the treatment levels. Although, TNPN repression in this grain resulted from repression of both small peptides and FAN, it would appear that the extent of FAN repression was higher than for small peptides at most of the treatment levels. These observations revealed that peptide accumulation in both ICSV 400 and KSV 8 is both cultivar and steep Ca^{2+} treatment related.

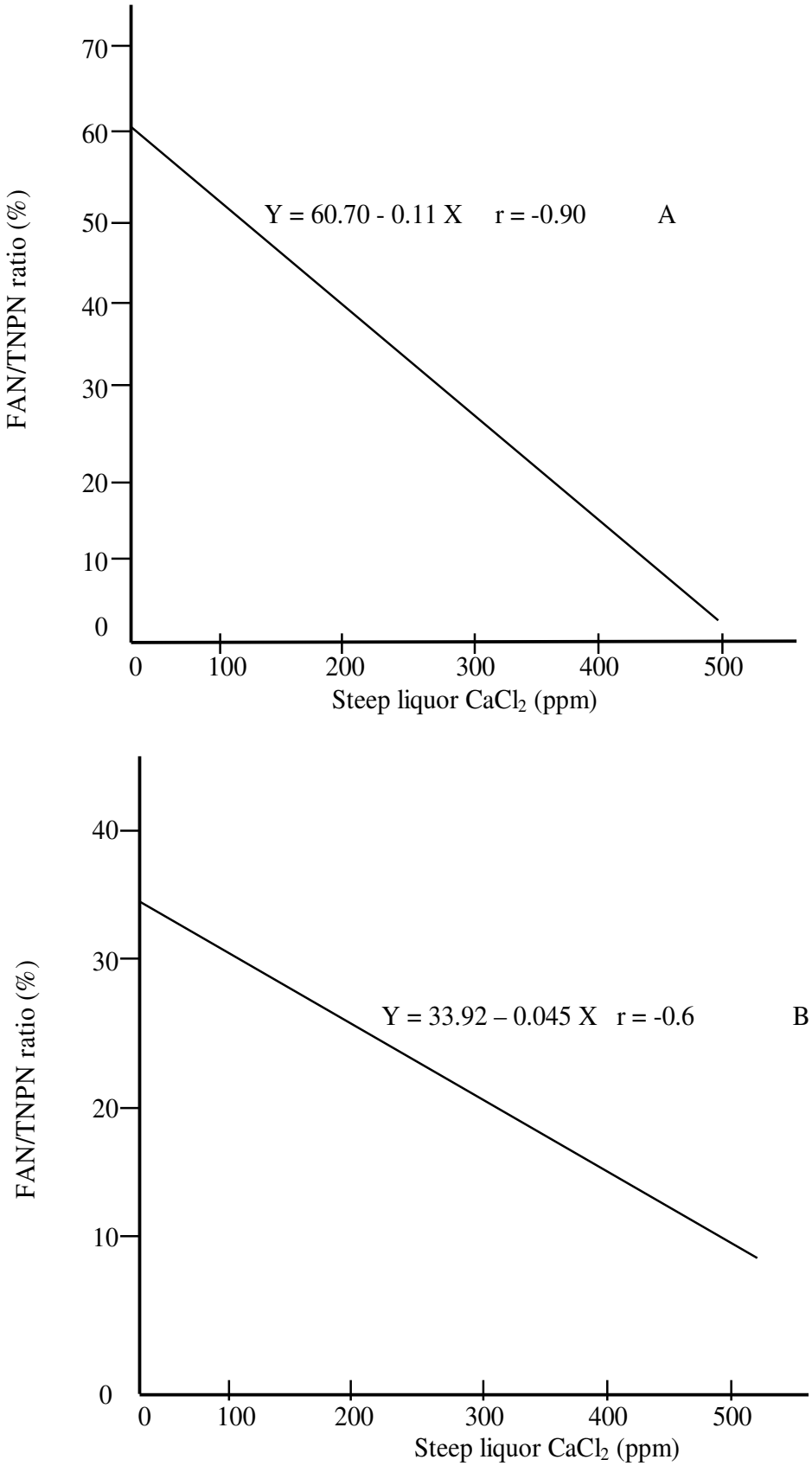

Figure 3. Correlation of steep liquor $CaCl_2$ concentration with FAN/TNPN ratio for sorghum cultivars (A) ICSV400 and (B) KSV 8.

Table 4. Effects of steep $CaCl_2$ level on the TNPN-FAN difference of malts from two improved Nigeria sorghum cultivars ICSV 400 and KSV 8 (means, n = 3).

Cultivar	Malt TNPN-FAN level (mg %) by $CaCl_2$ level (ppm)					
	0	100	200	300	400	500
ICSV 400	66.50	256.27	162.68	256.10	237.71	244.69
KSV 8	543.20	83.00	393.35	424.49	270.91	195.27

There was no obvious trend in the pattern of small pepetide development in response to Ca^{2+} treatment for both cultivars. This was confirmed by correlation analyses which revealed not a very significant positive relationship between the two factors for ICSV 400 at r = 0.65 and also a very weak inverse correlation for KSV 8 at r = 0.37 (Figure 4). This also indicates the existence of strong cultivar-independent differences in the ability of these grains to accumulate products of seed storage protein hydrolysis during seedling growth in response to Ca^{2+} treatment.

The enzymic conversion of water insoluble seed storage proteins to water soluble proteins at the onset of seed germination has been described as one of the most important steps in seed storage protein mobilization (Palmer, 1991; Poulle and Jones, 1988). The solubilisation of endospermal proteins is accompanied by increased accessibility and invariably improved susceptibility to starchy reserve materials to hydrolysis by malt amylolytic enzymes (Glennie et al., 1983; Palmer, 1991; Slack et al., 1979). Since proteins solubilised during germination form part of the total cold water soluble of malt (CWE), Holmes (1992) recommended the determination of soluble protein fraction of malt CWE as a measure of grain modification during malting.

The effects of Ca^{2+} treatment on cold water soluble protein (CWS-protein) development was therefore determined for the two sorghum cultivars ICSV 400 and KSV 8. As illustrated in Table 5, Ca^{2+} treatment in general, possibly inhibited solubilisation of storage proteins for both sorghum cultivars. For ICSV 400, Ca^{2+} treatment at 100 ppm level caused 37% decrease in amount of soluble protein detectable in the CWE relative to control values. Significant improvements in CWS-protein accumulation over the 100 ppm Ca^{2+} treated malts was observed on exposure of grains to 200 ppm Ca^{2+} level. Further increases beyond this level elicited progressive repression of CWS-protein development. Similarly, Ca^{2+} treatment caused highly significant repression of CWS-protein accumulation in KSV 8. However, the repression effect of 100 ppm Ca^{2+} treatment on protein solubilisation was clearly more pronounced in KSV 8 than in ICSV 400, at 42% decrease in detectable soluble proteins in CWE. The repression of CWS-protein development also appeared to increase progressively as Ca^{2+} treatment increased up to 300 ppm Ca^{2+} beyond, which further increase caused significant reductions in malt CWS-protein. ICSV 400 malts generally showed higher levels of CWS-protein compared with KSV 8 malts suggesting higher protein solubilising activity and CWS-protein accumulation in this cultivar and also indicating that both characteristics are cultivar related. These observed differences may therefore reflect major differences in composition and structural complexity and/or the nature and level of proteolytic enzymes responsible for seed protein solubilisation. Ca^{2+} has been reported to regulate the secretion of specific isoforms of cereal hydrolases during malting (Jones and Jacobsen, 1983), probably through occurrence of specific proteinase inhibitor compounds whose physiological roles are little understood (Mosolov and Shul'gin, 1986). Analyses of variance confirm that steep water Ca^{2+} treatment and cultivar as well as their pairwise interactions significantly influenced the pattern of malt soluble protein accumulation. Correlation analyses indicated a good inverse relationship between Ca^{2+} treatment and CWS-protein for both cultivars at r = - 0.81 for ICSV 400 and r = - 0.79 for KSV 8 (Figure 5).

The extent of grain endosperm protein solubilisation has been shown to correlate with level of starch modification during cereal malting (Glennie et al., 1983; Palmer, 1989; Palmer and Bathgate, 1976). Hence, simple analytical techniques were developed to determine protein solubilisation in malts as a measure of grain modification during malting (Smith and Gill, 1986). Holmes (1992) proposed the use of the term index of cold water protein solubilisation as a measure of grain modification. Since the degradation of grain endosperm reserve proteins during malting is accompanied by its solubilisation, the author argued that the soluble protein fraction of the total cold water soluble used as a proportion of total protein of the parent grain, in a ratio analogous to the Kolbach index (Recommended Methods of Analysis, 1986) would be a useful indicator of how much grain structure modification occurred during malting.

Data presenting indices of cold water soluble protein modification of the sorghum malts expressed as mg CWS-protein per g parent grain protein are depicted in Table 6. CWS-protein modification was clearly repressed in a very significant manner by Ca^{2+} treatment of both sorghum cultivars and at all levels of treatment. However, ICSV 400 malts seemed to be better modified and at all treatment levels than KSV 8 malts further confirming superior protein solubilisation potential for ICSV 400 malts. Furthermore, this observation presumably indicates the possibility of repression of the expression or activity of the major proteolytic enzymes involved in seed protein

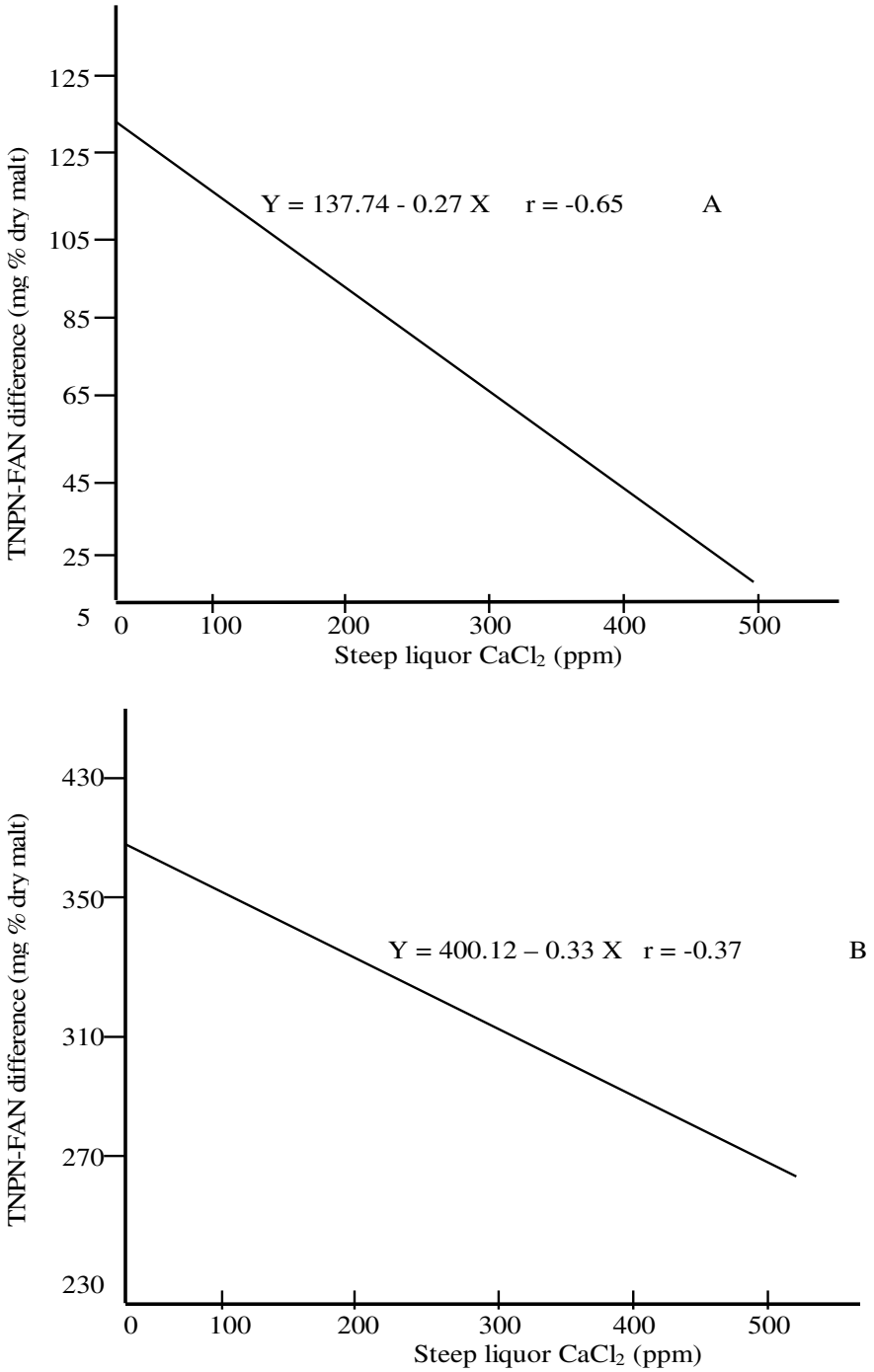

Figure 4. Correlation of steep liquor $CaCl_2$ concentration with TNPN-FAN difference for sorghum cultivars (A) ICSV400 and (B) KSV 8.

Table 5. Effects of steep $CaCl_2$ level on the CWS-protein value of malts from two improved Nigeria sorghum cultivars ICSV 400 and KSV 8 (means, n = 3).

Cultivar	Cold water soluble protein (mg % dry malt) by steep $CaCl_2$ level (ppm)					
	0	100	200	300	400	500
ICSV 400	1850	1170	1590	1249	1131	909
KSV 8	1700	990	1147	1305	949	601

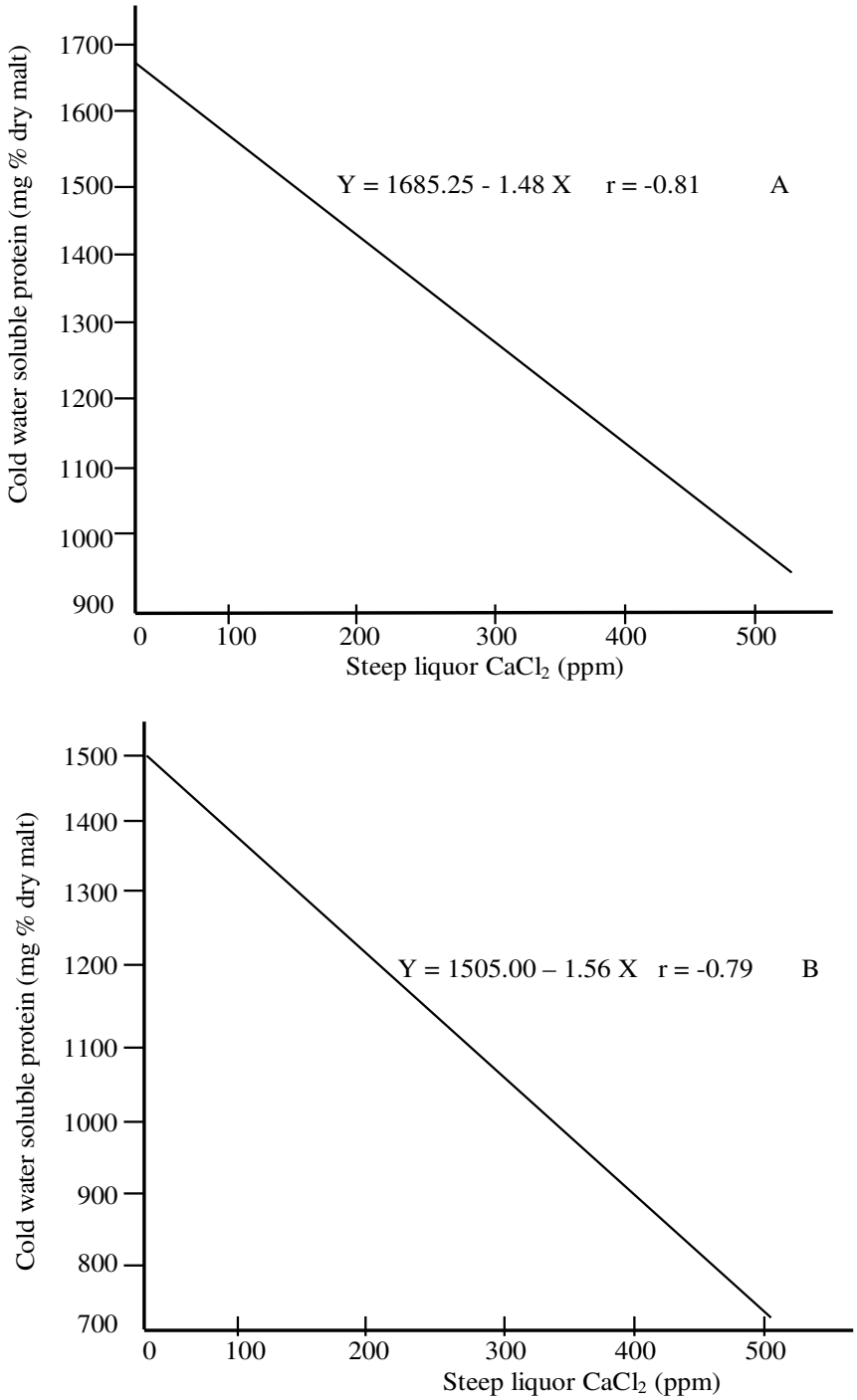

Figure 5. Correlation of steep liquor $CaCl_2$ concentration with cold water soluble protein for sorghum cultivars (A) ICSV400 and (B) KSV 8.

Table 6. Effects of steep $CaCl_2$ level on the CWS-protein modification index of two improved Nigeria sorghum cultivars ICSV 400 and KSV 8 (means, n = 3).

Cultivar	Cold water soluble protein modification index (mg/g dry malt) by $CaCl_2$ level					
	0	100	200	300	400	500
ICSV 400	168.18	106.36	144.55	113.55	102.82	82.64
KSV 8	147.83	86.09	99.74	113.49	82.52	52.26

Table 7. Effects of steep liquor $CaCl_2$ level on carboxypeptidase activity of malts from two improved Nigeria sorghum cultivars ICSV 400 and KSV 8 (means, n = 3).

Cultivar	Malt carboxypeptidase activity (µg FAN/3h/g dry malt) by $CaCl_2$ level (ppm)					
	0	100	200	300	400	500
ICSV 400	570	2137.5	570	427.5	1710	570
KSV 8	997.5	1425	1425	285	1140	2850

solubilisation. Analyses of variance data confirm that Ca^{2+} treatment and cultivar plus their pairwise interactions exerted very significant influences of CWS-protein modification of sorghum malts. Satisfactory correlation was obtained between CWS-protein modification and Ca^{2+} treatment for both cultivars (data not shown).

The effects of steep liquor $CaCl_2$ concentration on carboxypeptidase activity of sorghum cultivars ICSV 400 and KSV 8 are illustrated in Table 7, where it can be seen that Ca^{2+} significantly influenced carboxypeptidase development in both cultivars. There was no definite trend in the development of carboxypeptidase in ICSV 400. An activity of 570 µg FAN/3h/g dry malt was recorded for ICSV 400 in the absence of Ca^{2+} in steep liquor. Highest carboxypeptidase activity was attained in ICSV 400 on exposure of grains to 100 ppm Ca^{2+} treatment at 2137 units. A second high point in this enzyme activity was observed when the grains were subjected to 400 ppm Ca^{2+} treatment. Similar levels of carboxypeptidase activity as in the control malts were obtained at 200 and 300 ppm Ca^{2+} treatment while the lowest enzyme activity occurred at 300 ppm Ca^{2+} treatment. However, patterns of carboxypeptidase development in response to treatment to steep liquor containing $CaCl_2$ were different for KSV 8. Exposure of KSV 8 grains to 100 and 200 ppm Ca^{2+} steeps elicited a 43% enhancement of carboxypeptidase activity at 1425 units over control values. The enzyme activity obtained at 300 ppm Ca^{2+} treatment however, fell sharply to 285 µg FAN/3h/g dry malt. Nevertheless, further increase in Ca^{2+} treatment caused a similar sharp increase in carboxypeptidase activity at 1140 and 2850 units, respectively for 400 and 500 ppm Ca^{2+} treatment levels. Interestingly, the lowest activity was recorded for both grains at 200 ppm Ca^{2+} treatment levels. It is also noteworthy that KSV 8 exhibited highly significantly greater average malt peptidase activity than ICSV 400 malts. Moreover, the highest carboxypeptidase activity for both cultivars were attained at different Ca^{2+} treatment levels, 100 ppm Ca^{2+} for ICSV 400 and 500 ppm Ca^{2+} for KSV 8. Furthermore, both cultivars showed at least two high points of carboxypeptidase activity. These observations clearly indicate that cultivar and Ca^{2+} treatment played very highly significant roles in determining sorghum malt carboxypeptidase development. This opinion is supported by analyses of variance data which indicated that Ca^{2+} treatment cultivar as well as their pairwise interactions highly significantly ($P < 0.001$) affected sorghum carboxypeptidase activity. However, correlation analysis established very poor relationship between malt carboxypeptidase activity and steep liquor $CaCl_2$ for both cultivars at r = 0.10 for ICSV 400 and r = 0.46 for KSV 8 (Figure 6). It is very probable that the existence of multiple high points in carboxypeptidase activity in both sorghum cultivars reflect occurrence of multiple enzyme forms. In fact, polymorphism in cereal hydrolases and in particular peptidase has long been demonstrated (Enari and Sopanen, 1986; Mikola, 1983; Ranki et al., 1990). Therefore, Ca^{2+} treatment perhaps acting as possible selective factors presumably induced expression of specific carboxypeptidase isoforms is best suited for the condition of steeping. A similar selection of α-amylase isotype for secretion following treatments to specific steep conditions has been reported (Jones and Jacobsen, 1983; Mitsui and Akazawa, 1986). The release of FAN from solubilised proteins during grain malting is catalysed by carboxypeptidases (Ranki et al., 1990). No correlation was obtained in this study between FAN and carboxypeptidase activity for both cultivars suggesting roles for factors other than catabolic processes in determining the net FAN. This observation is in reasonable agreement with Taylor (1983) who advanced that the rates of FAN removal to new tissues in the growing embryo region of the young seedling during germination are possible determining factors in malt FAN. In actual fact, Enari and Sopanen (1986) demonstrated that the rate of endoproteolytic activities rather than rate of carboxypeptidase activity was the rate limiting step in the conversion of barley reserved protein to FAN during malting.

Ca^{2+} has long been associated with enhanced hydrolytic enzyme secretion in germinating cereal grains (Jones and Jacobsen, 1983; Stewart et al., 1988; Sticher et al., 1981; Taylor and Boyd, 1986). The effect of different steep liquor $CaCl_2$ concentrations on sorghum malt proteinase development was therefore investigated. As depicted in Table 8, Ca^{2+} clearly elicited widely varying responses by sorghum grains. For instance, steeping ICSV 400 grains in $CaCl_2$ containing liquor within the range of Ca^{2+}, 100 and 500 ppm examined produced malts with significantly reduced proteinase activity relative to grains malted in steep liquors containing no $CaCl_2$. The highest proteinase activity was obtained from grains steeped in 100 ppm Ca^{2+} liquor. Proteinase activity increased progressively from 339.3 µg N/3h/g dry malt at 100 ppm Ca^{2+} treatment to the highest activity of 950.3 µg N/3h/g dry malt at 400 ppm Ca^{2+}. A different pattern of proteinase development was observed for KSV 8.

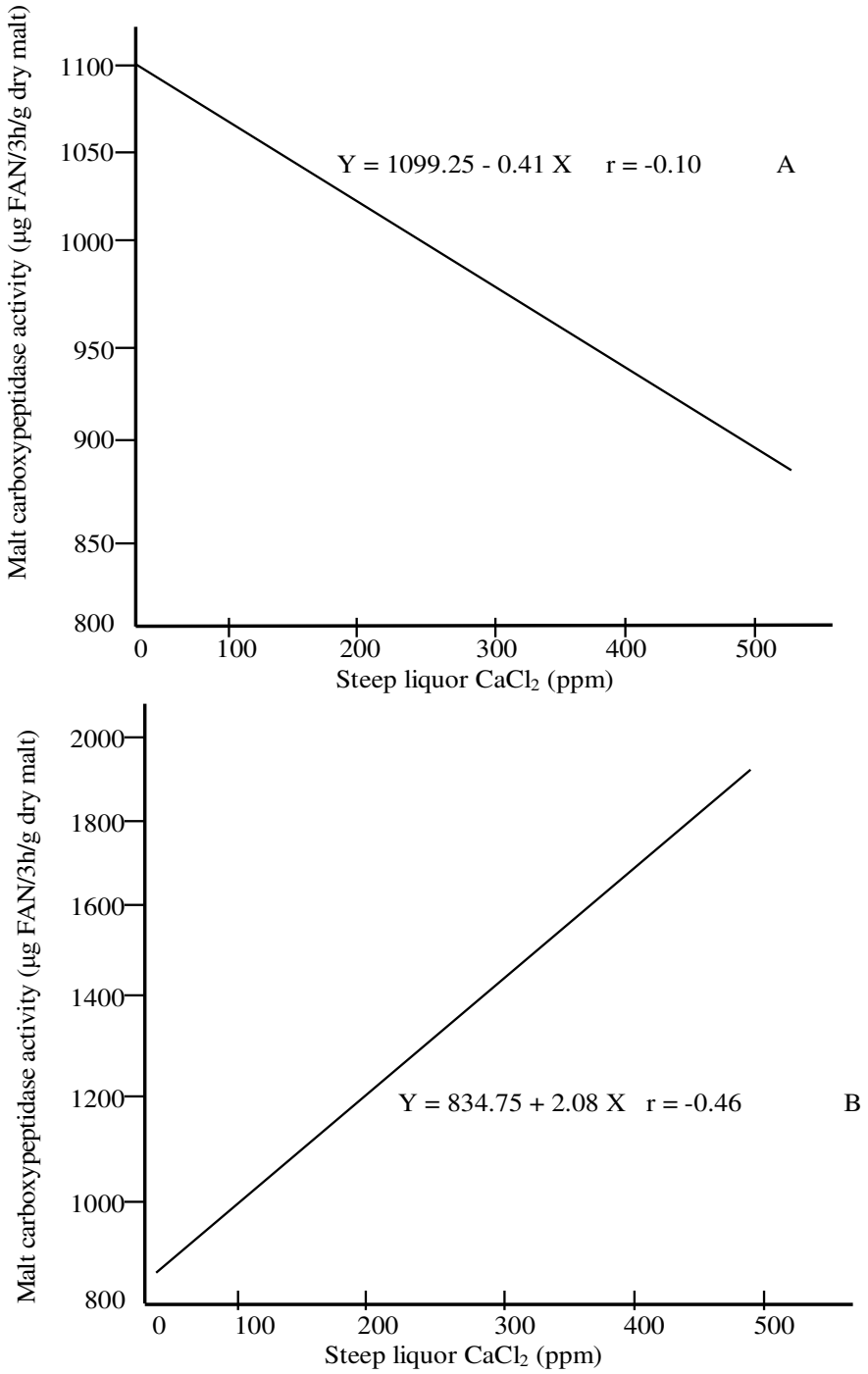

Figure 6. Correlation of steep liquor $CaCl_2$ concentration with carboxypeptidase activity for sorghum cultivars (A) ICSV400 and (B) KSV 8.

Table 8. Effects of steep liquor $CaCl_2$ level on proteinase activity of malts from two improved Nigeria sorghum cultivars ICSV 400 and KSV 8 (means, n = 3).

Cultivar	Malt proteinase level (µg N/3h/g dry malt) by $CaCl_2$ level					
	0	100	200	300	400	500
ICSV 400	1220.5	339.3	565.5	565.5	950.3	791.9
KSV 8	3206.4	3340.0	2137.6	1113.3	3500.7	1113.3

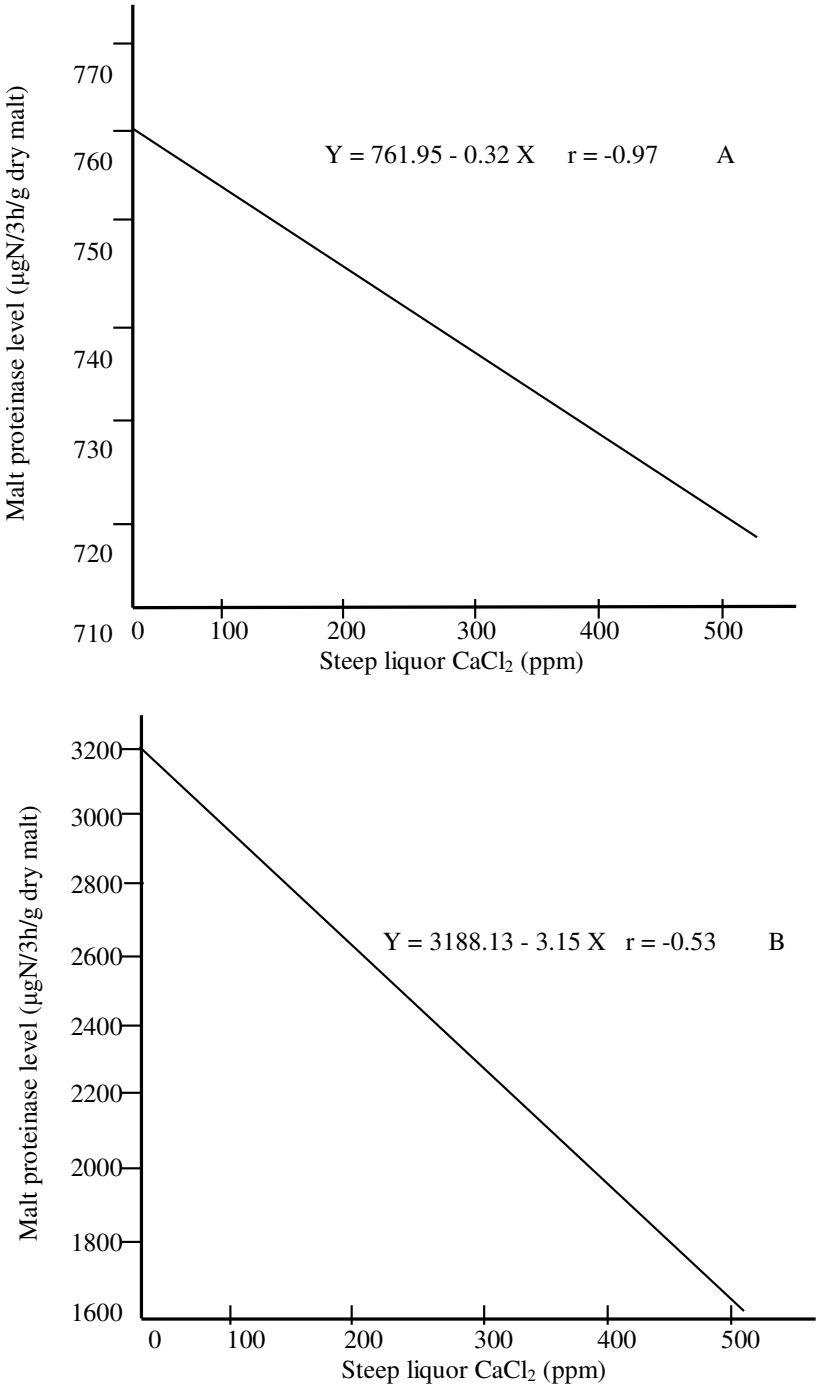

Figure 7. Correlation of steep liquor $CaCl_2$ concentration with proteinase activity for sorghum cultivars (A) ICSV400 and (B) KSV 8.

Steeping KSV 8 grains in 100 ppm Ca^{2+} containing liquor caused only a little enhancement of proteinase activity at 3340.0 units. Highest enzyme activity was recorded for grains exposed to 400 ppm Ca^{2+} as in ICSV 400. These observations are confirmed by analyses of variance data which revealed that cultivar and steep liquor $CaCl_2$ concentration as well as their pairwise interactions played highly significant ($P < 0.001$) roles in development of proteinase activity in sorghum malts. Furthermore, there was a poor correlation between proteinase activity and Ca^{2+} treatment at $r = 0.5$ for ICSV 400 and KSV 8 at $r = 0.53$ (Figure 7). The effects of Ca^{2+} on the synthesis and secretion of hydrolytic enzymes in cereal grains have been linked to the mediatory roles of gibberellic acid

particularly GA_3 (Aisien et al., 1983; Koehler, 1981; Palmer and Bathgate, 1976). It has also been unequivocally demonstrated that the response of sorghum to the stimulatory effects of GA_3 on the synthesis of hydrolytic enzymes during malting is cultivar dependent (Aisien et al., 1983; Koehler, 1981). It seems reasonable and most probable therefore that only those sorghum cultivars that respond positively to GA_3 induced enzyme synthesis would have salutary effects from steep liquor Ca^{2+} treatment. Koehler (1981), in fact showed that only large seeded sorghum grains possessed the capacity to respond positively to Ga_3 induced enzyme synthesis. Sorghum cultivar KSV 8 used in this study is large seeded in contrast to ICSV 400 and this might be responsible for the observed enhancement of proteinase activity in this cultivar, though at certain levels of treatment.

Interestingly, lower soluble protein obtained for KSV 8 malts in this study (Table 5) irrespective of significantly higher average malt proteinase activity might be a reflection of qualitative differences in the nature and complexity of endosperm proteins of various sorghum cultivars and/or differences in the nature of major isoforms of proteinase expressed by these grains. Significant differences in barley endosperm proteins from different cultivars which affect their maltability have been demonstrated (Rastogi and Oaks, 1986; Riggs et al., 1983). The susceptibility of proteins to enzyme degradation would invariably be affected by wide differences in grain protein character. These factors have been shown to influence the rate of proteolysis in cereal grains during malting (Enari and Sopanen, 1986; Riggs et al., 1983).

Conclusions

Generally, steep liquor treated with calcium has a great influence on sorghum endosperm protein modification in the sorghum varieties. The effect of calcium ion on all the key protein modification factors, including FAN, TNPN and CWE-P are varietal dependent. This study revealed that protein solubilisation, soluble protein accumulation and cold water soluble protein modification in both cultivars are highly repressed by the presence of calcium in the steep liquor.

REFERENCES

Aisien AO, Palmer GH, Stark JR (1983). The development of enzymes during germination and seedling growth in Nigeria sorghum. Starch/Starke, 35: 316-320.

Analysis committee of EBC (1975). Analytica-EBC. 3rd Edition, Zurich, pp. 59-60

Axcell B, Jankovsky D, Morral P (1983). Steeping: the crucial factor in determining malt quality. Brewers Digest, 58: 20-23.

Bathgate GN, Palmer GH (1974). The in vivo and in vitro degradation of barley and malt starch granules. J. inst. Brew. 79: 402-406.

Beck E, Ziegler P. (1989). Biosynthesis and degradation of starch in higher-plants, Ann. Rev. Plant Physiol. Plant Mol. Biol. 40: 95-117.

Brookes PA, Lovitt DA, McWilliam IC (1976). The steeping of barley. A review of the metabolic consequences of water uptake and their practical implications. J. Inst. Brew. 82: 14-26.

Bush DS, Cornejo MJ, Huang CN, RL (1986). Ca^{2+} stimulated secretion of amylases during developments in barley aleurone protoplasts. Plant Physiol. 82: 566-574

Chrispeels MJ, Varner JE (1967). Gibberellic acid-enhanced synthesis and release of alpha-amylase and ribonuclease by isolated barley and aleurone layers. Plant Physiol. 42(3): 398-406.

Cohen SS (1988). Practical Statistics, Edward Arnold, London, Melbourne, Auckland.

Enari TM, Sopanen T (1986). Centenary review: Mobilisation of endospermal reserves during the germination of barley. J. Inst. Brew. 92: 25-31.

Ezeogu LI, Okolo BN (1994). Effects of final warm water steep and air rest cycles on malt properties of three improved Nigerian sorghum cultivars. J. Inst. Brew. 100: 335-338.

Ezeogu LI, Okolo BN (1995a). Effects of air rest periods on malting sorghum response to final warm water steep. J. Inst. Brew. 101: 39-45.

Ezeogu LI, Okolo BN (1995b). Effects of air rest periods on the mobilization of sorghum reserve proteins. J. Inst. o Brew. 101: 465-468.

FAO (2004). Food outlook. http://www.fao-org/docrep/006/j2084c/j2084. Retrieved on 25th June, 2009.

Fincher GB (1989). Molecular and cellular biology associated with endosperm mobilization in germinating cereal grains. Ann. Rev. Plant Physiol. Plant Mol. Biol. 40: 305-346

Glennie CW, Harris J, Liebenberg NVW (1983). Endosperm modification in germinating sorghum grain. Cereal Chem. 60: 27-31.

Hach CC, Bowden BK, Kopelove AB, Brayton SV (1987). More powerful Kjeldahl digestion method. J. Assoc. Off. Anal. Chem. 70: 783-787.

Hii V, Herwig WC (1982). Determination of high molecular weight proteins in beer using Coomassie blue. J. Am. Soc. Brew. Chem. 40: 46

Holmes MG (1991). Cold water extract of malts: soluble carbohydrate and malting behaviour. J. Inst. Brew. 97: 445-451.

Holmes MG (1992). Studies on cold water extract of malts: soluble protein and malting behaviour. J. Inst. Brew. 98: 47-51.

Jones M (1969). The amino acid metabolism of germinating barley. Brewers' Digest, 44: 60-62, 64, 65, 74.

Jones RL, Jacobsen JV (1983). Calcium regulation of the secretion of α-amylase isoenzymes and other proteins from barley aleurone layers. Planta, 15: 1-9

Koehler DE (1981). Hydrolytic enzyme production during sorghum germination. Plant Physiology, 67: p. 218, 40.

Koehler SM, Ho TD (1988). Purification and characterization of gibberellic acid-induced cysteine endoproteases in barley aleurone layers. Plant Physiol., 87(1): 95-103.

Koehler SM, Ho TD (1990). A major gibberellic acid-induced barley aleuronic cysteine proteinase which digests Hordein: Purification and characterization. Plant Physiol. 94(1): 251-258.

Kuntz RJ, Bamforth (2007). Time course for the development of enzymes in Barley. J. Inst. Brew. 113(3): 196-205

Lewis MJ, Krumlan SC, Muhleman DJ (1979). Dye-binding method for measuring protein in wort and beer. J. Am. Soc. Brew. Chem. 38: 37-41.

Macleod AM (1978). Malting: quo vadis? (schwarz award lecture). Tech. Q. Master. Brew. Assoc. Am., 15(2): 65-78.

Mikola J (1983). Proteinases, peptidases, and inhibitors of endogenous proteinases in germinating seeds. In J Daussant, J Mosse, J Vaughan, eds, Academic Press, New York, Ann. Proc. Phytochem. Soc. Eur. 20: 35-52

Mitsui T, Akazawa T (1986). Preferential secretion of R-type α-amylase molecules in rice seed scutellum at high temperature. Plant Physiol., 82(3): 880-884.

Mitsuhashi W, Oaks A (1994). Development of endopeptidase activities in maize endosperms. Plant Physiol. 100: 401-407.

Morrall P, Boyd HK, Taylor JRN, Vander-Walt WH (1986). Effect of germination time, temperature and moisture on malting sorghum. J. Inst. Brew. 92: 439-445.

Mosolov VV, Shul'gin MN (1986). Protein inhibitors of microbial proteinases from wheat, rye and triticale. Planta, 167: 595-600

Ogbonna AC, Obi SKC, Okolo BN (2003). Modification of the methods

for the extraction of carboxypeptidase and proteinase activities from sorghum malts. J. Inst. Brew. 109: 51-56.
Okolo BN, Moneke AN, Ezeogu LI, Ire FS (2010) Calcium regulation of carbohydrate modification in sorghum. Afr. J. Biotechnol. 9: 3861-3873
Okolo BN, Ezeogu LI (1995a). Duration of final warm water steep as a factor influencing some carbohydrate related properties of sorghum malt. J. Inst. Brew. 100(4): 267-274.
Okolo BN, Ezeogu LI (1995b). Effects of air rest period on the mobilisation of sorghum reserve proteins. J. Inst. Brew. 101: 463-468.
Okolo BN, Ezeogu LI (1996). Promoting sorghum reserve protein mobilisation by steeping in alkaline liquor. J. Inst. Brew. 102: 277-284.
Okolo BN, Ezeogu LI (1996). Duration of final warm steep as a crucial factor in protein modification in sorghum malts. J. Inst. Brew. 102: 167-177
Palmer GH (1989). Cereals in malting and brewing. In Cereal Science and Technology, ed Palmer, G.H. Aberdeen: Aberdeen University Press. pp. 61-242.
Palmer GH (1991). Enzymic degradation of the endosperm cell walls of germinated sorghum. World J. Microbiol. Biotechnol. 7: 17-21.
Palmer GH (1993). Ultrastucture of endosperm and quality. Fermentation, 6: 105.
Palmer GH, Bathgate GN (1976). Malting and Brewing. In Advances in Cereal Science and Technology (Ed. Y. Pomeranz), St. Paul, Am. Assoc. Cereal Chem. 1: 263-274.
Pollock JRA (1962). In Barley and Malt: Biology, Biochemistry and Technology (Cook. A. H., edit.). London. Academic Press. p. 303.
Poulle M, Jones BL (1988). A proteinase from germinating barley. I. Purification and some physical properties of a 30 kD cysteine endoproteinase from green malt. Plant Physiol. 88: 1454-1460.
Rastogi V, Oaks A (1986). Hydrolysis of storage proteins in barley endosperms. 1 Analysis of soluble products. Plant Physiol. 81: 901-906.
Institute of Brewing (1986). Recommended Methods of Analysis. London.
Riggs TJ, Sanada M, Morgan AG, Smith DB (1983). Use of acid gel electrophoresis in the characterization of 'B' hordein protein in relation to malting quality and mildew resistance of barley. J. Sci. Food Agric. 34(6): 576-586.
Ranki H, Sopanen T, Voutilainen R (1990). Localization of carboxypeptidase I in germinating barley grain. Plant Physiol. 93: 1449-1452.
Shutov AD, Vaintraub IA (1987). Degradation of storage proteins in germinating seeds. Phytochemistry, 26: 1557-1566.
Slack PT, Baxter ED, Wainwright T (1979). Inhibition by hordein of starch degradation. J. Inst. Brew. 85: 112-114.
Stewart A, Nield H, Lott JNA (1988). An investigation of the mineral content of barley grains and seedlings. Plant Physiol. 86: 93-97.
Smith DB, Gill AA (1986). Quantitative changes in concentrations of the major components of four genotypes of barley grains during malting and mashing. J. Inst. Brew. 92: 360-366.
Sticher L, Penel C, Greppin H (1981). Calcium requirement for the secretion of peroxidases by plant cell suspensions. J. Cell Sci. 48: 345-353.
Taylor JRN (1983). Effect of malting on the protein and free amino nitrogen composition of sorghum. J. Sci. Food Agric. 34: 885-892.
Taylor JRN, Belton PS (2002). Sorghum. In: *Pseudocereals and Less Common Cereals.* Belton, P.S and Taylor, J.R.N (eds). Springer-Verlag Berlin. pp. 55-59.
Taylor JRN, Dewar J (2001). Developments in sorghum food technologies. Adv. Food and Nutr. Res. 43: 217-264
Taylor JRN, Boyd HK (1986). Free α-amino nitrogen production in sorghum beer mashing. J. Sci. Food Agric. 37: 1109-1117.
Varner JE, Mense RM (1972). Characteristics of the process of enzyme release from secretory plant cells 1. Plant Physiol. 49: 187-189.