Effect of Sorghum Type and Malting on Production of Free Amino Nitrogen in conjunction with Exogenous Protease Enzymes

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Abstract

BACKGROUND: Sorghum types suitable for brewing and bioethanol production are required. The effect of sorghum type (white non-tannin versus white type II tannin) on free amino nitrogen (FAN) production from sorghum grain and malt using exogenous protease enzymes was investigated over extended incubation at moderate temperature (45 ^{o}C).

RESULTS: With grain in the absence of exogenous proteases, white non-tannin sorghum produced substantially higher levels of FAN than white type II tannin sorghum, due to the tannins in the latter. Incubating sorghum grain with neutral proteinase and amino-peptidase in combination, improved FAN production. The two sorghum types produced similar FAN levels when malted and incubated in the absence of the exogenous proteases. When both sorghums were malted and incubated with neutral proteinase alone substantially more FAN yield (124-126 mg 100 g^{-1}) occurred than with grains (61-84 mg 100 g^{-1}). The combination of amino-peptidase and proteinase did not improve further. Neither, did malting influence wort free amino acid profile. Group B amino acids constituted the highest percentage (42-47%).

CONCLUSION: With grain, white non-tannin sorghum plus proteinase and amino-peptidase yields the highest FAN, with malt both white non-tannin and white type II tannin sorghums plus proteinase yield the highest FAN.

Keywords: sorghum; free amino nitrogen; exogenous proteases; malting, tannins

INTRODUCTION

The use of sorghum either as malt and/or raw grain for the large-scale brewing of lager and stout beers or malt nonalcoholic beverages is a major industry in several African countries.[1-3] In other countries, such as the USA, the market for sorghum beers is rising due to its "gluten free" advantage[3] and sorghum grain is a major feedstock in bioethanol production.[4] The use of sorghum, however, comes with challenges. Notable among these is insufficient free amino nitrogen (FAN), particularly when raw grain is used[5]. For example, FAN levels of up to 150 mg L^{-1} are required during high gravity brewing.[6] Low levels of FAN during fermentation may lead to incomplete or protracted fermentations[7] since nitrogen is utilized by yeast to synthesize new cellular and enzymic proteins.[8] Peptides are also consumed by yeast during fermentation depending on the peptide type[9] but generally at a slower rate than amino acids.[10]

The low FAN levels with sorghum grain are attributable to the low digestibility of the sorghum protein (kafirin) upon cooking. This is thought to be due to the cross-linking of kafirin by disulphide bonding.[11] This phenomenon is varietal as genetically modified sorghum types with reduced levels of gamma-kafirin yield higher levels of FAN than normal sorghums.[12] A number of approaches have been applied to improve the digestibility of sorghum protein[11, 13-14] and hence improve wort FAN.

Cultivar differences in barley malt brewing are major cause of beer wort quality variability[15] and the same has been reported for sorghum.[16] In different parts of Africa various different types of sorghum are used for the production of lager beers. For example, in Nigeria malted white Type II tannin sorghum is often used,[17] while white tan-plant sorghum is used in the Eastern and Southern Africa as in the form of raw grain.[2] A recent study in our laboratory reported wide variations (31–139 mg L^{-1}) in wort FAN from different sorghum grain.[18]

Similarly, malt FAN levels were found to be substantially influenced by variety in eleven Botswana sorghum cultivars.[19] White tan-plant is notably low in phenolics, whereas white type II tannin sorghum contains some condensed tannins (anthocyanidins), which are beneficial by being protective against biotic stresses during cultivation.[20]

The objective of this study was to compare how these sorghum types influence FAN production in raw grain and malt in conjunction with the addition of exogenous proteases, with the aim of supporting rapid fermentation.

MATERIALS AND METHODS

Grains and Malts

Raw grain and malt (with external roots and shoots removed) of white Type II tannin (WT) sorghum (cultivar Gadam el Hamam type) and of white-tan plant sorghum (W) (cultivar Macia) were sourced from Zimbabwe and kindly provided by the South African Breweries. The malt was produced commercially in a pneumatic malting, under standard sorghum malting conditions[21]. The moisture content of the sorghums was approx. 125 g kg^{-1}, dry weight basis. The grains and malts were hammer milled in a laboratory mill (Falling Number, Huddinge, Sweden) fitted with a 1.2 mm opening screen and the flour was stored in zip-lock type polythene bags at 6-8 °C.

Enzymes

Two types of commercial proteolytic enzymes: an amino-peptidase (Flavourzyme 500 MG) and a neutral proteinase (Neutrase 1.5 MG) were kindly provided by Novozymes SA, Benmore, Johannesburg, South Africa. The amino-peptidase had an enzyme activity of 500 Leucine Amino Peptidase Units/gram (LAPU/g)[22] while that of the neutral proteinase was 1.5 Anson Units/gram (AU/g)[23].

Incubation of sorghum

Milled sorghum (37.7 g, dry basis) was mixed with distilled water (360 mL) pre-heated to 45 °C contained in stirred stainless-steel beakers in a BRF Mashing Bath (Brewing Research Foundation, Nutfield, UK). The contents of the beakers were stirred continuously by magnetic stirring at a speed which maintained the grain/malt in suspension. The pH was measured and adjusted with phosphoric acid (Merck, Gauteng, South Africa) to ensure that it was between pH 5.5-6.0 for all the treatments. This range was within the pH optima of the neutral proteinase. Freshly prepared neutral proteinase (1 mg kg^{-1}, 5 mg kg^{-1} and 10 mg kg^{-1}) or amino-peptidase enzyme (1 mg kg^{-1}) solutions at different concentrations were added. The enzymes were prepared in water, as would be done in brewing, and the beakers were closed with watch glasses during the incubation period. The samples were incubated isothermally at 45 °C which is within the temperature optima for neutral proteinase.[23] The temperature was monitored within the grain/malt liquor and beakers were closed with watch glasses during incubation. At intervals, 0, 1, 2, 4, 6 and 24 h aliquots (20 mL) were removed, centrifuged at 6400 g for 10 min (4 °C) for FAN determination. In another experiment, a mixture of the two enzymes was added (1 mg kg^{-1}, in total) to determine if there was any synergistic effect since neutral proteinase is an endoprotease[23] enzyme while amino-peptidase is an exopeptidase[22] enzyme.

Analyses

DP, moisture and protein content

Malt diastatic power with peptone and water extraction was performed according to the South African Bureau of Standards Method 235 (SABS).[24] Protein content was determined by combustion analysis according to AACC Method 46-30.[25] Moisture content was determined according to AACC Method 44-15A.[25]

FAN

Free amino nitrogen was determined using the European Brewery Convention ninhydrin colorimetric assay with glycine as standard.[26] The results were expressed as mg FAN 100 g^{-1} sorghum (dry basis).

Free amino acids

Wort free amino acids were determined on freeze dried samples by using the PICO.TAG-method Bidlingmeyer *et al*.[27] The wort samples were not hydrolysed with HCl. Amino acids were analysed using a PICO.TAG C18 (3.9 mm x 300 mm) column for free amino acids (Waters, Millipore Corp., Milford, MA).

Total phenols and condensed tannins

Total phenolic content was determined using a modified (1% conc HCl in methanol) Folin-Ciocalteu method.[28] Condensed tannins were determined using the modified Vanillin HCl method (extraction with 1% conc HCl in methanol) of Price *et al*.[29]

Statistical analyses

All experiments were repeated at least once and the mean of closely agreeing replicates were reported. Single factor analysis of variance (ANOVA) was used to determine the effect of proteolytic enzymes on FAN production. Multifactor ANOVA was used to determine the effect of the malting, temperature and cultivar on FAN. ANOVA was performed using Statistica software for Windows, version 10 (Tulsa, OK).

RESULTS AND DISCUSSION

Sorghum type composition

Both sorghum types had a similar low protein content (60-70 g kg^{-1}) and were thus comparable for studying FAN production (Table 1). A higher protein content of up to 124 g kg^{-1} has been reported for regular white sorghum.[30] The W and WT malts both had lower protein contents than their corresponding raw grains, mainly as a result of removal of the external roots and shoots. The level of total phenols in WT raw grain and malt was 3-4 times that in W because of the tannins in the former. Further, as expected there were no detectable tannins in W raw grain but significant levels (approx. 2 g kg^{-1}) in WT. However, this level is substantially lower than that in high tannin (Type III) sorghum.[31] As has been reported elsewhere,[25] malting caused a substantial reduction in the assayable tannins in WT. The reduction in tannins during malting is thought to be due to the formation of highly polymeric compounds under moist conditions.[32] Notwithstanding this, the peptone extract DP (potential amylase activity) of the WT malt was less half that of the water extract DP (actual amylase activity). This shows that the tannins remaining still substantially inhibited the amylase activity of the malt. However, the water extract DPs of the WT and W were similar, also showing that the malts of the two sorghum types were comparable.

Effect of raw grain sorghum type on FAN production with neutral proteinase

Neutral proteinase was selected based on its pH optimum range of between 6 and 8 and its activity at low temperature (optimum at 50 °C)[18], enabling it to be active in mashing[33-34] and in fermentation applications.[35] In general, increasing the concentration of neutral protease resulted to a corresponding increase in FAN production with both the W and WT raw grain (Figure 1). Free amino nitrogen production from W was significantly higher than that from WT sorghum grain. Increasing the enzyme concentration from 1 mg kg^{-1} sorghum to 10 mg

kg^{-1} resulted to a 3 fold and 4 fold increases in FAN with W and WT sorghum grain, respectively. The total wort FAN for the WT sorghum was, however, substantially lower by approx. 35, 14 and 19% at 1 mg kg^{-1}, 5 mg kg^{-1} and 10 mg kg^{-1}, respectively, when compared to W grain.

The lower FAN from WT was expected since this sorghum variety contains tannins and had much higher total phenols than the W sorghum (Table 1). Tannins are associated with reduced protein digestibility of sorghum[36] due to binding irreversibly to proteins, probably through hydrogen bonding.[37] Such bonding will take place in the aqueous environment of incubation. In addition, as shown in Table 1 the tannins also inhibited enzymes[30] and thus impeded sorghum protein hydrolysis. In fact, this work shows that even a very high concentration of exogenous protease addition (10 mg kg^{-1}) did not overcome the tannin inhibition.

Effect of malted sorghum type on FAN production

During incubation, sorghum malt produced substantially more FAN (up to 59 mg 100 g^{-1}) than sorghum grain (up to 46 mg 100 g^{-1}) in the absence neutral proteinase. This was due to the action of endogenous malt proteinase and peptidase enzymes on the protein reserves of the sorghum malt.[21] For example, there is substantial carboxypeptidase activity in sorghum malt but not in the grain.[39] This enzyme is important in the hydrolyses of peptide products of endoprotease cleavage of prolamins into free amino acids.[40]

Incubating with the neutral proteinase enzyme increased FAN production to approx. 84 and 79 mg 100 g^{-1} with both W and WT sorghum malt, while up to 71 and 55 mg 100 g^{-1} increases in FAN occurred when W and WT sorghum grain was incubated with the enzyme,

respectively, (Figure 2). The higher FAN production that occurred with the malt compared to the raw grain is presumably because some of the available proteins had already been partially hydrolysed in the malt due to the action of endogenous proteolytic enzymes.[39] In work done on traditional sorghum opaque beer, it was found that mashing (2 h incubation at 60°C) accounted for about 30% of the FAN in the wort with the remainder pre-formed in the malt.[41]

Incubating W and WT sorghum malts with the neutral proteinase enzyme resulted in only small increases (25 and 26 mg 100 g^{-1}, respectively) in total FAN produced when compared to incubating the malts without the enzyme (Figure 2). This indicates that the use of the enzyme on malts did not substantially improve FAN production, because the malt endogenous proteases were active.

Effect of malted sorghum type on the free amino acid profile

Although malting significantly increased sorghum FAN content (Figure 2), it did not substantially influence the free amino acid profile of the worts after incubation with neutral proteinase (Table 2). This is probably because the malt protein substrate did not differ substantially in protein composition from the grain because the external roots and shoots had been removed from the malt. Group B amino acids constituted the highest percentage of the total amino acids (42-47%), followed by Group A amino acids (28-29%) in both sorghum grain and malt worts. The proportion of proline (Group D) in the sorghum malt wort was similar (8%) to that of the sorghum grain wort (10%).

Elsewhere, it has been reported that sorghum worts without acid hydrolysis, had a rather higher proportion of Group A amino acids than other groups when mashed with or without a proteinase enzyme.[5] This difference may be due to differences in sorghum (the sorghum used

in the study was not indicated) and the fact that different proteinase was used from *Bacillus subtilis* as opposed to *B. amyloliquefaciens* in this study.

In this study, the most abundant amino acids were leucine followed by glutamic acid/glutamine, alanine and isoleucine, in both sorghum grain and malt (Table 2). This is probably related to the amino acid composition of the sorghum kafirin proteins, which are rich in glutamine and non-polar amino acids including proline, leucine and alanine.[42] The high proportions of leucine (210-235 g $kg^{-1)}$, isoleucine (92-111 g kg^{-1}) and particularly valine (90-91 g kg^{-1}) in both sorghum grain and malt suggest that the wort produced would result in a beer with good flavour stability. A recent study reported that production of diacetyl (butane-2, 3-dione) during fermentation decreases with an increase in the uptake of valine.[43] Diacetyl produces a strong "butterscotch" or "toffee" aromas or tastes that cause objectionable flavours when present above its threshold of around 0.15 ppm.[44] The effect of leucine and isoleucine on diacetyl production is not clear. Other workers reported that leucine slightly influences production of diacetyl,[45] while opposite results of isoleucine having a slight influence and leucine not influencing diacetyl production, are also reported.[46]

Effect of sorghum type on FAN production when incubated with neutral proteinase and amino-peptidase enzymes

The effect of sorghum type on FAN production when incubated with neutral proteinase and amino-peptidase enzymes, in combination, was investigated to determine whether there was any synergistic effect since the two enzymes should work collaboratively to hydrolyse proteins into free amino acids[47]. The neutral proteinase is an endo-protease and preferably cleaves polypeptide chains within the chain at any exposed point away from the nitrogen and carbon termini.[40] In contrast, the amino-peptidase is an exopeptidase,[41] which hydrolyses

only the nitrogen terminal endo-polypeptides and in doing so, cleaves off either single amino acids or very short di- and tripeptides.[40]

Incubating W and WT sorghum grain with the combination of neutral proteinase and amino-peptidase enzyme increased FAN production by approx. 18% (to 87 mg 100 g^{-1}) and 13% (to 63 mg 100 g^{-1}), respectively (Figure 3). This indicates that incubating with the combination of the two enzymes had a synergistic effect on FAN production. In contrast, with sorghum grain, incubating sorghum malt with the two enzymes did not increase FAN production with either W or WT (Figure 3), when compared to incubating with neutral proteinase alone (Figure 2). In fact, FAN production decreased by approx. 15 and 18 mg 100 g^{-1}, in total, on both W and WT malts, respectively, when incubated with both enzymes. The lack of a synergistic effect with the malt is probably due to the additional influence of the malt endogenous proteases.

CONCLUSIONS

It has been proposed that FAN levels of about 150 mg L^{-1} are required during high gravity fermentation to avoid incomplete or protracted fermentations.[6] Based on this, sorghum grain wort has inadequate FAN levels for rapid and complete fermentation. In contrast, incubating sorghum malt with the neutral proteinase enzyme can produce sufficient FAN levels. Incubating sorghum grain with the neutral proteinase and amino-peptidase enzymes in combination increases FAN production when compared to incubating the grain with neutral proteinase only. However, the total FAN is still far less than that obtained with incubating sorghum malt with neutral proteinase enzyme only, presumably due to the presence of endogenous enzymes in the malt.

Concerning sorghum type, with grain white tan-plant sorghum produces substantially higher FAN levels than white type II tannin grain because of the tannins present in the latter. However, with malting, white type II tannin malt can produce similar FAN levels to that of white tan-plant malt, apparently as a result of the malt proteins binding with the tannins.

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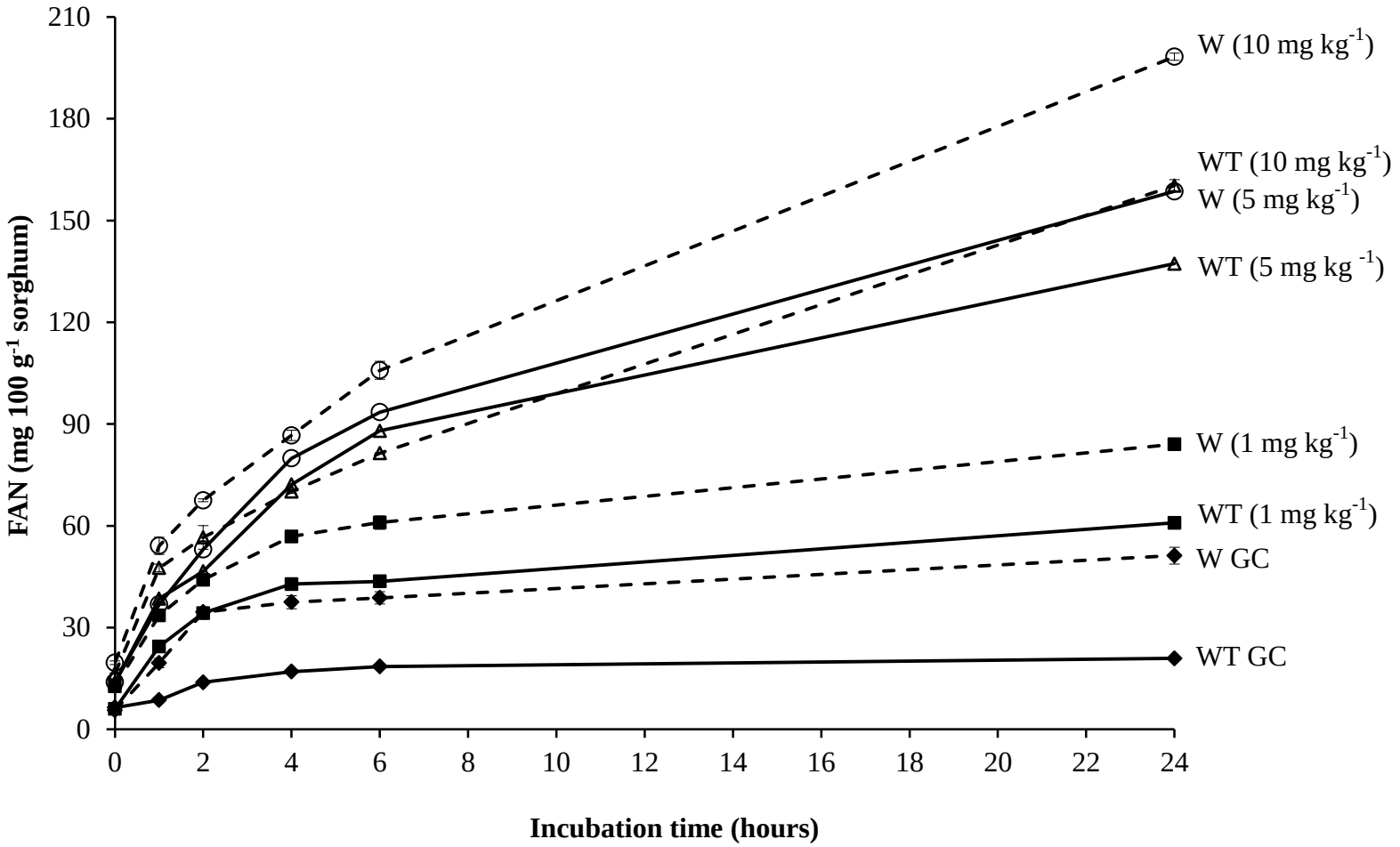

Figure 1. Effect of sorghum type on FAN production when incubating raw sorghum grain with neutral proteinase enzyme for up to 24 h at 45 °C. W - white tan-plant sorghum; WT - white type II tannin sorghum; GC – grain control. Error bars indicate standard deviations (n = 2).

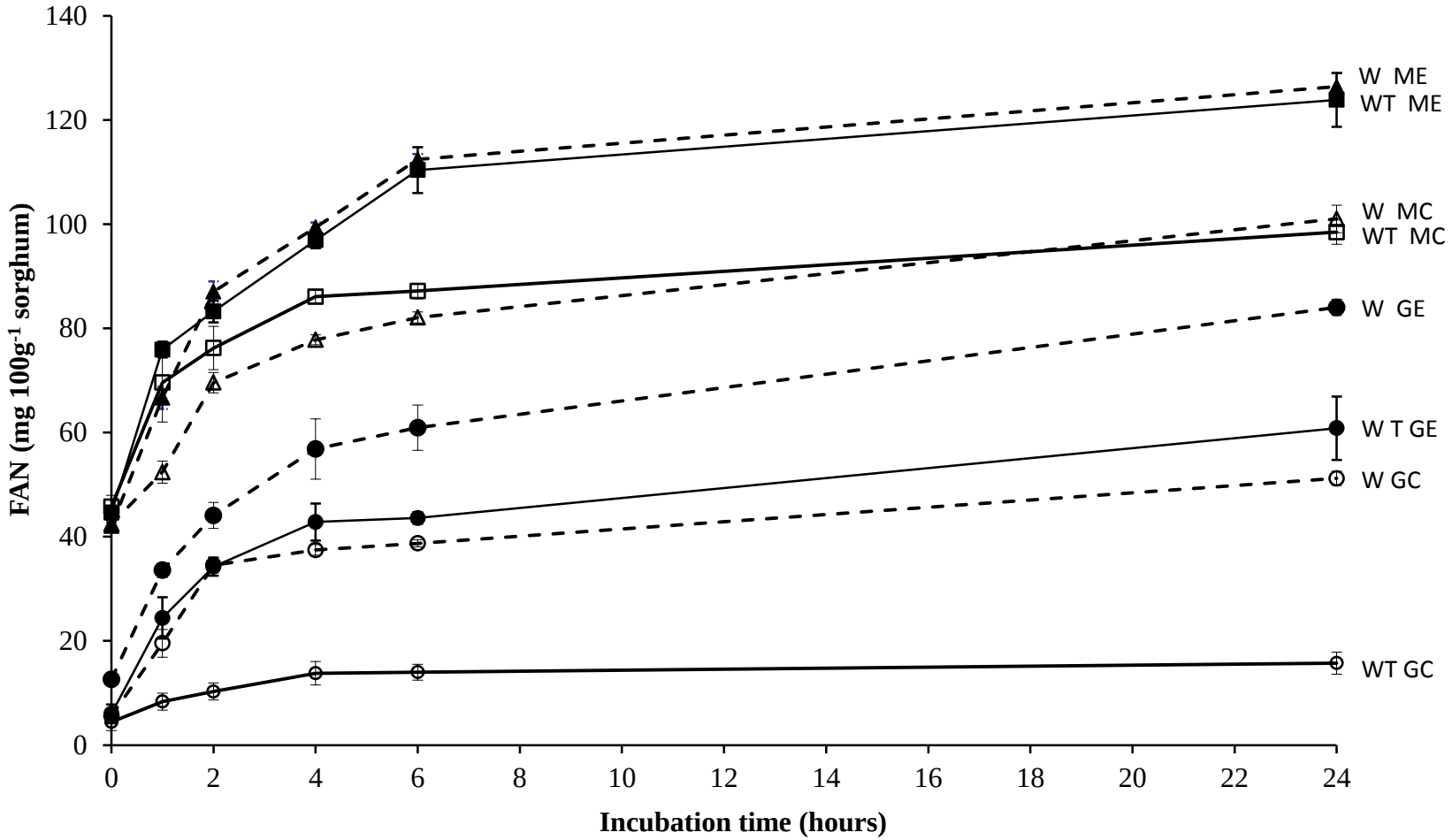

Figure 2. Effect of sorghum type and malting on FAN production when incubated with neutral proteinase enzyme (1 mg kg^{-1}) for up to 24 h at 45 °C. W – white tan-plant sorghum; WT – white type II tannin sorghum; GC – grain control; GE – grain enzyme; MC – malt control; ME – malt enzyme. Error bars indicate standard deviations (n = 2).

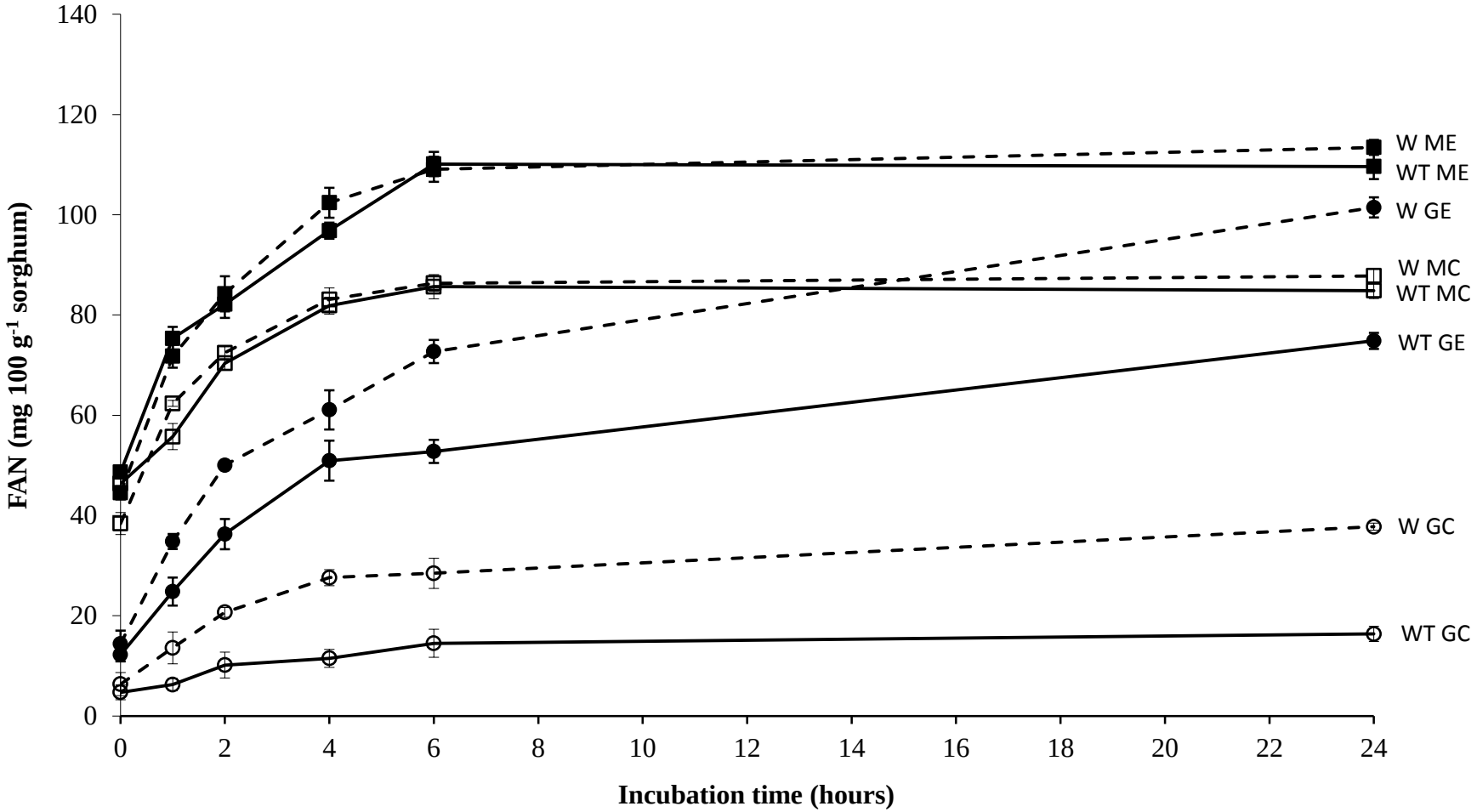

Figure 3. Effects of sorghum type and malting on FAN production when incubated with neutral proteinase and amino-peptidase (1 mg kg^{-1}, in total) in combination for up to 24 h at 45 °C. W – white tan-plant sorghum; WT – white type II tannin sorghum; GC – grain control; GE – grain enzyme; MC – malt control; ME – malt enzyme. Error bars indicate standard deviations (n = 2).

Table 1. Protein, total phenols, diastatic power and tannin contents of sorghum types

Sorghum type	Protein (N X 6.25) (g kg^{-1} dwb*)	Total phenols (g catechin equiv. kg^{-1} dwb)	Tannin (g catechin equiv. kg^{-1} dwb)	Diastatic Power
White tan-plant grain	65.8^{c} (0.4)	2.8^{a} (0.6)	ND	NA
White tan-plant malt	62.7^{b} (0.6)	2.2^{a} (0.2)	ND	11P, 10W
White type II tannin grain	70.4^{d} (0.3)	9.5^{c} (0.1)	19.81^{c} (0.12)	NA
White type II tannin malt	59.65^{a} (0.1)	7.0^{b} (0.1)	7.92^{b} (0.16)	21P, 8W

Figures in parentheses indicate standard deviations; ND – not detected; NA – not applicable; *dry weight basis; P- peptone extract; W – water extract; values with different letter superscripts in a column are significantly different (n=2)

Table 2. Free amino acid composition (g amino acid kg^{-1} amino acid analysed) of white tan-plant sorghum (Macia) grain and malt worts incubated with neutral proteinase (1 mg kg^{-1} sorghum)

Amino acid	**Raw grain**	**Malt**
***Group A**		
Glutamic acid/ Glutamine	160	183
Aspartic acid/ Asparagine	65	40
Serine	29	32
Threonine	15	23
Lysine	12	7
Total	***281 (28)***	***285 (29)***
Group B		
Valine	90	91
Methionine	34	30
Leucine	235	210
Isoleucine	111	92
Histidine	4	ND
Total	***474 (47)***	***422 (42)***
Group C		
Glycine	19	21
Tyrosine	ND	7
Tryptophan	ND	7
Alanine	150	159
Total	***169 (17)***	***194 (19)***
Group D		
Proline	**76 (8)**	**99 (10)**

Figures in parentheses represent relative percentages of each group; ND – not detected; *Pierce[8].