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FACTORS INFLUENCING BETA-AMYLASE ACTIVITY IN SORGHUM MALT

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An investigation into factors influencing beta-amylase activity in sorghum malt confirmed that ungerminated sorghum grain exhibited essentially no beta-amylase activity. Malted sorghum had beta-amylase activity less than 25% of the level in barley malt. Neither reducing agents nor papain affected beta-amylase activity in sorghum, indicating that the enzyme is not in a bound form, unlike in barley. Isoelectric focusing indicated that sorghum beta-amylase comprises just one major and one minor isozyme of pI approximately 4.4–4.5, unlike the many isozymes all of higher pI in barley. However, like barley, sorghum beta-amylase was more temperature-labile than its alpha-amylase. Beta-amylase activity in sorghum malt was increased by germination time, high germination moisture and over the germination temperature range investigated (24–32°C), 24°C gave the highest activity. The beta-amylase activity of sorghum malts was significantly correlated with malt diastatic power, despite the fact that alpha-amylase and not beta-amylase is the predominant diastatic enzyme in sorghum malt.

Key Words: *Beta-amylase, sorghum malt, germination conditions, isoelectric focusing.*

Introduction

Malted sorghum (*Sorghum bicolor* (L.) Moench) is used in Africa to brew traditional sorghum (opaque) beer[18]. In recent years there has been extensive research into using sorghum malt as a replacement for barley malt in the brewing of conventional lager beer[7,8,21]. The substitution of sorghum malt for barley malt would be of considerable advantage to countries in the semi-arid tropics where sorghum is indigenous and the climatic conditions are unsuitable for the cultivation of barley.

There are a number of differences between sorghum malt and barley malt, however, which may affect the quality of beer brewed with sorghum malt. A major difference is that sorghum malt is apparently much lower in beta-amylase activity than barley malt[16,21]. Beta-amylase progressively hydrolyses the penultimate alpha-(1−>4) glucosidic bond at the non-reducing end of starch molecules, releasing maltose. The low levels of beta-amylase in sorghum malt apparently adversely affect wort fermentability[21].

Despite the importance of sorghum beta-amylase, our knowledge about the enzyme is fragmentary. Purification has been carried out[2,19] and enzyme levels in sorghum malts have been measured[1,6,15,16,17,20]. A problem with some of this work appears to be that indirect assay methods have been used, whereby beta-amylase has been calculated from the difference between total amylase and alpha-amylase activity. Considerable doubts have been expressed about the implied assumption that alpha- and beta-amylase activity are additive[5]. The objective of the work described in this paper was to examine, using direct methods of assay, the influence of various factors on the beta-amylase activity of sorghum malt.

Experimental Methods

Materials

Sorghum grain of various cultivars (all low-tannin types) and barley grain *c.v.* Clipper were malted for 6 days at 25°C and 20°C, respectively, as described by Daiber *et al.*[4], then dried in a forced draught oven at 50°C. Sorghum grain *c.v.* Barnard Red, a traditional southern African malting variety of known good diastatic power, was steeped for 24 h at 25°C, then germinated for 6.5 days under a range of temperature and moisture conditions, as described by Morrall *et al.*[14]. A sample of commercial barley malt *c.v.* Clipper (kindly donated by the South African Wheat Board) was also used. All malts were milled to a fine flour using a water-cooled, beater-type coffee mill. Except in the case of the commercial barley malt sample, the milled malts included the seedling rootlets as well as the acrospire, as is the practise in commercial sorghum beer brewing.

Enzyme assays

Malt diastatic power was determined using the standard method for sorghum malt[25], with distilled water as the enzyme extractant. Beta-amylase was determined by two direct methods. The Betamyl assay (Biocon (Aust) Pty. Ltd., Boronia, Australia), which uses a mixture of para-nitro maltopentaoside and para-nitro maltohexaside as substrate[13], was carried out according to the manufacturer's instructions. The assay method of Delcour and Verschaeve[5], which involves inactivation of the alpha-amylase component of diastatic activity by chelation of Ca^{2+} ions with ammonium oxalate and measurement of the residual enzymic (beta-amylase) activity was also performed. The precise procedure used was that adapted specifically for sorghum beta-amylase[27]. To test for bound beta-amylase, grain and malt were extracted with papain or reducing agents. For the Betamyl assay, extraction was carried out with buffer A containing 20 mM cysteine.HCl or 1% (w/v) papain, overnight at room temperature[10]. For the Delcour and Verschaeve assay, a 90 min extraction at 30°C with 0.2 M ammonium oxalate[27], containing 10 mM mercaptoethanol or 1% papain was performed. The different extraction conditions were employed as they followed the literature procedures. However, McCleary and Codd[13] have shown that extraction time has little influence on beta-amylase extraction. More than 95% of barley beta-amylase is extracted in 2 hours.

Isoelectric focusing (IEF) was performed using an LKB Multiphor apparatus essentially according to the manufacturer's instructions. The pH range was 3.5–5.0. The anodic buffer was 0.1 M H_3PO_4 and the cathodic buffer 0.1 M NaOH. Sorghum malt was extracted with either water or

TABLE I. Beta-amylase activity in sorghum and barley, as determined by a modified Delcour and Vershaeve[a] method: Influence of extraction with mercaptoethanol and papain

	Sorghum Diastatic Units/g (dry basis)	
	Sorghum	Barley
Ungerminated grain		
Control	0[b]	55
Mercaptoethanol extraction	0	120
Papain extraction	0	120
Malt		
Control	23	68
Mercaptoethanol extraction	25	105
Papain extraction	23	104

[a]Ref. 27
[b]Means of duplicate assays

0.2 M ammonium oxalate with stirring at room temperature for 90 min, at a flour to extractant ratio of 1 to 40. After centrifugation to obtain a clear supernatant, a portion of the water extract was heated at 68°C for 15 min in the presence of 0.2% (w/v) calcium acetate. The three different samples were then freeze dried. For IEF, the freeze dried samples were dissolved in pH 3.5–5.0 Ampholine containing 15% (w/v) sucrose, at a powder to liquid ratio of 1 to 15. Samples (25 μl) equivalent to 40–50 μg protein, were applied on the anodic side of the gel. IEF was performed at 30 Watts constant power for 3 h at a temperature of 5°C. A counter gel of 2% (w/v) starch and 1% (w/v) agarose in 5 mM sodium acetate buffer, pH 5.4 was prepared. Directly after IEF, the starch gel was placed on the IEF gel and incubated for 20 min at room temperature (27°C). The counter gel was then removed and stained immediately with a 1:1 mixture of IKI (I_2 1% (w/v), KI 1% (w/v)) and ethanol. The stained gel was photographed immediately after staining.

The significance of treatment effects was evaluated by correlation and analysis of variance.

Results and Discussion

The beta-amylase activities in sorghum and barley, using the two different assay methods are shown in Tables I and II. It can be seen that ungerminated sorghum, unlike ungerminated barley[24], exhibited essentially no beta-amylase activity. This finding confirms the results of previous studies where less specific beta-amylase assays were used[15,17,20]. Treatment of the sorghum with either reducing agents or cysteine, hydrochlorine acid and mercaptoethanol, or with the proteolytic enzyme papain failed to yield any beta-amylase activity. In contrast, these reagents under the same conditions, resulted in an increase in beta-amylase activity in ungerminated barley, as would be expected from the literature[24]. This indicates that the reason there was no beta-amylase activity in ungerminated sorghum grain is not because the enzyme is in a bound form as is the case in barley[11]. This suggests that beta-amylase is in fact absent in ungerminated sorghum.

TABLE II. Beta-amylase activity in sorghum and barley, as determined by the Betamyl[a] method: Influence of extraction with cysteine.HCl and papain

	Sorghum Diastatic Units/g (dry basis)	
	Sorghum	Barley
Ungerminated grain		
Control	5[b]	310
Cysteine.HCl extraction	3	392
Papain extraction	3	387
Malt		
Control	79	414[c]
Cysteine.HCl extraction	79	427
Papain extraction	80	432

[a]Ref. 13
[b]Means of duplicate assays
[c]Malt without external rootlets

The sorghum malt exhibited significant beta-amylase activity (Tables I and II), although the level was only 22–24% of that measured in barley malt by the Delcour and Verschaeve assay and 18–19% by the Betamyl assay (as determined in the presence of a reducing agent or papain). Treatment of the sorghum malt with reducing agents or papain did not increase beta-amylase activity, unlike in barley malt, further supporting the proposal that sorghum beta-amylase is not present in a bound form. It is of interest that recent research has shown that concentrations of mercuric chloride which completely inhibit barley beta-amylase do not completely inhibit sorghum beta-amylase[23]. As mercuric chloride specifically inhibits enzymes in which sulphydryl groups are essential for activity, the above data are consistent with this finding.

IEF of a water extract from sorghum malt revealed a number of amylase bands of acidic pI (Fig. 1). These bands occurred in two distinct groups of pI, approximately 4.1–4.2 and 4.4–4.5. Initial work using a broad range IEF gradient (pH 3.5–9.5) did not reveal any other bands. IEF of a malt extract containing calcium acetate, which had been heated at 68°C for 15 min, revealed only the presence of the bands of more acidic pI, the pIs of which are similar to the literature value for sorghum alpha-amylase, pI 4.3[12]. As the addition of calcium ions helps to prevent thermal inactivation of sorghum alpha-amylase[26], this suggests that the bands of less acidic pI were due to the sorghum beta-amylase. The pattern of bands in lane 3, an aqueous extract of sorghum malt in the presence of ammonium oxalate, confirms this. The ammonium oxalate treatment, which inactivates alpha-amylase[5], resulted essentially in the appearance of only the bands of less acidic pI. The fact that there was slight decolourisation in the position of the alpha-amylase bands suggests that the ammonium oxalate treatment did not completely inactivate the alpha-amylase. That the bands of less acidic pI were due to beta-amylase was also indicated by the fact that when the starch gel was initially stained with IKI, the bands of less acidic pI stained reddish-purple, showing the presence of beta-limit dextrin, the product of beta-amylase hydrolysis of starch. In contrast, the bands of more acidic pI did not stain indicating more complete hydrolysis of the starch due to alphaamylase action.

The IEF data show that sorghum beta-amylase, like barley beta-amylase[22], is more heat-labile than its alpha-amylase.

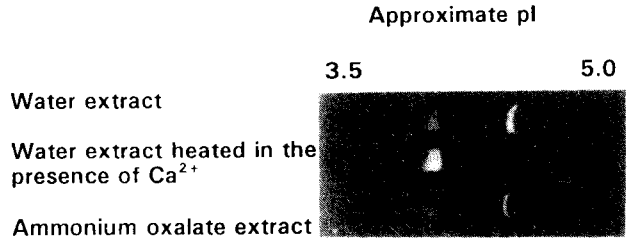

FIG. 1. Isoelectric focusing of aqueous extracts from sorghum malt, starch counter gel stained with IKI solution.

The fact that complete beta-amylase inactivation was achieved by heating at 68°C for 15 min has important implications with regard to brewing with sorghum malt. Elevated temperatures which are commonly employed in temperature-programmed lager mashing processes[3], would further reduce the already limited beta-amylase activity.

IEF also indicated that sorghum beta-amylase comprised one major and one minor isozyme, in contrast to the many beta-amylase isozymes in barley malt[9]. The pI of sorghum beta-amylase, approximately 4.4–4.5 is much more acidic than that of barley malt beta-amylases which have pIs in the range 4.6–6.9[10]. Comparison of the amino acid composition of purified sorghum[2] and barley beta-amylase[28] shows that the sorghum enzyme is richer in the acidic amino acids threonine and aspartic acid, which is consistent with it having a more acidic pI.

Table III shows the influence of germination time and temperature, under the same green malt moisture conditions, on sorghum malt beta-amylase activity. There was a significant effect ($P < 0.001$) of both germination time and temperature on beta-amylase activity and significant interaction ($p < 0.001$) between these factors. Beta-amylase activity increased with germination time throughout the 6.5 days germination, at all 3 germination temperatures (24, 28 and 32°C) investigated. Activity increased rapidly over the first 2 days of germination after which the rate of increase declined with time. A similar pattern of development of sorghum beta-amylase activity with time of germination was observed by Okon and Uwaifa[20] working with 2 Nigerian sorghum varieties. Sorghum malt beta-amylase activity was inversely related to germination temperature over the 24–32°C range investigated. Malt beta-amylase activity was significantly affected ($p < 0.001$) by green malt malt moisture (Table IV). Highest malt beta-amylase activity was obtained when malting under the high green malt moisture conditions. There was also significant interaction ($p < 0.001$) between green malt moisture and germination time.

TABLE III. Influence of germination time and temperature on sorghum malt beta-amylase activity, under medium green malt moisture conditions

Beta-amylase (Sorghum Diastatic Units/g dry malt)			
Time from steep-out (days)	Temperature (°C)		
	24	28	32
0.5	2.3[a]	1.3	0.9
1.0	3.0	4.3	5.3
1.5	9.4	10.3	8.5
2.0	12.3	10.5	9.9
2.5	14.4	12.0	10.5
3.0	16.8	13.5	12.5
3.5	18.8	15.0	13.2
4.0	20.3	16.1	13.3
4.5	22.8	18.3	14.5
5.0	24.8	20.3	14.9
5.5	24.5	20.5	15.8
6.0	25.1	20.8	16.1
6.5	25.3	20.8	16.9

Analysis of variance table

Source of variation	Degrees of freedom	Mean square	F-value	Probability
Time	12	241	1000	<0.001
Temperature	2	176	1000	<0.001
Time × Temp.	24	7	1000	<0.001
Residual	39	<1		

[a]Means of duplicate assays

TABLE IV. Influence of germination time and moisture on sorghum malt beta-amylase activity, at a germination temperature of 24°C

Beta-amylase (Sorghum Diastatic Units/g dry malt)			
Time from steep-out (days)	Moisture		
	Low	Medium	High
0.5	2.1[a]	2.3	2.9
1.0	4.1	3.0	9.9
1.5	6.4	9.4	12.8
2.0	10.1	12.3	17.8
2.5	12.0	14.4	18.1
3.0	14.0	16.8	22.3
3.5	15.2	18.8	25.7
4.0	16.8	20.3	28.1
4.5	16.9	22.8	27.8
5.0	16.2	24.8	27.9
5.5	16.9	24.5	28.0
6.0	16.9	25.1	28.4
6.5	16.9	25.3	28.4

Analysis of variance table

Source of variation	Degrees of freedom	Mean square	F-value	Probability
Time	12	316	1000	<0.001
Temperature	2	496	1000	<0.001
Time ×	24	8	1000	<0.001
Temp.Residual	39	<1		

[a]Means of duplicate assays

The effects of germination time, temperature and moisture on sorghum malt beta-amylase activity are very similar to their effects on malt diastatic power. The diastatic power of sorghum malt increases with germination time[1,14,17,20]. High germination temperatures (greater than 30°C) result in lower diastatic power than lower temperatures (24 and 28°C)[14,17] and diastatic power is highest in malts produced under a high germination moisture regime[14]. These parallels are probably to be expected because beta-amylase along with alpha-amylase are the major enzymes contributing to malt diastatic power.

Table V shows the relationship between diastatic power, and beta-amylase activity in a range of sorghum malts produced from South African sorghum cultivars, under the same malting conditions. All the cultivars exhibited substantial beta-amylase activity when compared with their diastatic power (56–71% of diastatic power), and there were significant differences in beta-amylase activity between cultivars. The former finding is in apparent contradiction with that of Dufour and Melotte[6]. These authors found that nearly 60% of the sorghum malts they examined had very low beta-amylase activity and none was detected in a few of the malts. However, the method of assay used by Delcour and Melotte, selective inactivation of beta-amylase with a mercury salt, has recently been called into question for sorghum[23]. As suggested by the germination study, beta-amylase activity was significantly correlated with malt diastatic power, $p < 0.001$ for the Delour and Verschaeve assay and $p < 0.05$ for the Betamyl assay. This is despite the fact that in comparison to barley, alpha-amylase and not beta-amylase, is the predominant diastatic enzyme[16,17]. The correlation between the beta-amylase activity of the malts measured by the two different methods, although significant, was not particularly high ($p < 0.05$), accounting for 44% of the variance

TABLE V. Relationship between sorghum malt diastatic power and beta-amylase activity

	Diastatic power SDU/g[ab]	Beta-amylase activity: Delcour & Verschaeve[cd] SDU/g	Beta-amylase activity: Betamyl[ef] U/g
Malt			
1	18.2	10.2	25.5
2	20.7	11.9	16.6
3	22.6	16.1	28.8
4	24.9	14.6	41.7
5	26.3	15.4	49.3
6	31.8	20.3	33.1
7	32.9	20.4	52.6
8	34.4	20.4	26.4
9	38.8	23.1	44.8
10	39.4	24.3	37.5
11	40.9	25.7	57.2
12	44.1	27.1	56.8

Correlation coefficients

	Beta-amylase (Delcour & Verschaeve)	Beta-amylase (Betamyl)
Diastatic power	0.9855[g]	0.6700[h]
β-Amylase (Betamyl)	0.6638[h]	

[a]Sorghum Diastatic Units/g
[b]Means of triplicate assays
[c]Ref. 27
[d]Means of duplicate assays
[e]Ref. 13
[f]Means of triplicate assays
[g]Significant at the 0.1% level
[h]Significant at the 5% level

(r^2 = 0.4406). The relatively poor correlation is possibly due to the differences between the substrates used in the two assay methods. The Delcour and Vershaeve assay uses soluble starch as substrate[5] and the Betamyl assay uses a dye-labelled maltopentaoside as substrate[13]. It is unlikely that contamination by the malt alpha-amylase is the cause. In the case of the Betamyl assay the small amount of alpha-amylase activity against the maltopentaoside is subtracted to give the true beta-amylase activity. With the Delcour and Verschaeve assay, residual alpha-amylase activity was found to be less than 1% of the original, using both the Phadebas[27] and the Ceralpha (Robbins unpublished data) alpha-amylase assays.

Conclusions

By the use of direct enzyme assays, this investigation confirms that beta-amylase activity in sorghum malt is much lower than in barley malt, and is absent in the ungerminated grain. It shows that sorghum malt beta-amylase activity, unlike barley beta-amylase, is not affected by the application of reducing agents or papain, indicating that the sorghum enzyme is not in a bound form. The sorghum malt beta-amylase, like the barley enzyme, is more temperature-labile than its alpha-amylase. This implies that if conventional lager mashing processes are used with sorghum malt, the already limited beta-amylase activity would be further reduced. Beta-amylase activity in sorghum malt can be manipulated by germination time, temperature and moisture conditions. Sorghum malt beta-amylase activity is significantly correlated with malt diastatic power. Thus in a breeding programme, initial selection of sorghums for high beta-amylase activity could simply be done on the basis of diastatic power.

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