

Assessment of Enzymatic Endosperm Modification of Malting Barley Using Individual Grain Analyses

Roberta M. de Sá^{1,2} and G. H. Palmer¹

ABSTRACT

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Enzymatic modification of the endosperm of malting barley is a main feature of the malting process. Uneven enzymatic modification of the endosperm (heterogeneity) can cause brewhouse problems. Although there is a general correlation between endosperm modification, beta-glucan breakdown and endo-beta-glucanase development, it is based on average results from sample analyses and may conceal heterogeneity. The primary aim of this work was to use individual grain analyses to investigate factors that control endosperm modification and beta-glucan breakdown. In terms of beta-glucan breakdown and physical modification, the barley variety Chariot malted faster than Decanter. However, both varieties showed similar distribution of endo-beta-glucanase in individual grains during malting. Further work on individual grains showed that the correlation between beta-glucan breakdown and endo-beta-glucanase activity was not significant. Surprisingly beta-glucan breakdown did not always correlate with the physical modification of the endosperm. Both these findings suggest that sample analyses of beta-glucan levels and malt beta-glucanase activities are not reliable indicators of the degrees of which malt samples are modified during malting. Since the distribution of beta-glucan in individual grains of the unmalted barley varieties was similar, the total beta-glucan levels of the original barley did not determine the rate at which beta-glucan was broken-down during malting. Although protein studies are at a preliminary stage, the rate of protein breakdown was not correlated with the rate at which beta-glucan was broken down in the malting grain. It is possible that the physico-chemical properties of endosperm storage proteins may limit the rate of beta-glucan breakdown during malting.

Key words: Beta-glucan, endo-beta-glucanase, enzymatic modification, homogeneity, malting barley, protein.

¹International Centre for Brewing and Distilling, School of Life Sciences, Heriot-Watt University, Riccarton, Edinburgh, UK, EH14 4AS.

²Corresponding author. E-mail: r.marins_de_sa@hw.ac.uk

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INTRODUCTION

Barley (*Hordeum vulgare*) is the most important cereal used for malt production. For example, it produces enzymes that hydrolyse (modify) the starchy endosperm and undergoes physico-chemical changes which cause flavour, foam and colour to develop during the brewing process.

Barley endosperm cell walls are composed of about 70% mixed linkage (1,3),(1,4)-D-glucan (hereafter referred to as beta-glucan), which are non-starch polysaccharides, formed by glucose residues linked by $\beta(1,3)$ and $\beta(1,4)$ glucosidic linkages^{4,15,20}. Extensive cell wall breakdown by endo-beta-glucanase is an important feature of high quality malts used for making beer or Scotch Whisky. During the malting process, beta-glucans are degraded, from approximately 3.0–4.5% to 0.2–1.0%²⁰.

The term enzymatic modification is used to describe changes in the friability of the endosperm of barley grains during the malting process¹. The rate of modification depends on (a) the distribution of moisture through the starchy endosperm; (b) the rate of synthesis of hydrolytic enzymes; (c) the extent of release of these enzymes into the starchy endosperm and (d) structural features of the starchy endosperm that determine its resistance to digestion⁴.

Undegraded proteins and cell walls of the endosperm will limit the movement of all the endosperm degrading enzymes in the starchy endosperm, producing an uneven pattern of modification of the endosperm^{19,20}. This results in diminished extraction of storage reserves, low yields of brewer's extract, in addition to slow wort separation (filtration) and reduced rates of beer filtration. Hazes may also develop in the stored beer^{1,6,15,20,22}.

Some beta-glucanases have been identified in malt. These are endo- β -1,3-glucanase (EC 3.2.1.39), endo- β -1,4-glucanase (EC 3.2.1.4) and endo-1,3;1,4- β -glucanase (EC 3.2.1.73). Of these, endo- β -1,4-glucanase is associated with the husk. Endo-1,3;1,4- β -glucanase and endo- β -1,3-glucanase are produced in the aleurone layer and, with a non-specific enzyme called β -glucan solubilase, hydrolyse the cell walls of the starchy endosperm^{5,6,9,10,22}.

Endo-1,3;1,4- β -glucanase is the main cell wall degrading enzyme and is almost absent in raw barley, but increases, to maximum levels in five to six days after the initiation of the germination phase of malting^{6,9}. Gibberellic acid is produced in germinated embryos and induces the production of endo-1,3;1,4- β -glucanase in the aleu-

rone layer²⁰. The amount of endosperm degrading enzyme produced is related to levels of gibberellic acid and the responsiveness of aleurone layers of different barley varieties to the hormone^{7,17}. Endo-1,3;1,4- β -glucanase is heat sensitive. About 40% of this enzyme is lost during kilning⁶. Endo-1,3;1,4- β -glucanase is inactivated rapidly during mashing (i.e. 65°C). If beta-glucans are not broken down during malting, they will not be broken down during mashing¹⁸.

Cell wall modification (beta-glucan breakdown) is regarded as a key step in extract development (endosperm modification) during malting¹. It has been suggested that the rate of modification of different samples of barley is principally determined by their content of cell wall beta-glucan and their ability to synthesise endo-beta-glucanase⁴. Therefore, low residual beta-glucan in malted barley is regarded as an indicator of the grain capacity to synthesise high levels of endo-beta-glucanase during malting⁹.

However, malt samples with low total beta-glucan levels and high endo-beta glucanase activity can cause brew-house problems. These problems might be related to heterogeneity (inhomogeneity^{18,20}) of endosperm modification, where a small but significant number of grains had limited breakdown of cell wall (beta-glucan) material. This kind of heterogeneity cannot be detected using traditional malt analysis, which are based on averages^{11,12,19,21}.

The purpose of this study was to analyse malt beta-glucanase activity and beta-glucan breakdown in individual grains of malt, and determine if these parameters were correlated with overall physical modification of the starchy endosperm.

MATERIALS AND METHODS

Barley quality and malting

Barley, varieties Decanter and Chariot were harvested in Scotland in 2000 and 2001, respectively. Extraneous matter and small grains (<2.2 mm) were removed. The germinative energy and total nitrogen content of the barley varieties were determined. Barley samples (300 g) were steeped at 16°C. A first steep of 8 h was followed by 16 h air rest and a 24 h final steep. A Seeger micro-malting unit was used. Germination was for four days, at 16°C. Germinative percentage was checked daily. Samples were collected after 48, 72 and 96 h of germination. Part (~100 g) of the resulting green (unkilned) malt was frozen and the rest was kilned at 65°C, for 16 h.

Assay of total beta-glucan content and malt endo-beta-glucanase activity

Barley and the resulting malt samples were analysed for their total beta-glucan content² and malt beta-glucanase activity, using the Azo-Barley Glucan Method¹⁶. All assays were performed in triplicate and results are expressed on a dry basis.

Individual grain analyses

In order to evaluate individual grains, the enzymatic method for determination of beta-glucan content² was scaled down as follows: each grain was crushed using mortar and pestle and transferred to a 15 mL polypropylene

tube. To each tube 0.4 mL 50% ethanol was added; the content was mixed using a vortex stirrer and the mixture was incubated for 2 min in a boiling water bath. A further 0.4 mL 50% ethanol was added, mixed and centrifuged for 10 min at 1000 \times g. The supernatant was discarded and the pellet was resuspended in 0.8 mL 50% ethanol, mixed and centrifuged as above. The supernatant was discarded and the remaining pellet was resuspended in 0.4 mL of sodium phosphate buffer (20 mM, pH 6.5) and incubated in a boiling water bath for 2 min, mixed and followed by another incubation for 3 min. To reduce viscosity of the mixture, 0.4 mL of distilled water was added, before adding 16 μ L of lichenase (EC 3.2.1.73, 50 U/mL in 20 mM phosphate buffer, pH 6.5). The tubes were incubated at 40°C for 1 h. The volume was then adjusted to 2.4 mL with the addition of 1.5 mL distilled water, thoroughly mixed and centrifuged at 1000 \times g for 10 min. The resulting supernatant was assayed with β -glucosidase (EC 3.2.1.21, 2U/mL in 50 mM sodium acetate buffer, pH 4.0) and analysed for glucose content according to the original method². This procedure was routinely calibrated against a barley flour standard supplied by Megazyme International Ireland Limited (Bray, County Wicklow, Ireland).

In order to analyse single grains, the Azo-Barley Glucan method¹⁶ had to be miniaturized and another calibration curve was necessary. The enzymes most effective in releasing dye-labelled fragments from Azo-barley glucan that are soluble in the presence of the precipitant are malt beta-glucanase and lichenase. These enzymes have very similar action patterns and their standard curves are very similar¹⁶.

To prepare the calibration curve lichenase (endo-1,3-1,4- β -D-glucanase – EC 3.2.1.73, Megazyme International) was diluted in 40 mM sodium acetate and 40 mM sodium phosphate, pH 4.6, obtaining activities ranging from 0 to 0.15 U/mL. Using Azo-barley glucan (Megazyme International) as substrate, the resultant equation for the curve was:

$$\text{Activity (U)} = 0.0069 + 0.2702 \times \text{Abs}$$

(where Abs = Absorbance at 590 nm minus blank, with $S = 0.004$ and $R^2 = 97.8\%$). The method has a *Detection Limit* (L_D) of 0.072 and *Determination Limit* (L_Q) of 0.135³.

Samples of green (unkilned) malt were defrosted and used immediately. Each grain was ground using mortar and pestle and transferred to a polypropylene tube with 4.0 mL of extraction buffer solution (40 mM sodium acetate/40 mM sodium phosphate, pH 4.6). The content was stirred thoroughly using a vortex mixer and the enzyme was extracted at room temperature over exactly 15 min, with occasional mixing. The tubes were centrifuged at 1000 \times g for 10 min and the extract was assayed for malt beta-glucanase activity according to the Azo-barley glucan method¹⁶. Control malt, supplied by Megazyme International, was used to correct the activity obtained for each grain. The values were also corrected for the volume as follows: $U/\text{grain} = U/0.5 \text{ mL of extract} \times 8$ (from a total extract of 4 mL, 0.5 mL is used in the assay).

To assess the relationship between beta-glucan levels in individual grains and their corresponding overall degree of modification, 100 grains of kilned malt samples were

selected at random and classified visually after hand sectioning as follows: UM: Undermodified; PM: Partly Modified; M: Modified; WM: Well Modified¹⁹. After classification, each grain was analysed for its beta-glucan content. One hundred single grains of the barley samples from which the malts were made were also analysed for their beta-glucan content.

Dumas combustion method² was used to analyse total nitrogen in individual grains. A combustion nitrogen analyser (Leco FP-528 Protein/Nitrogen analyser) was calibrated with nitrogen standards and EDTA. Individual grains (100) of each barley sample were crushed, weighed and analysed for their individual total nitrogen content. The analyses were performed at the quality assurance laboratory at Simpsons Malt (Craigswalls, Duns, Scotland, UK).

Mashing

The grains were ground using the Buhler-Miag mill, at setting 7. The flour (10 g) of each sample was weighed into mashing beakers, in duplicate. The flour was mashed with 72 mL of distilled water at 65°C for 1 h. After cooling to 20°C the mash was washed into a 100 mL volumetric flask and a further 3 mL distilled water was added, making up the final volume to 103 mL. The content was then filtered and the wort collected for subsequent analyses.

Wort analyses

The analyses performed on the worts were hot water extract, beta-glucan, free amino nitrogen and total soluble nitrogen (spectrophotometric method). Wort analyses were carried out in triplicate, as described in Analytica-EBC².

Statistical analyses

Statistical analyses of data were performed using Minitab (Minitab, Inc., release 13.1). The methods used were One Way ANOVA for total nitrogen and beta-glucan in individual barley grains and General Linear Model for beta-glucan and endo-beta-glucanase in malt samples and individual malt grains, as well as for the wort analyses, using variety and time of germination as fixed factors. Comparisons of means, descriptive statistics, distribution and correlation were also carried out.

RESULTS AND DISCUSSION

Sample analyses

Comparative analyses of barley and malt samples are presented in Table I. Although germination percentages were similar, beta-glucan breakdown was faster in Chariot than in Decanter. The faster breakdown in Chariot was as-

sociated with a faster development of endo-beta-glucanase. The friability of the endosperm of malted barley is an indication of the action of the endosperm degrading enzymes, such as endo-beta-glucanase. Table I shows that as beta-glucan declined, the physical modification of the endosperm, as indicated by friability scores, increased. These results confirm previous studies^{19,20} which propose that enzymatic breakdown of beta-glucans of endosperm cell walls is an essential feature of normal enzymatic conversion of the hard endosperm of barley into the friable endosperm of malt.

The correlation between total beta-glucan content and endo-beta-glucanase activity was highly significant, with $r = -0.90$ ($p = 0.00$). Chariot had lower levels of beta-glucan and higher levels of endo-beta-glucanase at the end of 96 h of germination, than Decanter.

Individual grain analyses

Beta-glucan. The results presented in Table I are based on traditional analyses of samples of barley and malt. In general, they give no indication of the distribution (homogeneity) of endo-beta-glucanase development and beta-glucan breakdown, and the different degrees of physical modification, which may exist in each grain of the malt sample^{19,21}. In order to assess the homogeneity of beta-glucan breakdown, the existing enzymatic method for beta-glucan determination² was adapted to analyse individual grains rather than normal samples of barley and malt.

In this way, it was possible to observe the physiological response of each variety of barley during germination, in terms of beta-glucan breakdown between individual grains. Fig. 1 shows the distribution of beta-glucan content (mg/grain) in 100 grains of each malt sample.

After 48 hours of germination a wide distribution of beta-glucan content within the grains was observed, indicating that degradation of beta-glucan was already in progress. Decanter presented a wider distribution of beta-glucan content between individual grains than Chariot. After 72 h the drift towards lower levels of beta-glucan was more evident for Chariot, suggesting a faster breakdown of beta-glucan in certain grains. At the end of 96 h Chariot presented a distinctly skewed distribution of beta-glucan breakdown, with most of the grains having very low levels of beta-glucan, whereas Decanter still had many grains with higher levels.

Based on the observation that there was a significant correlation between total beta-glucan breakdown and total endo-beta-glucanase activity in malt samples (Table I), it was possible that the faster breakdown of beta-glucan in Chariot reflected faster development of endo-beta-glucanase activity in the malt grains of Chariot.

Table I. Comparative analyses of barley and malt samples.

Parameter	Decanter				Chariot			
	Barley	48H	72H	96H	Barley	48H	72H	96H
Germination (%)	97	96	97	99	98	98	99	99
Total nitrogen (%)	1.8				1.7			
Total beta-glucan (%)*	3.2 ± 0.2	1.7 ± 0.04	0.9 ± 0.03	0.5 ± 0.03	3.3 ± 0.05	0.9 ± 0.02	0.4 ± 0.01	0.2 ± 0.01
Beta-glucanase (U/kg)*	23.4 ± 0.3	103.0 ± 9.6	181.3 ± 2.4	239.0 ± 10.8	10.6 ± 1.4	214.8 ± 5.17	387.1 ± 28.6	411.1 ± 26.6
Friability (%)		45.8	68.3	80.0		59.0	76.5	84.0

*Mean ± SD ($n = 3$)

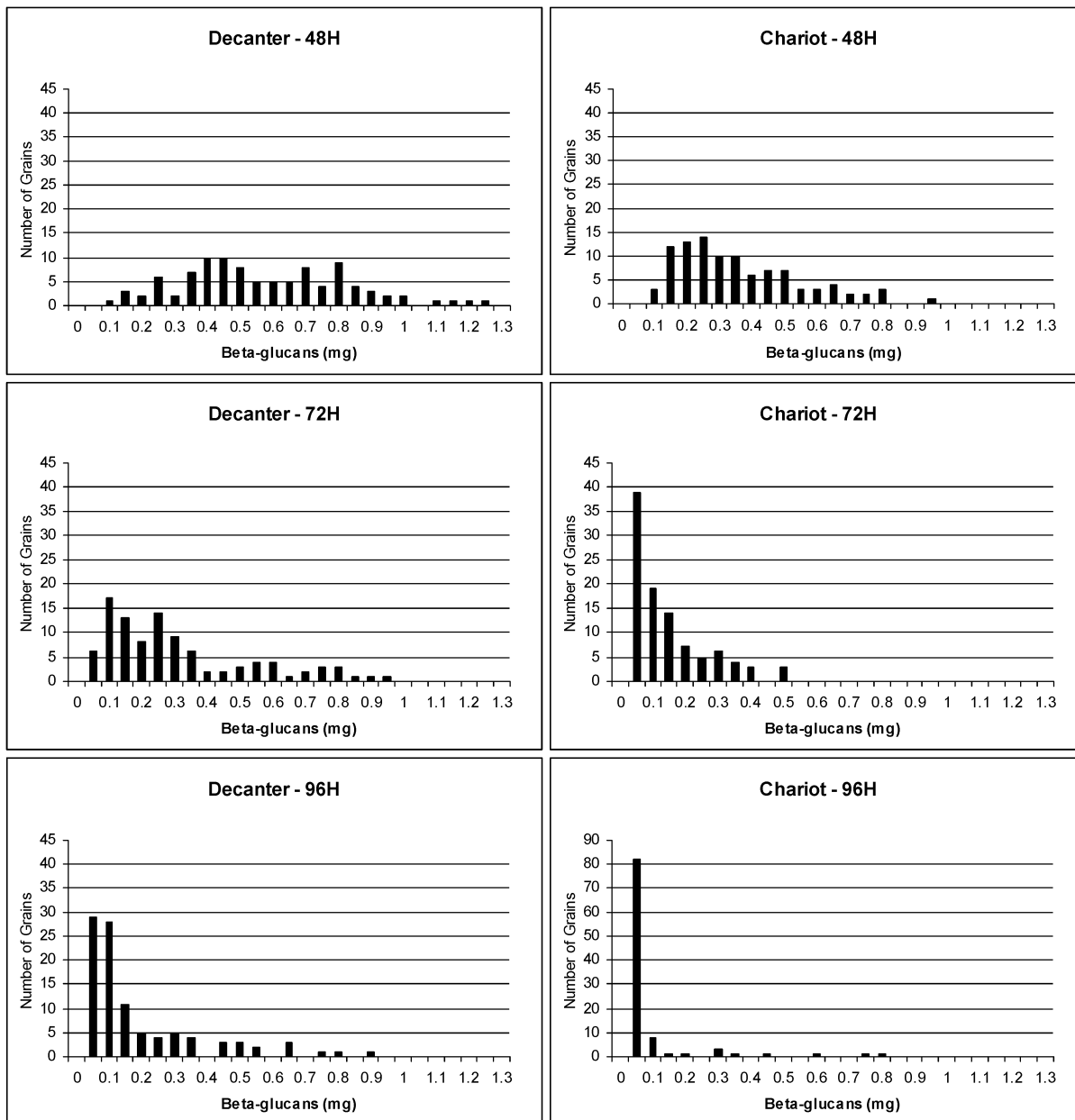


Fig. 1. Distribution of beta-glucan content (mg/grain) in 100 individual grains of malt (varieties Decanter and Chariot) after 48, 72 and 96 h of germination (kilned malt).

Endo-beta-glucanase activity. To assess the relationship between beta-glucan breakdown and beta-glucanase development, 100 individual grains of each malt sample were analysed for their endo-beta-glucanase levels. The levels of endo-beta-glucanase in individual grains of kilned malt were very low, and could not be assessed accurately. In this regard, the enzyme levels of kilned malts do not reflect physiological levels of enzymes which break down the endosperm during malting. Therefore, to assess the distribution of endo-beta-glucanase activity in individual grains, green (unkilned) malt was used (Fig. 2). Values below the *Determination Limit* (D_Q) were considered as 0 (zero).

At 48 and 72 h of germination, the distributions of endo-beta-glucanase in individual grains were very similar for the two varieties studied. The main difference ob-

served was after 96 h of germination. At this germination time 55% of the Decanter grains had endo-beta-glucanase levels between 0.5 and 0.7 U/grain and 36% had levels between 0.8 and 1.2 U/grain, while Chariot had 31% and 60% of grains with these levels, respectively.

The correlation between beta-glucan content and endo-beta-glucanase activity in individual grains was not significant ($p < 0.05$). The weak relationship observed between beta-glucan breakdown and endo-beta-glucanase development in individual grain analyses did not confirm the strong correlation between beta-glucan breakdown and beta-glucanase levels observed when samples of malt were analysed.

The differences observed, when comparing standard sample analyses with individual grain analyses may relate to the wide variations of beta-glucan levels and endo-beta

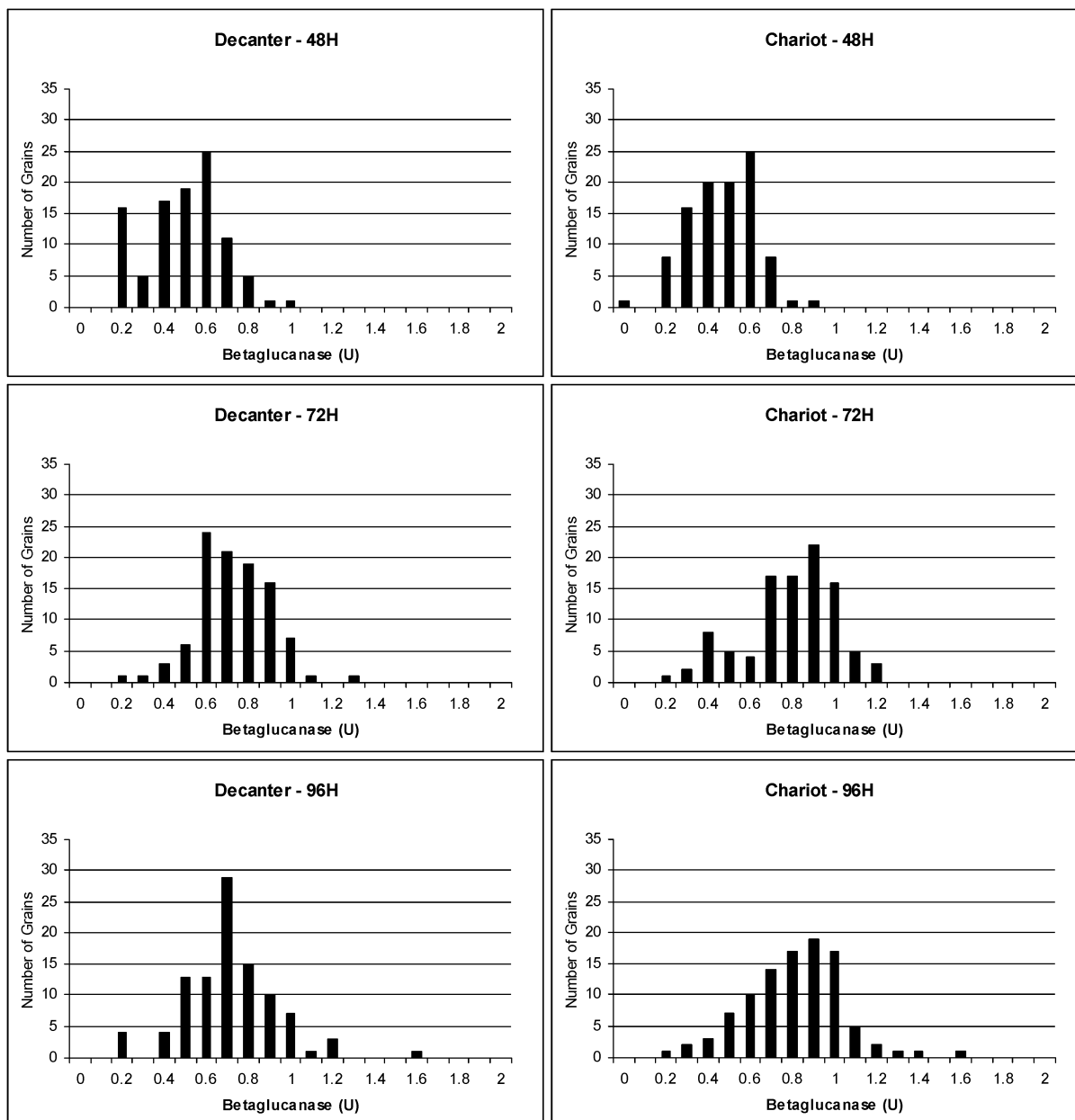


Fig. 2. Distribution of endo-beta-glucanase activity (U/grain) in 100 individual grains of malt (varieties Decanter and Chariot) after 48, 72 and 96 h of germination (unkilned malt).

glucanase activity when individual grains of each sample are analysed. Using standard methods of analyses an amount of sample is milled and extracted, turning an inhomogeneous sample of grains into a homogenous mixture, which conceals original inhomogeneity. Furthermore, the weak relationship between beta-glucan content and endo-beta-glucanase activity in individual grains could be due to the structure of the endosperm, which may limit enzyme activity^{8,16,19,21}.

Assessment of physical modification of the endosperm

After hand sectioning¹⁹, 100 grains of each malt sample were classified visually according to their degree of physical modification. Fig. 3 presents the evaluation of physical

modification in each variety, according to time of germination. The varieties studied presented different patterns of physical modification. Chariot modified faster, and at the end of 96 h of germination 17% of its grains were under-modified, whereas 41% of the corresponding grains of Decanter were under-modified.

After visual inspection and classification of the grains according to their degrees of modification¹⁹, the same grains were analysed for their content of beta-glucan. The data of beta-glucan content and degree of physical modification is presented at Table II.

In Table II it is evident that well-modified grains had very low beta-glucan content. However, taking into consideration the mean beta-glucan values for under-modified grains, the relationship between beta-glucan content and degree of physical modification is not clear, because the

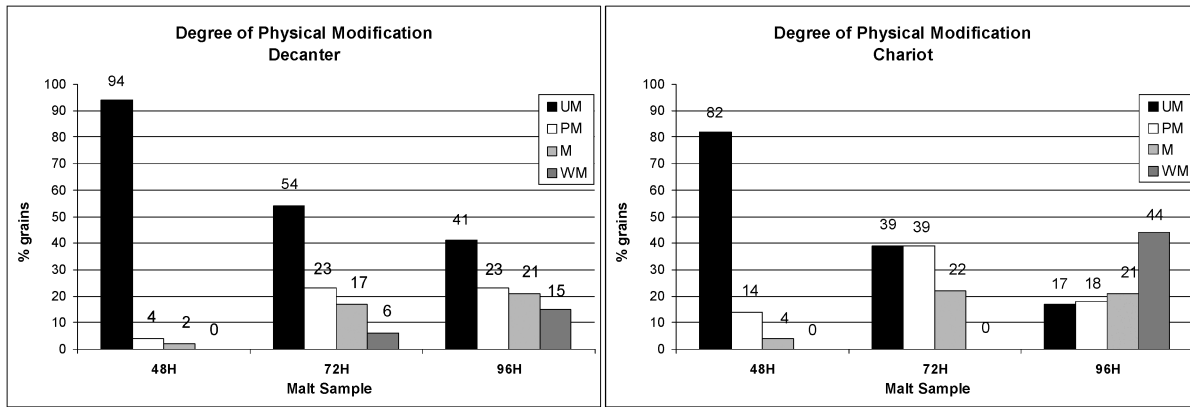


Fig. 3. Visual percentages of physical modification in malt samples of the varieties Decanter and Chariot (kilned malt). UM = undermodified; PM = partly modified; M = modified; WM = well modified.

Table II. Relationship between beta-glucan content (mg/grain) and degree of physical modification within malt samples.

	Decanter						Chariot					
	48H		72H		96H		48H		72H		96H	
	<i>n</i>	β	<i>n</i>	β	<i>n</i>	β	<i>n</i>	β	<i>n</i>	β	<i>n</i>	β
UM	94	0.58	54	0.40	41	0.30	82	0.36	39	0.16	17	0.19
PM	4	0.18	23	0.20	23	0.09	14	0.25	39	0.12	18	0.06
M	2	0.15	17	0.11	21	0.08	4	0.17	22	0.05	21	0.05
WM	0	nd	6	0.16	17	0.07	0	nd	0	nd	44	0.02

UM = undermodified; PM = partly modified; M = modified; WM = well modified.
n = Number of grains; β = mean beta-glucan content (mg/grain); nd = not detected.

Table III. Mean beta-glucan (mg/grain) and total nitrogen (%) of individual grains of barley, varieties Decanter and Chariot.

	Beta-glucan (mg/grain)				Nitrogen (%)			
	Mean	SD	Min.	Max.	Mean	SD	Min.	Max.
Decanter	0.86	0.18	0.38	1.23	1.42	0.20	0.89	1.98
Chariot	0.96	0.20	0.54	1.49	1.42	0.29	0.83	2.61

Mean, standard deviation (SD), minimum and maximum of 100 barley grains.

beta-glucan contents of under-modified grains are very variable. It appears that degree of physical modification is not based only on beta-glucan content.

The discrepancies between the two varieties studied, in terms of beta-glucan breakdown and degree of physical modification, may reflect differences in protein breakdown during malting of barley. The random distribution of grain nitrogen (protein) in the endosperm could result in uneven breakdown of beta-glucan^{19,21}.

Relationship between barley nitrogen (protein) and beta-glucan breakdown

The beta-glucan (mg/grain) and total nitrogen content (%) of individual grains of barley samples were analysed. Table III presents the mean values of barley samples, which consisted of 100 grains each.

Chariot had higher mean individual beta-glucan content than Decanter (95% confidence intervals for mean based on pooled standard deviation). In terms of distribution of the beta-glucan content of individual grains (Fig. 4), Chariot had a tendency towards the higher levels of beta-glucan, while Decanter had more grains with lower

levels of beta-glucan. These results suggested that the higher beta-glucan levels in the grains of Chariot did not limit beta-glucan breakdown during malting (see Fig. 1).

In terms of individual grain total nitrogen, there was no statistical difference between the Chariot and Decanter (95% confidence intervals for mean based on pooled standard deviation). However, there were substantial differences as regards the distribution of nitrogen between individual grains. Fig. 5 shows that Chariot had a more uniform and wider distribution of total nitrogen (%) in 100 barley grains. This distribution might be associated with the differences in beta-glucan breakdown and degree of physical modification observed for the two varieties. In addition to this possibility, the nitrogen (proteins) of Decanter may have physico-chemical properties that limit the activity of malt beta-glucanase during malting^{19,21}.

Wort properties of Decanter and Chariot malts

The results for wort analyses are presented at Fig. 6. As expected, Chariot gave a higher extract yield and lower wort beta-glucan after 96 h of germination than Decanter. The beta-glucan levels in the worts reflect beta-glucan

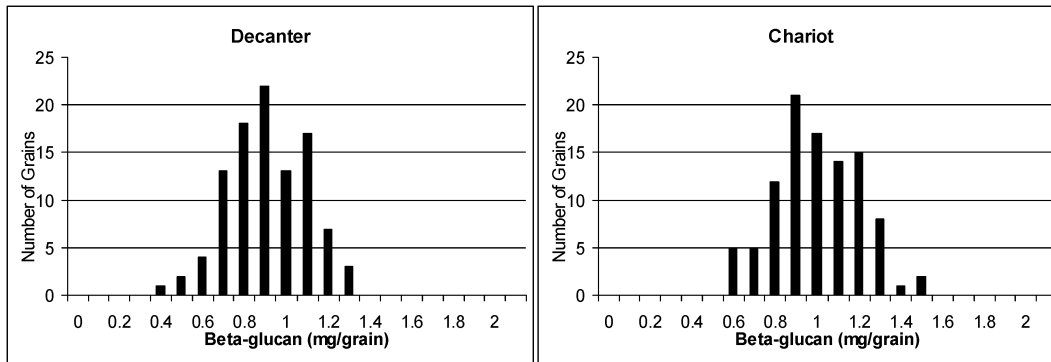


Fig. 4. Distribution of beta-glucan content (mg/grain) in 100 individual grains of barley, varieties Decanter and Chariot.

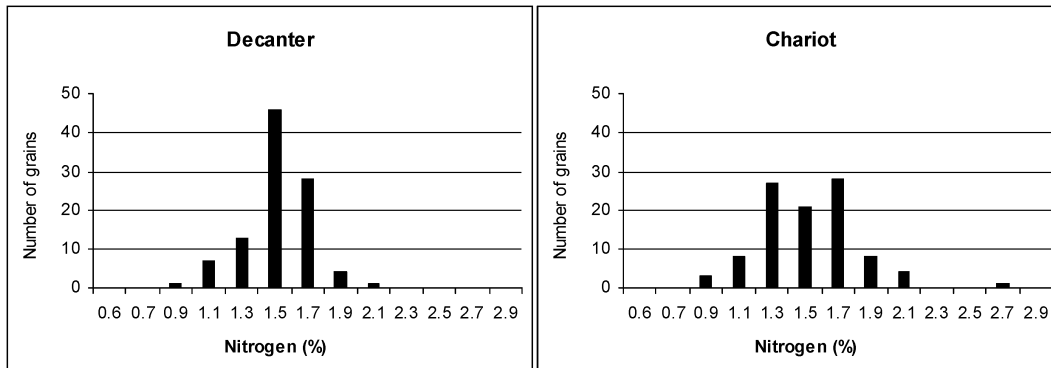


Fig. 5. Distribution of total nitrogen (%) in 100 individual grains of barley, varieties Decanter and Chariot.

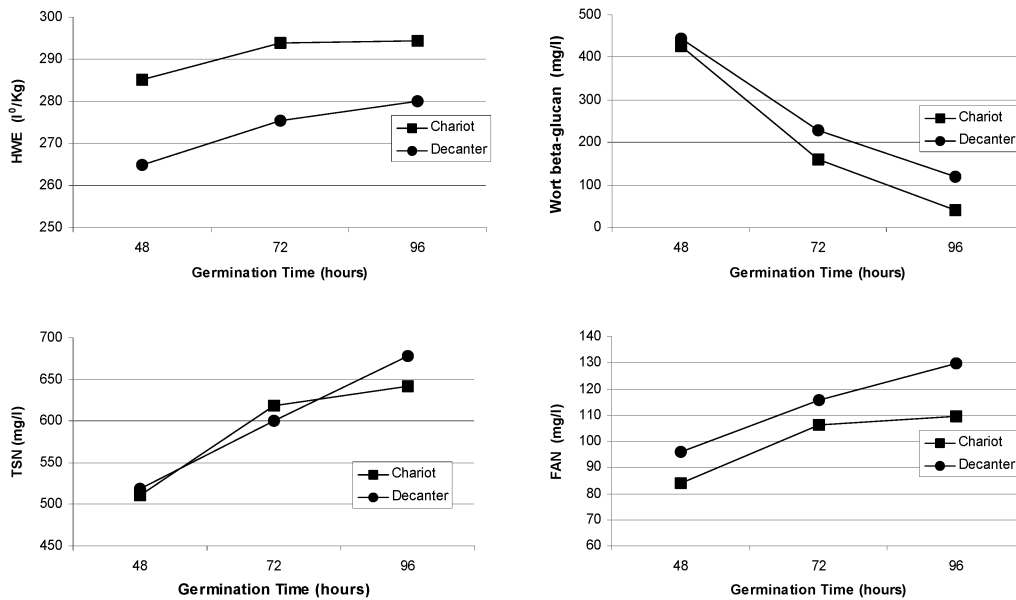


Fig. 6. Hot Water Extract (HWE, 1°K/g), beta-glucan (mg/L), Total Soluble Nitrogen (TSN, mg/L) and Free Amino Nitrogen (FAN, mg/L) in wort samples, varieties Decanter and Chariot.

levels in the malts. Although protein breakdown in Decanter was faster than Chariot at 96 h, beta-glucan breakdown was slower, as regards higher levels of beta-glucan both in malt and wort.

Although preliminary, the results suggest that the rate of protein solubilization in Decanter (Fig. 6) was not asso-

ciated with the rate of beta-glucan breakdown (Fig. 1). However, if protein breakdown is associated with beta-glucan in the endosperm during malting, in Decanter protein breakdown may have been localised. Although differences in endosperm structure can cause both localised over-modification of protein and under-modification of

beta-glucan²⁰, future work is required to establish that the endosperm of Decanter contains such variations in structure^{19,21}.

CONCLUSIONS

The faster rate of development of friability and brewer's extract in Chariot, in contrast to Decanter, seem to reflect the faster and more uniform rate of beta-glucan breakdown in the endosperm during malting. Since this faster rate of beta-glucan breakdown in individual grains was not always linked to high levels of endo-beta-glucanase in individual grains, it seems that the structural properties of the endosperm of the Chariot sample might have caused this barley to modify faster than Decanter. These results confirm studies carried out on these varieties^{13,14} and the industrial expectations, as Chariot has higher malting grade than Decanter.

In general, the structure of beta-glucan and protein of the endosperm of Decanter may be less homogenous than the corresponding properties of the endosperm of Chariot. Indeed, earlier studies have shown that variations in endosperm structure can cause differences in the rates at which the endosperm is modified enzymatically during malting. Finally, results derived from the analyses of individual grains of barley and malt suggest that traditional analysis of samples of barley and malt give very little indication of the distribution (homogeneity) of beta-glucan breakdown, endo-beta-glucanase development and physical modification which occur during the production of malt. Without knowledge of this distribution, brewhouse problems may occur using malt samples that are expected to process satisfactorily.

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