MBAA TQ vol. 42, no. 3 • 2005 • pp. 184–198

PEER-REVIEWED PAPER

Mashing with Unmalted Barley—Impact of Malted Barley and Commercial Enzyme (*Bacillus* spp.) Additions

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ABSTRACT

This paper reports on the effects that the addition of both malted barley and commercial enzymes (*Bacillus* spp.) has on the processability and quality of worts when brewing with inclusions of raw barley. Increased inclusion of malted barley resulted in increases in extract recovery levels, wort α-amino nitrogen levels, and fermentability and in decreases in wort viscosity and β-glucan levels. While increases in wort amino acid levels resulted from inclusions of high levels of malt, the endogenous malt enzymes were found to exhibit very poor raw barley protein-hydrolyzing ability. Likewise, the endogenous malt amylases were found to exhibit very poor raw barley starch-hydrolyzing ability. As the level of malt was increased, its raw barley hydrolytic effects decreased. When mashing with 100% raw barley substrate and commercial enzymes, exogenous protease (*B. subtilis*) additions yielded increases in total soluble nitrogen levels, α-amino nitrogen levels, wort color, and extract recovery levels. However, the protease efficiency decreased as the level of protease was increased. Exogenous β-glucanase (*B. subtilis*) had little impact on mash filtration, but it reduced high-molecular-weight wort β-glucan levels. Exogenous α-amylase (*B. subtilis*) was found to have the greatest positive impact on mash separation. Likewise, exogenous α-amylase level increases resulted in higher wort glucose and maltotriose levels and lower maltose levels. Optimal addition of an exogenous high-heat thermostable α-amylase (*B. licheniformis*) in combination with the exogenous α-amylase (*B. subtilis*) was found to be necessary for complete starch conversion and maximum extract recovery from the raw barley substrate.

Keywords: adjunct, barley, commercial enzymes, enzymes, mashing, wort quality

SÍNTESIS

Se presentan los efectos que tiene la adición de cebada malteada o de enzimas comerciales (*Bacillus* spp.), sobre la procesabilidad y la calidad de mostos elaborados con la inclusión de cebada no malteada. Un aumento de la cantidad de malta agregada resultóa en un aumento del rendimiento de extracto, de la fermentabilidad y del contenido de alfa amino nitrógeno, a la vez que disminuyó la viscosidad y los niveles de β-glucanos del mosto. Si bien la utilización de altas cantidades de malta aumenta los niveles de amino ácidos en el mosto, las enzimas endógenas de la malta exhibieron una muy pobre capacidad de hidrolización de las proteínas y del almidón de la cebada. Al aumentar la proporción de malta, disminuye su efecto hidrolízante sobre la cebada. Al macerar con 100% cebada cruda junto con enzimas comerciales, el aumento de proteasa exógena (*B. subtilis*) resultó en un aumento de nitrógeno soluble total, alfa amino nitrógeno, color del mosto y mayor rendimiento de extracto. Sin embargo, la eficiencia de la proteasa disminuyó al aumentar la proporción de proteasa agregada. La β-glucanasa exógena (*B. subtilis*) tuvo poco impacto sobre la filtración del macerado, pero redujo los niveles de β-glucanos de alto peso molecular en el mosto. La α-amilasa exógena (*B. subtilis*) tuvo su mayor impacto sobre la filtración del macerado, pero resultó en mayores niveles de glucosa y maltotriosa, y menores niveles de maltosa, en el mosto. Fue necesario optimizar la adición de una α-amilasa exógena termoestable a altas temperaturas (*B. licheniformis*) en combinación con una α-amilasa exógena (*B. subtilis*) para conseguir una conversión total del almidón y una recuperación máxima de extracto de la cebada cruda.

Palabras claves: adjuntos, cebada, enzimas comerciales, enzimas, maceración, calidad del mosto

Introduction

In traditional brewing, malted barley is the grain of choice. It is preferred because it acts as a raw material supplying starch and protein, while also contributing a sufficient supply of cytolytic, proteolytic, and amylolytic enzymes, which are necessary for the efficient production of wort. In addition, the presence of a husk aids mash filtration, while the malt kilning process produces flavor and color compounds, which can significantly add to the character of beer.

It has been reported (5) that when the total cost of beer production is taken into consideration (from raw material purchase and processing through to packaging, sales, and taxation), malt costs in general have been estimated to represent just ~3.5% of the total cost. Therefore, it becomes apparent that grain costs represent only a proportionately minor contribution to the total cost of beer production. Then, why replace malted barley with an unmodified substrate "adjunct"? In less developed countries, malting facilities and malting conditions are quite often less than optimal. Therefore, because of its lower price, locally produced adjunct material can be used to

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Based on a paper presented at the World Brewing Congress 2004, San Diego, CA, July 2004.

DOI: 10.1094/TQ-42-0184

supplement malted barley grain (35). Apart from the direct cost of raw materials, indirect costs (much greater than the direct costs) can also influence raw material selection. In Kenya, for example, beer made from unmalted grain is taxed at 60% the rate of beer made from malted grain (19). Kenyan brewers are therefore encouraged to develop beer from exclusively nonmalted grain (mainly, raw barley). Likewise, in Japan, a much lower rate of taxation is applied to products containing less than 50% malt (e.g., happoshu) (15,87). Therefore, Japan's brewers have a great incentive to brew products from grists containing adjunct levels in excess of 50%. Likewise, in Nigeria, a government economic decision to ban the importation of malted barley forced local brewers to develop alternative brewing procedures to utilize locally grown sorghum and maize crops (36,57). Additionally, factors associated with product quality, tradition, and consumer product expectations can be the decisive reason to use adjuncts, such as the impact that rice has on the flavor, color, and colloidal stability of an American pale lager. Likewise, much of the distinct flavor profile of an Irish whiskey can be attributed to the traditional use of high proportions of raw barley in its manufacture (12).

The use of barley over other adjuncts offers significant advantages to the brewer. Since its starch has a gelatinization temperature similar to malted barley, it can be easily incorporated into conventional malted barley mashing procedures (78). Its endogenous β-amylase (64) ensures maltose production during mashing. Likewise, the presence of a husk can aid mash filtration through a traditional lauter tun (19). However, because of its low levels of essential enzymes (e.g., α-amylase, proteases, and glucanases), together with a relatively inaccessible starchy endosperm, high inclusions of unmalted barley (>20%) in the mash (without the aid of commercial enzymes) can lead to problems, such as low extract yields, high wort viscosities, decreased rates of lautering, fermentation problems, and beer haze problems (84,95). The cell walls in the starchy endosperm of barley are reported (28) to be composed of 75% β-(1-3)(1-4)-glucan, 20% arabinoxylan, and 5% protein, plus traces of other constituents, including ferulic acid. There is a significant body of knowledge relating to the structures of the β-glucan (45) and pentosan (22) components of these walls and their degradation during malting and brewing. Many of the problems associated with barley β-glucan are due to the high propensity of the polysaccharide to form aqueous solutions of high viscosity (43). This can impede the passage of liquid through the grain filter bed, resulting in increased lautering times (13,45,55,70,85). High levels of wort β-glucan are reported to impair beer filtration (8,40,45,73,74,83) and decrease the colloidal stability of beer (3). It has been shown that xylanases are capable of releasing β-glucan (53), which suggests that there is some masking of glucan by pentosan in the cell wall. Likewise, xylanases are capable of releasing all the pentosan components, thus indicating that, in comparison to glucan, it is fully accessible to enzymatic attack (53).

While hydrolysis of adjunct is often achieved by using the enzyme capacity of malted barley, high adjunct levels may dilute the malt enzymes to a limiting level. They are then required to be augmented or replaced by commercial enzyme preparations. The enzyme preparations are usually available as single products or as part of mixed-enzyme cocktails (17,78,82). Exogenous proteolytic activity is required to modify endosperm structure, facilitate saccharification, release bound β-amylase, and adjust the ratio of soluble nitrogen necessary for yeast growth (78,82). The most suitable preparation reported (78,82) is one containing only bacterial neutral protease from *Bacillus subtilis*. Addition of β-glucanase enzyme from *Bacillus*, *Aspergillus*, *Penicillium*, or *Trichoderma* sources has been found to improve filtration when undermodified malt or unmalted barley is present in the mash (56,77). High-heat thermolabile α-amylase derived from *B. subtilis* is widely used for the degradation of gelatinized starch and high-molecular-weight dextrins to lower-molecular-weight dextrins and fermentable sugars (17,62,78,82). An alternative is to use high-heat thermostable bacterial α-amylase from *B. licheniformis*, which is reported (62) to require a shorter contact time at higher temperatures, lower enzyme dose rates, and lower calcium ion concentrations. This enzyme has an optimum temperature range of 85–87°C and is inactivated only at temperatures close to boiling. Thus, it can hydrolyze a difficult starch even though swelling and gelatinization occur at higher temperatures than usual (62).

While many reviews (6,11,17,62,63,78,82,88,96) have been published on the use of commercial enzymes in brewing, a moderate volume of research literature (14,18,19,35,58,75,97) is available on the specific area of brewing with high levels of raw barley adjunct. The overall purpose of this study was to determine the effects on mash processability, mash filtration, and wort quality when mashing with (i) grists containing different inclusion levels of malted barley and unmalted barley, and (ii) grists containing 100% raw barley supplemented with different types and dosage levels of commercial enzyme preparations (neutral protease, β-glucanase, thermolabile α-amylase, and thermostable α-amylase, all from *Bacillus* spp.).

Materials

Unmalted barley (cultivar Optic, Irish harvest 2001) and malted barley (cultivar Optic, Irish harvest 2001) were kindly provided by the Malting Company of Ireland (Cork, Ireland) (Table 1). The commercial enzyme preparations Promalt (cocktail consisting of bacterial protease and thermolabile α-amylase and β-glucanase enzymes [all from *B. subtilis*]; IUB/EINECS/CAS nos.: 3.2.1.6/263-462-4/62213-14-3), Bioprotease N100L (bacterial [*B. subtilis*] neutral protease; IUB/EINECS/CAS

Table 1. Analysis of barley and malt samples

Analysis	Unmalted barley	Malted barley
Homogeneity (%)	n/a[a]	98.5
Friability (%)	n/a	96.3
Diastatic power (WK[b])	n/a	205
Moisture (%)	10.09	1.76
Nitrogen (%, dry wt)	1.50	1.62
Protein (%, dry wt)	9.38	10.13
Total soluble nitrogen (mg/L)	470.75 ± 4.52[c]	621 ± 5.89
Free amino nitrogen (mg/L)	72.97[c]	106 ± 8.9
β-Glucan (%)	2.84[c]	0.27 ± 0.03
Extract (%, dry wt)	77.39 ± 0.19[c]	79.66 ± 0.54
pH	6.09 ± 0.005[c]	6.11 ± 0.009
Color (EBC)	3.34 ± 0.197[c]	3.32 ± 0.067
Viscosity (mPa·s)	1.87 ± 0.008[c]	1.64 ± 0.04
Filterability	Slow[c]	Normal
α-Amylase (Ceralpha[d] U/g of dry grain)	0.061 ± 0.008	149.58 ± 8.66
β-Amylase (Betamyl[d] U/g of dry grain)	774.21 ± 4.73	606.50 ± 15.12
β-Glucanase (U/kg of dry grain)	20.59 ± 13.88	213.96 ± 0.28

[a] n/a = Not applicable.
[b] WK = Windisch Kolbach units.
[c] Results obtained by using the EBC Congress mashing procedure on a grist of 50% malted barley and 50% unmalted barley.
[d] Megazyme Ltd., Bray, Ireland.

nos.: 3.4.24.4/232-991-1/9080-56-2), Bio-BAA (α-amylase [*B. subtilis*]; IUB/EINECS/CAS nos.: 3.2.1.1/232-565-6/9000-90-2), Bioglucanase B10L (β-glucanase [*B. subtilis*]; IUB/EINECS/CAS nos.: 3.2.1.6/232-462-4/62213-14-3), and Hitempase 2XL (thermostable bacterial α-amylase [*B. licheniformis*]; IUB/EINECS/CAS nos.: 3.2.1.1/232-565-6/9000-90-2) were kindly provided by Kerry Bio-Science (Carrigaline, Co. Cork, Ireland). Details regarding the enzymatic activities of the enzyme preparations are outlined in Table 2.

Throughout the paper, Promalt will be referred to as "enzyme cocktail", Bioprotease N100L as "protease", Bio-BAA as "thermolabile α-amylase", Bioglucanase B10L as "β-glucanase", and Hitempase 2XL as "high-heat thermostable α-amylase". The definitions of their respective enzymatic units are as follows. NPU: One neutral protease unit is the quantity of enzyme required to produce the equivalent of 1 µg of tyrosine per minute from casein in acetate buffer (pH 5.8–6.2). BG: One glucanase unit is defined as the amount of enzyme that produces 1 mg of maltose per milliliter from β-D-glucan at pH 5.0 and at 50°C. BAA: One bacterial α-amylase unit is the amount of enzyme that breaks down 5.26 mg of starch per hour at pH 6.0 and 40°C.

Methods

Grain Analysis/Enzyme Activity Determination

The malted barley and unmalted barley grains (Table 1) were characterized by using the standard methods of the European Brewery Convention (EBC) (26). The enzymatic activity levels of the grains were determined by using enzyme assay kits (Megazyme International Ireland Ltd., Co. Wicklow, Ireland) for α-amylase (66,86), β-amylase (64), and β-glucanase (65).

Milling/Mashing

All grain samples were milled with a Bühler Miag laboratory-scale disc mill (Bühler GmbH, Braunschweig, Germany) set at a fine grind setting of 0.2 mm. Milled grain (100 g) was weighed into a stainless steel mashing beaker and mixed with mash-in liquor (300 mL) to give a liquor/grist ratio of 3:1. Five different mashing programs were used in these studies (Fig. 1). Mashing was carried out in a mash bath (LB 8 Electronic; Funke-Dr. N. Gerber Instruments GmbH, Leipzig, Germany) as outlined in the EBC method 4.5.1 (26). At the end of the mashing period, the total weights of the mashes were made up to 500 g with distilled water. Mash separation was then immediately performed.

Evaluation of Different Grist Proportions of Malted Barley and Unmalted Barley

Using mashing program A, five grist combinations with malted barley to unmalted barley proportions of 0:100, 20:80, 40:60, 60:40, 80:20, and 100:0 were assessed.

Evaluation of Commercial Enzymes on 100% Raw Barley Substrate

In the following experiments, mashes consisting of 100% unmalted barley and commercial enzyme additions were assessed. Unless otherwise stated, the commercial enzymes were added at mash-in.

*Evaluation of the enzyme cocktail (*B. subtilis*).* Using mashing program B, the enzyme cocktail was added at rates ranging from 0 to 1.0% (vol/wt) of grain.

*Evaluation of protease (*B. subtilis*).* Using mashing program B, with α-amylase (171 BAA U/g of grist) and β-glucanase (1 BG U/g of grist) additions, protease levels were tested over the range of 0 to 1,000 NPU U/g of grist.

*Evaluation of β-glucanase (*B. subtilis*).* Using mashing program B, with protease (50 NPU U/g of grist) and α-amylase (171 BAA U/g of grist) additions, β-glucanase levels were tested over the range of 0 to 20 BG U/g of grist.

*Evaluation of thermolabile α-amylase (*B. subtilis*).* Using mashing program B, with protease (50 NPU U/g of grist) and β-glucanase (1 BG U/g grist) additions, α-amylase levels were tested over the range of 0 to 2,850 BAA U/g grist.

*Evaluation of a high-heat thermostable α-amylase (*B. licheniformis*).* For evaluation of the high-heat thermostable

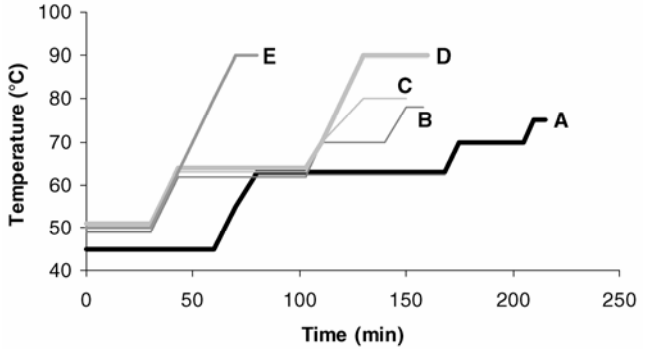

Figure 1. Mashing programs A, B, C, D, and E.

Table 2. Commercial enzyme preparations used in this study and their enzymatic activities

Enzyme type	Trade name (derivative organism)	Optimum pH	Optimum temperature	Principal activity[a]	Side activity[a]
Enzyme cocktail	Promalt (*Bacillus subtilis*)	6.0	55°C	Protease: 23,000 NPU U/mL α-Amylase: 80,000 BAA U/mL β-Glucanase: ~2,000 BG U/mL	None
Protease	Bioprotease N100L (*B. subtilis*)	6.0	55°C	Protease: 100,000 NPU U/mL	β-Glucanase: 100 BG U/mL α-Amylase: 800 BAA U/mL Xylanase: not detected
α-Amylase	Bio-BAA (*B. subtilis*)	6.0	65°C	α-Amylase: 700,000 BAA U/mL	Protease: ~3,000 NPU U/mL β-Glucanase: 130 BG U/mL
β-Glucanase	Bioglucanase B10L (*B. subtilis*)	5.5–7.0	60°C	β-Glucanase: 10,000 BG U/mL	None
Heat-stable α-amylase	Hitempase 2XL (*B. licheniformis*)	4.0–8.0	90°C	α-Amylase: 120,000 BAA U/mL	β-Glucanase: trace

[a] BAA = bacterial α-amylase, BG = β-glucanase, and NPU = neutral protease unit, with further details in text.

α-amylase (in combination with protease [50 NPU U/g of grist] and β-glucanase [1 BG U/g of grist], as outlined in Table 3), a number of different mashing programs (programs B, C, D, and E [Fig. 1]) were carried out. Both the thermolabile α-amylase (*B. subtilis*) and the high-heat thermostable α-amylase (*B. licheniformis*) were added at different dosage rates and at different addition times/temperatures to achieve starch-negative mashes as outlined in Table 3.

Determination of Filtration Performance

After completion of the mashing procedures (without mash cooling), to determine the filtration performance of the mashes, two different mash filtration techniques were carried out.

Filter paper technique. This was carried out on all mash formulations with the mash filtration apparatus described in the EBC method 4.5.1 (26). Runoff amounts were recorded every 15 min over a 90-min period. The slopes of the filtration curves were then calculated.

Laboratory-scale lautering apparatus. Mashes with grist inclusions of 0, 20, 40, 60, 70, 80, 85, 90, 95, and 100% unmalted barley, together with malted barley, were tested for lautering performance with a laboratory-scale lautering apparatus developed and built at University College Cork (58,94). The slopes of the lautering curves were then calculated.

Determination of Wort Quality

The specific gravity of the wort sample was measured with a Servo Chem Automatic Beer Analyzer (SCABA) (Tecator AB, Hoganos, Sweden). Following this, the worts were brought to a specific gravity of 1.048 by the addition of distilled water. The following wort analyses were carried out by using the standard methods of the EBC (26); color (method 8.5), pH (method 8.17), free amino nitrogen (method 8.10), viscosity (method 8.4), fermentability (method 8.6), high-molecular-weight β-glucan (method 8.13.1), and total soluble nitrogen (method 8.9.2). High-molecular-weight protein (HMWP) levels were determined by using a modified version (29,32) of the Mitteleuropaische Brautechnische Analysenkommission (MEBAK) procedure (68). Amino acid analysis was carried out according to the method of Fenelon et al. (27). Wort sugar analysis was carried out according to the method outlined by Goode and Arendt (30).

Experimental Procedure

The results quoted are the means of three repeated experiments with standard deviations.

Results and Discussion

Mash Filterability and Wort Quality When Mashing with Grists Containing Various Ratios of Malted to Unmalted Barley

Figure 2 shows the effects on mash filterability when the ratio of raw barley to malted barley grist was increased. The negligible decrease in filtration rate from the 0 to 40% unmalted barley mashes would be expected because of increasing levels of undegraded β-glucan and arabinoxylan compounds, which are reported (22,45,78) to increase the water-holding capacity of the grain bed and thus obstruct mash filtration. However, the increase in filtration at raw barley levels of 40 to 85% is more difficult to explain since it contradicts conventional brewing logic (10,13,70,85), which suggests that an increase in the level of unmodified grain in the grist results in a decrease in the rate of mash filtration. Indeed, the results do represent laboratory-scale filtration tests (two types) and may not accurately represent industrial-scale performance. However, the results do indicate the inherent structural differences that may exist between the different mash filtration beds (in the absence of commercial enzymes). Even though big differences did not exist in the milled grist particle sizes of the different mashes, it is likely that the suspended mashed grist particle sizes varied quite considerably. It has been shown (38) that mash particle

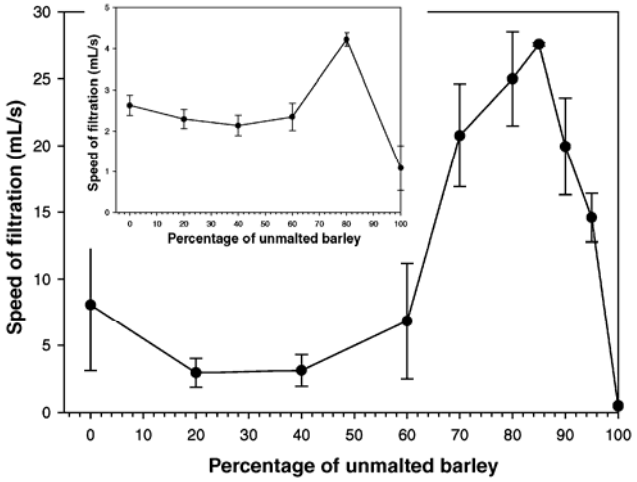

Figure 2. The filtration performance of mashes consisting of different levels of unmalted barley and malted barley (no commercial enzymes) using the filter paper technique (inset) and the laboratory-scale lautering apparatus.

Table 3. α-Amylase enzyme dosages (thermolabile and thermostable) and mashing programs required to bring the mash[a] to a starch-negative state

Mashing program	Level of thermolabile α-amylase (*Bacillus subtilis*) added at 50°C	Level of thermostable α-amylase (*B. licheniformis*) and temperature of addition	Minimum time/temperature for starch-free mash
B	171 BAA[b] U/g of grist	1,200 BAA U/g of grist added at 50°C	Till end of program B
C	171 BAA U/g of grist	240 BAA U/g of grist added at 80°C	30 min at 80°C
C	171 BAA U/g of grist	600 BAA U/g of grist added at 80°C	20 min at 80°C
D	171 BAA U/g of grist	90 BAA U/g of grist added at 90°C	20 min at 90°C
D	171 BAA U/g of grist	120 BAA U/g of grist added at 90°C	10 min at 90°C
E	57 BAA U/g of grist	120 BAA U/g of grist added at 63°C	30 min at 90°C
C	171 BAA U/g of grist	120 BAA U/g of grist added at 63°C	35 min at 80°C

[a] Mash is 100% barley, protease at 50 NPU U/g of grist and β-glucanase at 1 BG U/g of grist. BG = β-glucanase, and NPU = neutral protease unit, with further details in text.
[b] BAA = bacterial α-amylase, with further details in text.

size distributions can differ greatly from the particle size distributions of their respective dry grist sieve fractions. This is due to further disintegration of the constituents of the endosperm during water mixing and mash enzymatic processes, thus increasing the fine fraction portions of the mash. Likewise, the fragments of husk material swell as a result of water uptake, thus increasing the coarse fraction portion. Hence, the increase in filtration performance due to an increase in the level of unmodified grain may simply be because of increased particle size distributions as a result of lower levels of endogenous malted barley hydrolytic enzymes and higher levels of unmodified endosperm. As predicted by the modified version of Darcy's Law (37), this leads to the formation of a mash filter bed of increased porosity. Anderson (1) suggested that variations in a flocculent present in some malts could be a factor in determining lautering quality. In these trials, addition of polycationic material (proteins and polysaccharides) to mashes was found to greatly improve malt filtration performance. Mash bed porosity was increased because of the cationic flocculent overcoming the mutual repulsion between negatively charged particles by binding to a positively charged site on the flocculent, thus forming a flocculent with greater mean particle size (1). It is plausible to suggest in the current experiments that mashes consisting of increased inclusions of raw barley may have increased levels of polycationic material (because of increased levels of undegraded proteinaceous and polysaccharide materials), thus resulting in increases in mash bed porosity. However, currently, these reasons are just hypothetical.

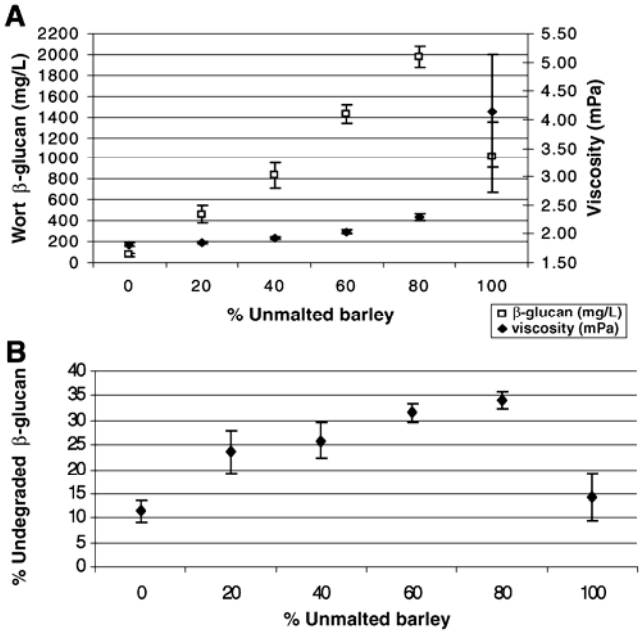

Figure 3. The total **A,** β-glucan and viscosity; and **B,** percent undegraded high-molecular-weight β-glucan values of worts separated from mashes containing increasing levels of unmalted barley.

The first stage of β-glucan degradation is reported to be the volatilization of the polymer from the endosperm cell walls. The main solubilizing enzyme is β-glucan solubilase. Its activity is reported (45) to be very heat stable and is usually present in large quantities in malt and even raw barley. Its activity level increases 1.5–1.7 times during germination and decreases a little during kilning (7). Therefore, this enzyme is unlikely to be a limiting factor in any mash in which barley adjunct is used. Endo-β-glucanase plays an important role in the degradation of β-glucan polymers. Its activity can be found in raw barley (44,45), although its levels are reported (44,45) to be 4.5 times less than those of germinated barley. Because of its heat sensitivity, very little activity can be detected after 5 min of mashing at 55°C, while at higher temperatures, no activity is detected (2,41). Consequently, when mashing with undermodified malt or high adjunct levels, problematic high-molecular-weight β-glucan viscous worts can result. In Figure 3A, as the percentage of unmalted barley was increased, a corresponding increase in wort high-molecular-weight β-glucan levels and wort viscosity was observed. Results depicted in Figure 3B show that the post-mash wort undegraded β-glucan percentage also increased with an increased level of unmalted barley. This reflects the decrease in the level of endogenous enzymes capable of releasing and hydrolyzing the β-glucan molecules.

It is assumed that brewing yeast is only capable of assimilating simple amino acids (51,52,79) and peptides (60) but not proteins. Therefore, the amount of free amino nitrogen (FAN) is important to guarantee a stable fermentation process (24, 81). For a conventional wort (specific gravity of 1.048, 12°P), FAN levels of ~150 mg/L are reported to be necessary for a healthy fermentation (59,75,80). In the current study, as the level of malted barley was increased, the amount of total soluble nitrogen (TSN) and FAN fractions also increased (Table 4),

Table 4. The analytical properties of worts separated from mashes composed of different levels of unmalted barley

	% Malted barley/% Unmalted barley					
Property	**0/100**	**20/80**	**40/60**	**60/40**	**80/20**	**100/0**
α-Amylase[a]	5.44	2,943	5,881	8,919	11,757	14,695
β-Amylase[b]	69,609	67,604	65,599	63,594	61,588	59,583
β-Glucanase[c]	1,852	5,685	9,518	13,352	17,186	21,020
Extract (%, dry wt)	56.2 ± 11.3	78.1 ± 0.78	79.6 ± 1.08	80.4 ± 0.70	81.9 ± 0.39	82.0 ± 0.85
Color (EBC)	4.45 ± 0.7	4.22 ± 0.21	4.65 ± 0.38	4.95 ± 0.22	5.42 ± 0.18	5.96 ± 0.17
pH	6.09 ± 0.01	5.92 ± 0.03	5.98 ± 0.03	5.99 ± 0.04	6.0 ± 0.02	6.01 ± 0.02
TSN[d] (mg/L)	579 ± 40	658 ± 11	725 ± 27	810 ± 15	888 ± 28	927 ± 38
HMWP[d] (mg/L)	258 ± 24	262 ± 11	249 ± 19	250 ± 3	254 ± 14	261 ± 7
FAN[d] (mg/L)	65 ± 9	54 ± 9	88 ± 22	121 ± 21	141 ± 15	177 ± 20
Fermentability (%APP[e])	53.9 ± 0.07	72.9 ± 0.21	77.2 ± 0.24	80.5 ± 0.53	84.1 ± 0.13	87.5 ± 0.32

[a] Total α-amylase activity in the mash (Ceralpha units; Megazyme Ltd., Bray, Ireland).
[b] Total β-amylase activity in the mash (Betamyl units; Megazyme Ltd.).
[c] Total glucanase activity in the mash (glucanase units).
[d] FAN = free amino nitrogen, HMWP = high-molecular-weight protein, and TSN = total soluble nitrogen.
[e] %APP = percent apparant.

while the HMWP fraction stayed the same. Only at levels greater than 80% malted barley was sufficient FAN produced. The amino acid composition of the wort FAN plays an important role (76). It was found that, as the malted barley levels were increased, the levels of all the individual amino acids (Table 5) increased with the exception of cysteine, which was observed to decrease. Proline was found to exist at the highest percentages in worts, particularly when high levels of malt were used. Its contribution to total wort amino acid levels increased as the level of malt was increased. Cysteine was the highest contributor to total amino acid levels in the wort void of malted barley, while leucine and valine were high contributors to wort total amino acid levels irrespective of the level of malted barley.

The addition of malted barley was found to bring in higher levels of assimilable nitrogenous material than was unmalted barley. Since unmalted barley contains little endoproteolytic activity (98), it is important to consider whether the endogenous malt proteolytic enzymes can bring extra assimilable nitrogen from the raw barley substrate. With regard to the total level of amino acids (Table 6), the malt proteolytic enzymes were found to have a negligible impact on bringing extra amino acids into the wort. However, when the individual wort amino acids are considered, it can be seen that the levels of cysteic acid negatively correlates with increased levels of malted barley. At a malt addition level of 40%, the highest level of protein hydrolytic effects can be seen with regard to the amino acid categories of class 1 and class 2. However, as a total level of amino acids, this accounts for just a 5.9% increase (Table 6). The rate of protein hydrolysis during mashing is regulated by the activities of endoproteinases, which are known to be rate-limiting enzymes (16,89). The presence of these endoproteinases is not sufficient, however, to ensure that the barley proteins are rendered soluble. This is because there are low-molecular-weight proteins in both raw barley (67) and malted barley (48). When these are present in solution, they interact with the cysteine-class endoproteinases (the most important protein-solubilizing class in malt [46]) to form enzyme-inhibitor complexes and thus inhibit their activities (48–50). Two such inhibitors that have been purified and characterized are named lipid transfer protein 1 (LTP1) and lipid transfer protein 2 (LTP2) (47). It is therefore likely in these experiments that the resistance to raw barley protein hydrolysis by the endogenous malt endoproteinases is in some way being controlled by such inhibitors. The total amino acid content of wort is important in determining the extent of yeast growth, while the individual amino acid spectrum of the wort influences beer flavor (90). Therefore, the lower amounts of amino acids soluble in worts containing low levels of malted barley may result in fermentation difficulties (affecting the extent of yeast growth), while the altered spectrum of amino acids may result in the production of beers of a different flavor and aroma

Table 6. The percent increase or decrease in amino acid levels from the previous malt percentage

	Malt inclusion			
Amino acid[a]	**20%**	**40%**	**60%**	**80%**
Cysteic acid	651	487	169	65
Aspartic acid[A, C1]	30.7	72	–6.9	–7.8
Threonine[A, C1]	–3.27	1	1.3	–0.2
Serine[A, C1]	–16.7	–34.2	34.3	15.7
Glutamic acid[A, C1]	29.1	61.2	–9.7	–21.7
Proline[D, C1]	–3.0	–12.1	–2.8	–0.1
Glycine[C, C2]	–10.4	–6.7	–6.7	–0.9
Alanine[C, C2]	14.2	19.7	10.6	7.6
Cysteine	–31.4	–39.8	–62.9	–66.2
Valine[B, C2]	–10.4	–11.2	–17	4.6
Methionine[B]	0.6	9.7	0.5	15.7
Isoleucine[B, C2]	6.7	20.2	8.1	9.7
Leucine[B, C3]	0	7	0.9	1
Tyrosine[C, C2]	2.9	10	2.7	–0.5
Phenylalanine[C, C2]	4.1	7.2	0.2	–2.3
Histidine[B, C3]	–15.1	12	–2.1	–3.4
Lysine[A, C3]	40.2	38.3	–12	0
NH_3[C1]	–40.2	–38.9	–62.9	–66.2
Group A	9.2	20.8	4.8	–3
Group B	–4.3	3.2	–4.6	3
Group C	4.3	9.7	3.1	1.4
Group D	–3	–12	–2.8	–0.1
Class 1	3.7	18	0.9	–3
Class 2	1	5.7	–1.3	3.1
Class 3	9.7	16.4	7.4	–0.9
Total	0.6	5.9	–3.3	–4.2

[a] Amino acid groupings (27): [A] = group A amino acid, [B] = group B amino acid, [C] = group C amino acid, and [D] = group D amino acid. Amino acid classes (27): [C1] = class 1 amino acid, [C2] = class 2 amino acid, and [C3] = class 3 amino acid.

Table 5. Selected amino acid composition of worts[a] (mg/L) separated from mashes containing various proportions of unmalted barley and malted barley

	Malt inclusion[c]					
Amino acid[b]	**0%**	**20%**	**40%**	**60%**	**80%**	**100%**
Proline[D, C1]	31.53 (9.1)	85.25 (13.9)	128.49 (13.7)	198 (17.8)	260.62 (19.1)	318.3 (18.8)
Cysteine	32.65 (9.4)	21.32 (3.4)	17.77 (1.9)	18.74 (1.7)	18.95 (1.4)	24.86 (1.5)
Valine[B, C2]	27.9 (8.0)	44.32 (7.1)	63.07 (6.7)	76.89 (6.9)	119.44 (8.8)	135.76 (8.0)
Leucine[B, C3]	33.25 (9.6)	55.88 (9.0)	84.02 (9.0)	102.03 (9.2)	125.07 (9.2)	146.42 (8.7)
Group A	119 (34.2)	213.3 (34.4)	328.2 (35)	364.7 (32.7)	411.6 (30.2)	500.5 (29.6)
Group B	75 (21.6)	133.5 (21.5)	210.1 (22.4)	255.5 (22.9)	341.8 (25.1)	396.1 (23.4)
Group C	83.8 (24.1)	143.7 (23.2)	210.3 (22.5)	253.3 (22.7)	303.9 (22.3)	353.6 (20.9)
Group D	31.5 (9.1)	86.2 (13.9)	128.5 (13.7)	198 (17.8)	260.6 (19.1)	318.3 (18.8)
Class 1	100.5 (28.9)	169.8 (27.4)	268.0 (28.6)	293.1 (26.3)	343.1 (25.2)	417.1 (24.7)
Class 2	121.4 (34.9)	209.1 (33.7)	309.2 (33.0)	373.4 (33.5)	478.2 (35.1)	549.4 (32.5)
Class 3	56.1 (16.1)	111.4 (18.0)	171 (18.3)	207 (18.6)	236.0 (17.3)	283.7 (16.8)
Total	347.89	613.72	936.47	1,114.58	1,381.22	1,689.25

[a] All worts were standardized to a specific gravity of 1.048 (12°P). Mean values are shown.
[b] Amino acid groupings (27): [A] = group A amino acid, [B] = group B amino acid, [C] = group C amino acid, and [D] = group D amino acid. Amino acid classes (27): [C1] = class 1 amino acid, [C2] = class 2 amino acid, and [C3] = class 3 amino acid.
[c] The amino acid fractions are expressed as a percentage of the total wort amino acid and are represented in parentheses.

profile. For example, diacetyl formation is influenced by the wort concentrations of the amino acids valine, isoleucine, and leucine (69). In addition, the higher levels of sulfur-containing cysteine at the lower malted barley levels could be expected to have an influence on the fermentation beer flavor by-products hydrogen sulfide and sulfur dioxide (90).

Table 4 shows that increases in malted barley levels resulted in linear increases in extract recovery percentages. While extract recovery differences between 0% raw barley (100% malt) and 80% raw barley mashes were not large, the apparent fermentabilities of the worts were found to greatly differ (Table 4). A clear correlation was found between the level of malted barley and both the fermentable (DP1–DP3; DP = degree of polymerization) and the unfermentable wort sugar percentage profiles (DP4–DP10+) (Table 7). In relation to the fermentable sugar profiles (Table 7), a positive linear correlation was found between the level of glucose and the level of malt at addition levels of 0 to 60%. At malt levels of 80% and greater, the glucose wort percentage was observed to plateau, irrespective of the level of added malt. In all worts, maltose represented the sugar in the highest concentration (52.4–55.6%), irrespective of the level of malted barley. No clear correlations were found between the malted barley addition and the wort maltose compositional percentage of total sugars. However, when calculated as a percentage of the fermentable sugar concentration (i.e., the level of each fermentable sugar as a percentage of the total fermentable sugar levels in each wort) (Fig. 4), maltose was observed to linearly decrease from a high of 83.9% (0% malt) to a low of 69.9% (100% malt). Maltose is reported to account for 50–60% of the fermentable sugar in conventional wort (90). The higher levels of maltose in this case (100% malt; Fig. 4) can be attributed to increased mashing time at our β-amylase stand of 63°C. In addition, wort sucrose levels were not determined and were therefore not included in the total sugar calculation. The higher percentages of maltose, at the lower levels of malted barley addition, can be attributed to the increasing importance of barley soluble β-amylase levels as the unmalted barley levels were increased. Wort maltotriose percentages were found to linearly correlate with the level of malt addition. Likewise, when calculated as a percentage of total fermentable sugars, both the glucose and the maltotriose levels were observed to increase with an increase in malt levels (Fig. 4). At higher malt levels, malt provides a rich source of α-amylase, and it has a greater effect than does β-amylase, thus resulting in lower levels of maltose and higher levels of glucose and maltotriose. While it is clear that the addition of malted barley brings in higher levels of soluble sugars, and also that the addition of unmalted barley brings in a certain level of carbohydrate, it is also important to consider the hydrolytic effects, which the endogenous malt starch-degrading enzymes bring into the system. Overall, it was observed (Table 8) that, in contrast to the proteolytic enzymes, the endogenous malted barley amylolytic enzymes were capable of hydrolyzing the unmalted barley substrate. The contribution that the malt hydrolytic enzymes had on fermentable sugar increases was observed to be at its highest when the level of unmalted barley substrate was at its highest. At malt addition levels of 10–60%, the percent increase of wort sugars was observed to negatively correlate with an increase in the level of malted barley. At these addition levels, the percent increase of wort sug-

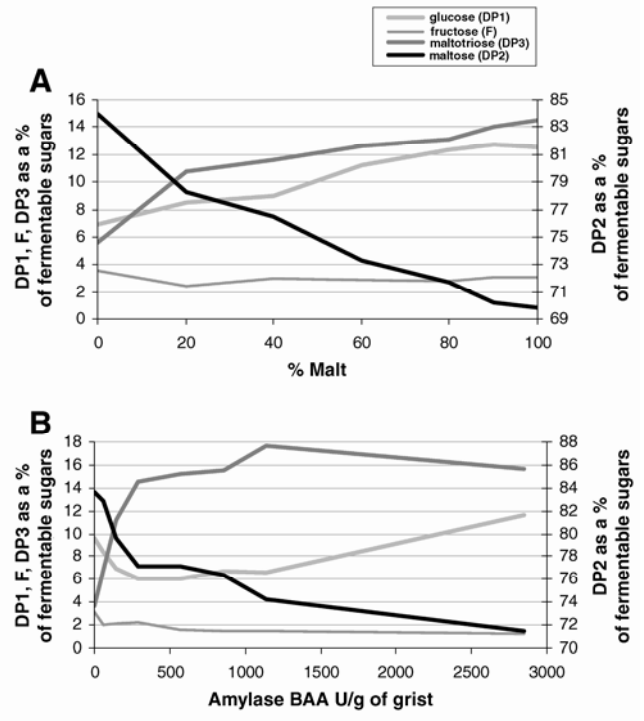

Figure 4. The influences of **A,** malted barley additions; and **B,** commercial α-amylase additions (100% barley mash, protease at 50 NPU U/g of grist, and β-glucanase at 1 BG U/g of grist) on the amounts of wort glucose, fructose, maltose, and maltotriose expressed as a percentage of total fermentable sugars. BAA = bacterial α-amylase, BG = β-glucanase, and NPU = neutral protease unit, with further details in text.

Table 7. The sugar profiles (g/100 mL) of worts separated from mashes composed of different levels of malt inclusions[a]

	Malt inclusion					
Sugar[b]	**0%**	**20%**	**40%**	**60%**	**80%**	**100%**
Fructose	0.21 (2.22)	0.25 (2.11)	0.24 (2.09)	0.22 (2.18)	0.29 (2.43)	0.28 (2.48)
Glucose	0.41 (4.33)	0.75 (6.33)	0.94 (8.19)	0.96 (9.51)	1.18 (9.90)	1.12 (9.94)
Maltose	4.96 (52.4)	6.41 (54.1)	6.16 (53.7)	5.58 (55.3)	6.52 (54.7)	6.26 (55.6)
Maltotriose	0.33 (3.48)	0.97 (8.19)	1.06 (9.23)	1.02 (10.1)	1.3 (10.91)	1.3 (11.54)
DP1–DP3	5.7 (60.19)	8.13 (68.7)	8.17 (71.2)	7.56 (74.9)	8.99 (75.4)	8.68 (77.0)
DP4–DP7	0.29 (3.06)	0.47 (3.97)	0.61 (5.31)	0.5 (4.96)	0.68 (5.70)	0.65 (5.77)
DP8–DP10+	3.27 (34.53)	2.99 (25.25)	2.47 (21.51)	1.81 (17.94)	1.95 (16.36)	1.66 (14.73)
DP4–DP10+	3.56 (37.6)	3.46 (29.2)	3.08 (26.8)	2.31 (22.9)	2.63 (22.1)	2.31 (20.5)
DP10+	3.05 (32.2)	2.42 (20.4)	2.06 (17.9)	1.55 (15.3)	1.67 (14.0)	1.47 (13.0)

[a] The results represent the absolute mean value of one wort sample. The sugar fractions are expressed as a percentage of the total wort carbohydrate are represented in parentheses. Mean values are shown.
[b] DP = Degree of polymerization.

ars DP1–DP3 and DP8–DP10+ decreased with an increase in malted barley levels, while no correlations could be established between the lower-molecular-weight unfermentable sugar fractions (DP4–DP7) and the level of malt addition. At malted barley levels of 60–80%, an increase in the contribution of malt enzymes to all sugar fractions could be observed. However, these increases were much lower than those observed at malted barley levels of 10–40% (Table 8).

In summary, malted barley brings an array of preformed soluble substances, such as native enzymes, sugars, and amino acids, into the mashing system. Its premodified grain structure, resulting in predegraded β-glucan and protein matrices, means a more accessible form of barley starch. A full-balanced complement of total amino nitrogen and amino acids are offered, which reduces the likelihood of fermentation difficulties and inferior beer flavor. Malted barley supplies endogenous enzyme systems, which are capable of further hydrolyzing their native malted grain substrates. However, in this study, the endogenous enzyme systems were found to have a very limited ability of converting the nonnative unmalted barley macromolecular compounds, such as β-glucan and proteins. The malted barley amylolytic enzymes were capable, to a limited extent, of hydrolyzing unmalted barley gelatinized starch. However, starch conversion difficulties and poor fermentability continue to occur in the absence of commercial enzymes.

Influence of an Enzyme Mixture on Mash Filterability and Wort Quality When Mashing with 100% Unmalted Barley

Brewers normally use a mixture of enzymes referred to as a "cocktail" (78). Figure 5 shows the influence of an enzyme cocktail (α-amylase, β-glucanase, and protease) at certain dosage rates (0–1.000% volume of enzyme per weight of grist) on the filtration performance of a mash consisting of 100% unmalted barley. At addition rates of 0.025 to 0.2% (vol/wt of grist), an exponential increase in filtration rate due to the amount of added enzyme cocktail was observed. A linear decrease in filtration rate was obtained when the enzyme cocktail levels were raised from 0.2 to 1.0% (vol/wt of grist). The optimum rate of enzyme dosage to achieve maximum filterability was observed to be 0.2% (vol/wt) of grist (equivalent to β-glucanase at 1 BG U/g of grist, protease at 50 NPU U/g of grist, and α-amylase at 171 BAA U/g of grist). Increases in the dosage rate of the enzyme resulted in a corresponding exponential increase in extract recovery levels. In addition (Table 9), a corresponding exponential increase in solubilized nitrogenous fractions (TSN) was observed. This included increases in both FAN (61.3–153.3 mg/L) and high-molecular-weight nitrogen (292–327 mg/L) fractions. Wort FAN levels showed an exponential correlation with the level of enzyme cocktail. A positive linear relationship was established between the color of the worts (4.55–10.5 EBC) (Table 9) and the level of enzyme addition. Correlations could also be established between the wort colors and both the soluble extract levels and the soluble nitrogen levels of the worts. This agrees with previous work reported (29–33), whereby commercial enzyme addition resulted in a deeper wort color because of increased proteolysis and amylolysis. It is, therefore, important to take these color increases into account when designing brewhouse recipes for the production of paler worts. As the enzyme levels were increased, a resultant decrease in wort viscosity was observed (Table 9). This could be weakly correlated ($R^2 = 0.8363$) to the dosage levels of the enzyme and can be attributed to increased amylolysis of starch and high-molecular-weight dextrins, together with hydrolysis of high-molecular-weight β-glucan, at the higher enzyme level additions. Dextrins, arabinoxylans, and β-glucans are all known to contribute greatly to the viscosity of wort and beer. While dextrins are found to be the primary determinant of viscosity, lower concentrations of both arabinoxylan and β-glucan have been found to have a more pronounced impact on increasing wort and beer viscosity (86). A clear correlation was found between the level of added enzyme and both the fermentable (DP1–DP3) and the unfermentable (DP4–DP10+) wort sugar percentage profiles (Table 10). In relation to the fermentable sugar profiles, positive correlations were made between the commercial enzyme dosage level and the levels of the wort sugars glucose and maltotriose. At enzyme addition levels of 0.15% and lower, a linear positive correlation was observed with wort maltose levels. At enzyme levels of 0.2% and greater, no correlations could be established with maltose levels. In relation to the unfermentable sugar percentage profiles, a positive correlation could be established between the middle-molecular-weight dextrins (DP4–DP7) and the level of added enzyme, while the high-molecular-weight sugar fraction (DP8–DP10+) was found to negatively correlate with an increasing amount of added enzyme (Table 10). These results give an indication of the nature of the enzyme cocktail's α-amylolytic component in randomly cleaving the α-1,4 glycosidic linkages, resulting in increases in the levels of low- and medium-molecular-weight sugars and decreases in the levels of high-molecular-weight dextrins.

Table 8. The percent increase or decrease in sugar levels, due to enzymatic hydrolysis of raw barley, from the previous malt percentage

	Malt inclusion				
Sugar[a]	10%	20%	40%	60%	80%
Fructose	–3.2	11.6	0.84	–12.7	9.0
Glucose	53.8	35.9	35.4	14.8	20.7
Maltose	33.2	22.8	12.4	–2.8	8.7
Maltotriose	117.8	85.1	47.6	11.8	17.5
DP1–DP3	40.9	29.1	18.5	1.00	11.2
DP4–DP7	22.7	29.8	40.6	–1.2	17.6
DP8–DP10+	7.8	1.4	–5.9	–21.4	–1.6
DP4–DP10+	9.2	4.5	0.7	–17.8	2.7
DP10+	–9.8	–11.5	–14.8	–26.3	–6.5
Total	28.6	20.4	12.7	–4.4	9.3

[a] DP = Degree of polymerization.

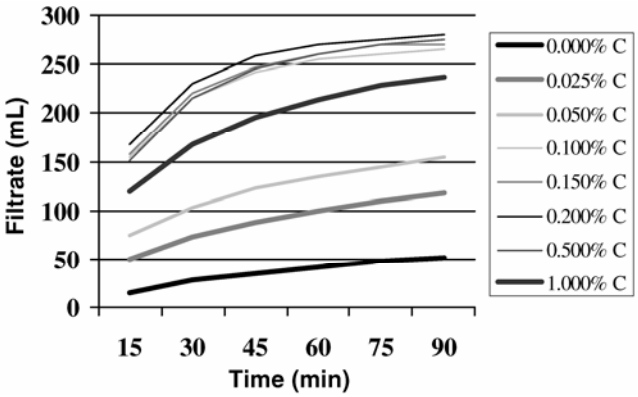

Figure 5. The filtration performance of mashes (100% unmalted barley with different additions of enzyme cocktail [C]) determined by using the filter paper method.

Influence of a Commercial Protease (*B. subtilis*) on Mash Filterability and Wort Quality When Mashing with 100% Unmalted Barley

With an increase in protease levels from 0 to 1,000 NPU U/g of grist (at optimal levels of β-glucanase and α-amylase), no significant effects on mash filterability were observed. The increased levels of protease resulted in an increase in extract recovery levels (Fig. 6). This may partly be because of the secondary enzymatic α-amylolytic side activity of the protease preparation (Table 2), resulting in increased breakdown of the starch. It may also be because of the increased proteolytic activity, breaking down cell wall proteins and making the starch molecules more accessible to amylolytic breakdown.

Table 9. Commercial enzyme additions to mashes containing 100% unmalted barley and their effects on wort characteristics

Enzyme	Addition[a]			TSN[b] (mg/L)	FAN[b] (mg/L)	HMWN[b] (mg/L)	Extract (%, dry wt)	Color (EBC)	Viscosity (mPa·s)
	Protease	α-Amylase	β-Glucanase						
Enzyme cocktail dosage level[c]									
0.025	5.75	20	0.125	688	61.3	292	74.9	4.55	2.20
0.05	11.5	40	0.25	733	70.9	290	76.4	4.67	1.97
0.10	23	80	0.5	799	81.8	297	77.7	5.01	1.85
0.15	34.5	120	0.75	847	96.6	315	78.7	5.48	1.83
0.20	46	160	1.0	868	105.2	318	79.3	5.64	1.78
0.50	115	400	2.5	1,003	122.3	323	81.4	6.77	1.76
1.0	230	800	5.0	1,104	153.3	327	83.9	10.5	1.72
Protease (*B. subtilis*)									
	0			593	53.1	268	79.9	4.57	1.80
	25			905	103.9	327	80.9	5.16	1.77
	50			984	122.8	329	81.4	5.64	1.76
	100			1,086	151.3	330	81.9	5.97	1.78
	200			1,236	170.7	326	82.6	6.26	1.77
	500			1,498	210.6	348	83.4	6.91	1.76
	1,000			1,637	240.8	343	84.3	7.65	1.76
β-Glucanase (*B. subtilis*)									
			0	903	111.4	313	79.3	5.16	1.90
			0.5	915	102.5	310	79.0	5.25	1.79
			1	922	102.0	319	79.4	5.41	1.76
			2.5	924	117.4	306	79.7	5.53	1.77
			5	901	107.0	292	80.3	5.27	1.78
			10	900	107.4	304	79.8	5.23	1.74
			15	900	101.5	301	80.5	5.47	1.74
			20	907	106.3	305	80.5	5.49	1.74
α-Amylase (*B. subtilis*)									
		57		963	128	333	80.2	5.36	2.09
		143		975	129	330	80.9	5.63	1.78
		285		883	132	329	81.4	6.19	1.75
		570		970	128	339	82.5	8.57	1.82
		855		956	132	342	83.3	9.29	1.79
		1,140		931	117	335	83.6	9.86	1.77
		2,850		946	113	339	84.2	13.58	1.76

[a] Enzyme units: protease, NPU U/g of grist; α-amylase, BAA U/g of grist; and β-glucanase, BG U/g of grist. All dosage levels are assumed. BAA = bacterial α-amylase, BG = β-glucanase, and NPU = neutral protease unit, with further details in text.
[b] FAN = free amino nitrogen, HMWN = high-molecular-weight nitrogen, and TSN = total soluble nitrogen.
[c] Dosage level = % (vol/wt) of grist.

Table 10. The sugar profiles (g/100 mL) of worts separated from mashes (100% unmalted barley) composed of different levels of the enzyme cocktail (% [vol/wt] of grist)[a]

Sugar[b]	Cocktail level					
	0.025%	0.050%	0.100%	0.150%	0.200%	0.500%
Fructose	0.19 (1.49)	0.15 (1.20)	0.19 (1.49)	0.16 (1.25)	0.21 (1.62)	0.2 (1.56)
Glucose	0.42 (3.29)	0.49 (3.91)	0.51 (3.99)	0.52 (4.07)	0.57 (4.39)	0.64 (5.01)
Maltose	6.78 (53.05)	6.69 (53.35)	6.89 (53.87)	6.92 (54.15)	6.9 (53.12)	6.9 (53.99)
Maltotriose	0.44 (3.44)	0.48 (3.83)	0.74 (5.79)	0.66 (5.16)	0.98 (7.54)	1.13 (8.84)
DP1–DP3	7.65 (59.86)	7.66 (61.08)	8.14 (63.64)	8.1 (63.38)	8.46 (65.13)	8.68 (67.92)
DP4–DP7	0.23 (1.80)	0.31 (2.47)	0.46 (3.60)	0.71 (5.56)	0.72 (5.54)	0.76 (5.95)
DP8–DP10+	4.72 (36.93)	4.42 (35.24)	4.00 (31.27)	3.81 (29.81)	3.61 (27.79)	3.15 (24.65)
DP10+	4.44 (34.74)	4.09 (32.62)	3.53 (27.60)	3.09 (24.18)	2.89 (22.25)	2.53 (19.8)
DP4–DP10+	4.95 (38.73)	4.73 (37.72)	4.46 (34.87)	4.52 (35.37)	4.33 (33.33)	3.91 (30.59)

[a] The results represent the absolute mean value of one wort sample. The sugar fractions as a percentage of the total wort carbohydrate are represented in parentheses. Mean values are shown.
[b] DP = Degree of polymerization.

Increasing the level of neutral protease resulted in an increase in the levels of solubilized nitrogenous fractions (Table 9). This included increases in TSN (593–1,637 mg/L), FAN (53.1–240.8 mg/L), and HMWP (268–343 mg/L) fractions. Wort FAN levels showed an exponential correlation with the level of added protease. No clear correlation was found between the wort HMWP levels and the dosage levels of this enzyme. A linear positive correlation was found between the wort TSN levels and the extract recovery levels (R^2 = 0.9886). An increase in TSN did not directly translate to an equally proportional increase in FAN (Fig. 7). A positive relationship was found between wort color (4.57–7.65 EBC) and the level of protease addition (Table 9). Linear correlations were detected between the wort color and both the soluble extract levels and the soluble nitrogen levels of the worts. As the level of protease was increased from 0 to 25 NPU U/g of grist, a decrease in viscosity was observed. At protease levels of greater than 25 NPU U/g of grist, the wort viscosity remained constant.

The enzyme dosage levels, as well as the wort properties, need to be taken into consideration when the impact of protease addition is evaluated. While an increase in protease level in this study resulted in increased levels of wort TSN and FAN, this, in turn, results in higher enzyme costs for the brewer. Therefore, it is useful to consider the yield of TSN or FAN per unit of added protease per gram of grain. Figure 8 shows that, as the protease levels were increased, the yields of TSN and FAN per unit of protease addition decreased. Therefore, the efficiency of the protease in hydrolyzing the unmalted barley substrate decreased. The protease efficiency yield was found to negatively correlate with the level of added protease for both TSN and FAN levels. With reference to Table 11, protease as 100 NPU U/g of grist yielded a FAN level of 151.3 mg/L. This is a FAN efficiency yield of just 1.51 mg/L per unit of added protease per gram of grain. By increasing the protease levels from 25 to 100 NPU U/g of grist (a 300% increase in protease levels), the FAN level increased by just 20.01%, while the efficiency yield decreased by 63.6%. With an increase in the protease level from 25 to 1,000 U/g (a 3,900% increase in protease levels), the FAN level increased by just 80.95%, while the efficiency yield decreased by 94.2%. Therefore, in deciding protease addition levels or indeed comparing different proteolytic products, it is important to take into consideration not only the yielded wort property but a combination of wort properties, efficiency yield of the enzyme (at the required level to achieve the desired wort property), and the unit cost of the enzyme.

Influence of a Commercial β-Glucanase (*B. subtilis*) on Mash Filterability and Wort Quality When Mashing with 100% Unmalted Barley

Barley and *Bacillus* (1,3)(1,4)-β-glucanases are reported to have identical substrate specificities (71) and their hydrolysis of β-glucan follow the same stereochemical course (20,61). Microbial β-glucanases have been reported to improve filtration, to increase extract yield, and to prevent β-glucan hazes

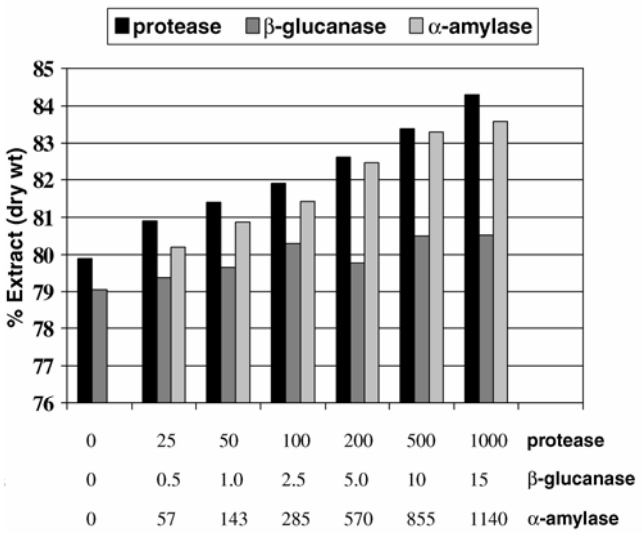

Figure 6. The percent extract recovery (dry wt) of mashes (100% unmalted barley) consisting of different levels of protease (α-amylase at 171 BAA U/g of grist, and β-glucanase at 1 BG U/g of grist), β-glucanase (protease at 50 NPU U/g of grist, and α-amylase at 171 BAA U/g of grist), and α-amylase (protease at 50 NPU U/g of grist, and β-glucanase at 1 BG U/g of grist). BAA = bacterial α-amylase, BG = β-glucanase, and NPU = neutral protease unit, with further details in text.

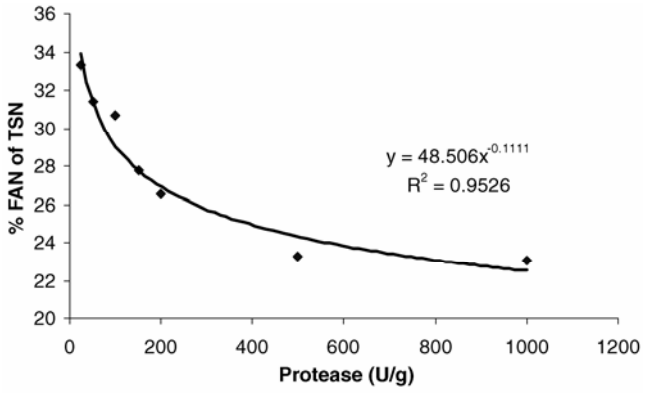

Figure 7. Free amino nitrogen (FAN) expressed as a percentage of total soluble nitrogen (TSN) at the different levels of added protease.

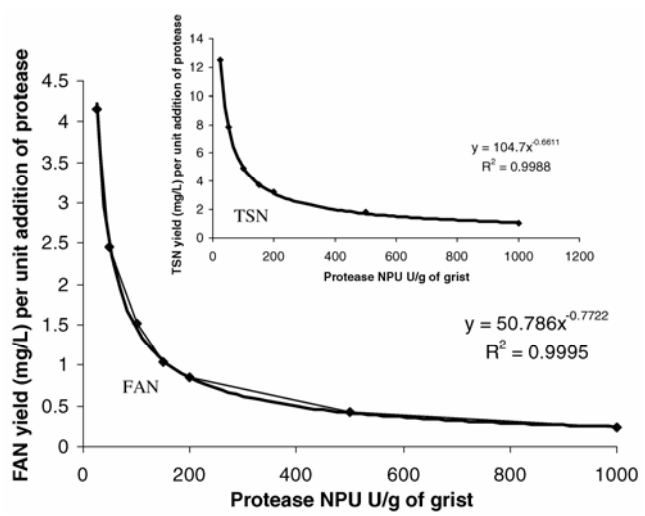

Figure 8. Effect of protease enzyme addition on the level of wort total soluble nitrogen (TSN) and free amino nitrogen (FAN). Calculated as the TSN or FAN yield per NPU U/g of grist. NPU = neutral protease unit, with further details in text.

(96). In these experiments, at optimized levels of α-amylase and protease, increasing the β-glucanase levels was found to have no significant impact on mash filterability, extract recovery (Fig. 6), or the nitrogenous fractions of TSN, FAN, and HMWP (Table 9). At addition rates of greater than 0.5 BG U/g of grist, the β-glucan fraction was reduced to levels that were undetectable when using the standard wort assay (Fig. 9). This is in agreement with previous studies (25,42,54,77,93), whereby addition of a heat-stable β-glucanase during mashing reduced the wort β-glucan level (Fig. 9A and B). Therefore, the optimum commercial β-glucanase addition rate when brewing with 100% unmalted barley was judged to be 0.5 BG U/g of grist. In addition, endo-β-glucanase preparations of *B. subtilis* have been used to hydrolyze beer β-glucan during fermentation, thus improving beer filtration performance (39,91). However, the addition of heat-stable β-glucanases during mashing is preferred because of the enhanced action of the enzymes at higher mash temperatures and also the inactivation of the added enzymes during wort boiling (4).

Influence of a Commercial α-Amylase (*B. subtilis*) on Mash Filterability and Wort Quality When Mashing with 100% Unmalted Barley

α-Amylase levels (0–2,850 BAA U/g of grist) were found to have the greatest impact on mash filterability (Fig. 10). At optimal levels of β-glucanase and protease enzymes, an increase in α-amylase from 0 to 143 BAA U/g of grist resulted in an increase in the mash filtration rates. At α-amylase levels ranging from 285 to 570 BAA U/g of grist, slight decreases in mash filterability were observed. At α-amylase levels ranging from 855 to 2,850 BAA U/g of grist, major decreases in mash filterability were observed. Therefore, it would appear that it is the α-amylase proportion of the enzyme cocktail that inhibits mash separation at high dosage concentrations. However, with an increase in α-amylase levels, significant increases in extract recovery levels were observed (Fig. 6; Table 9) and could be correlated with the added level of α-amylase. With an increase in the level of α-amylase, given a sufficient supply of underhydrolyzed substrate and adequate application time, the rate of extraction of dextrins and simple sugars into the wort solution increases. The color of the wort was also found to be related to the level of α-amylase over the dosage rate of 0 to 2,850 BAA U/g of grist (Table 9). A linear relationship was also established between wort color and wort extract. Comparing the color results with the level of color imparted by malt addition and amylase addition (Tables 4 and 9), it can be observed that the level of commercial amylase had the greatest influence on

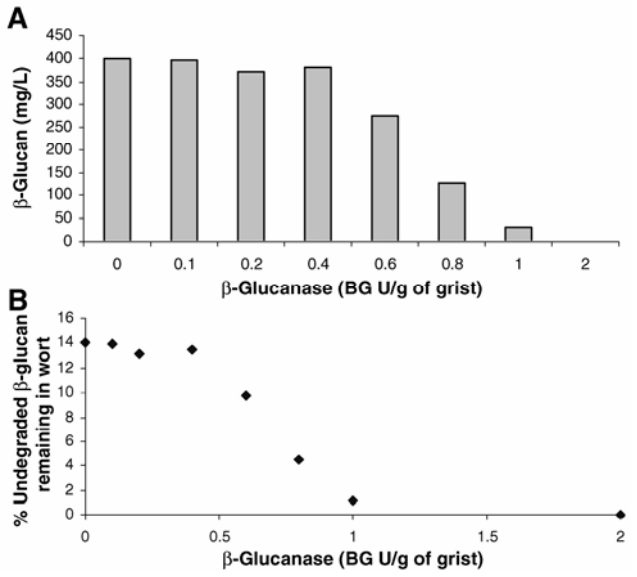

Figure 9. The **A,** β-glucan and **B,** percent undegraded β-glucan values of worts separated from mashes (100% unmalted barley) containing increasing levels of a β-glucanase preparation (protease at 50 NPU U/g of grist, and α-amylase at 171 BAA U/g of grist). BAA = bacterial α-amylase, BG = β-glucanase, and NPU = neutral protease unit, with further details in text.

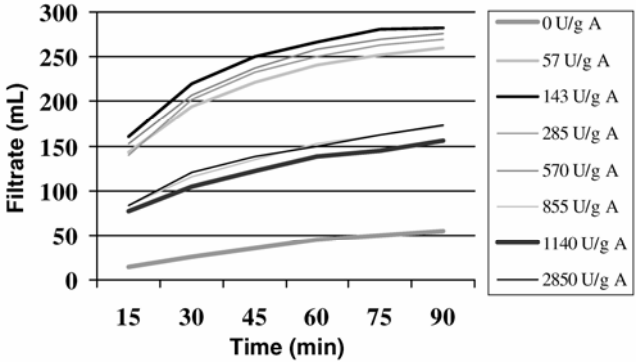

Figure 10. The filtration performance of mashes (100% unmalted barley, protease at 50 NPU U/g of grist, and β-glucanase at 1 BG U/g of grist) at different inclusion levels of the α-amylase enzyme (A = BAA U/g of grist) determined by using the filter paper method. BAA = bacterial α-amylase, BG = β-glucanase, and NPU = neutral protease unit, with further details in text.

Table 11. Protease efficiency evaluation

	Nitrogenous fraction[a]	Protease dosage[b]/Dosage increase (%)[c]						
		25/0	50/100	100/300	150/500	200/700	500/1,900	1,000/3,900
Nitrogen (mg/L)	TSN	904.8	984.4	1,085.9	1,158.5	1,235.5	1,498	1,637
	FAN	103.9	122.8	151.3	157.1	170.7	210.6	240.8
Nitrogen increase (%)	TSN	0	8.8	20.01	28.04	36.56	65.57	80.95
	FAN	0	18.19	45.62	51.2	64.29	102.69	131.76
Yield (mg/L)	TSN	12.48	7.82	4.93	3.77	3.22	1.81	1.04
	FAN	4.16	2.46	1.51	1.05	0.85	0.42	0.24
Yield increase/decrease (%)	TSN	0	–37.3	–60.5	–69.8	–74.2	–85.5	–91.6
	FAN	0	–40.9	–63.6	–74.8	–79.5	–89.9	–94.2

[a] FAN = free amino nitrogen, and TSN = total soluble nitrogen.
[b] Protease units: NPU U/g of grist. NPU = neutral protease unit, with further details in text.
[c] Calculated as the percent increase in protease dosage level, when the 25 NPU U/g of grist dosage level is 100%.

color formation for a given level of extract recovery. In addition, commercial protease addition had a greater impact on color than did the level of malt addition for a given level of extract recovery.

A clear correlation was found between the level of added enzyme and both the fermentable (DP1–DP3) and the unfermentable (DP4–DP10+) total sugar percentage profiles (Table 12). In relation to the fermentable sugar profiles, no clear correlations were found between the levels of glucose and maltose and the level of added enzyme. However, the percentage of maltotriose was positively correlated with the level of added α-amylase. In relation to the unfermentable total sugar percentage profiles, no clear correlation was found between the middle-molecular-weight dextrins (DP4–DP7) and the level of added enzyme, while the high-molecular-weight sugar fraction (DP8–DP10+) was found to negatively correlate with an increasing amount of added α-amylase. In all worts, maltose represented the sugar in the highest concentration (48.1–59.1%), regardless of the level of α-amylase. When calculated as a percentage of the total fermentable sugar concentration (Fig. 4B), maltose was observed to linearly decrease from a high of 83.6% (0 BAA U/g of grist) to a low of 71.4% (2,850 BAA U/g of grist). In this case, the higher levels of maltose, at the lower amylase levels, can be attributed to the effects of the endogenous barley β-amylase. Wort sucrose levels were not determined and were therefore not included in the total sugar calculation. However, wort sucrose levels would not be expected to seriously dilute any of these values. Likewise, when calculated as a percentage of total fermentable sugars, both the glucose and the maltotriose levels were observed to increase with an increase in α-amylase levels (Fig. 4B).

Influence of a Commercial High-Heat Thermostable α-Amylase (*B. licheniformis*) on Mash Filterability and Wort Quality When Mashing with 100% Unmalted Barley

Despite achieving starch-negative worts and adequate levels of filterability, wort β-glucan, FAN, and fermentable sugars by using optimal levels of commercial protease, α-amylase, and β-glucanase, the spent grains were starch positive. Barley starch granules are reported (92) to be of two different granular sizes. The smaller granules (B type) have diameters ranging from 2 to 3 μm and are reported to constitute 80–90% of the total number of starch granules but generally only 10–15% of the total starch weight. On the other hand, the larger A-type granules are reported to have diameters ranging from 12 to 32 μm but constitute a small proportion (10–20%) of the total number of starch granules and a high proportion (85–90%) of the total weight of starch. In barley starch, the smaller B-type granules paste at a higher temperature and over a wider temperature range than do the larger A-type granules (72). The reported negative impacts of small starch granules are that, because of higher temperatures of gelatinization, they are less digestible during mashing (23). In addition, they can impede wort filtration by cross-linking with other polymers (8). In most cases, raw unmalted barley has a higher propensity to have higher levels of small starch granules than does malted barley since, during the malting process, the small granules are preferentially degraded (9). Therefore, application of a high-heat thermostable α-amylase (Fig. 11) has many benefits when brewing with high levels of unmalted barley, since the enzyme has the ability of hydrolyzing these smaller starch granules at higher temperatures than usual (62).

Table 3 reveals that when a thermostable α-amylase (*B. licheniformis*) was added at mash-in (50°C, program B) in the presence of the thermolabile α-amylase (*B. subtilis*), it was only at the highest level of thermostable α-amylase addition (1,200 BAA U/g of grist) that a starch-free mash was obtained. The level of thermostable α-amylase was found to have no effect on filtration at addition rates ranging from 0 to 240 BAA U/g of grist. However, at levels of 600 to 1,200 BAA U/g of grist, a reduction in filtration rate was observed. This is in agreement with the previous result related to the reduction in filterability when high levels of the thermolabile α-amylase (*B. subtilis*) were added. Likewise, increases in the high-heat thermostable α-amylase resulted in increases in extract recovery levels (Fig. 11). The dosage level of the thermostable α-amylase was very much dependent on its temperature and time of application (Table 3). Lower enzyme dosage was required at higher temperatures (Table 3). Also, a longer time of application at any respective temperature required less enzyme addition (Table 3). Under these conditions, the conversion rate of the substrate was higher than the degradation rate of the heat-stable α-amylase. While the filterability of the mashes were very good at 90°C (because of increased temperature of separation), the separated worts were observed to be very turbid. In addition, the wort viscosities were considerably higher at the mash-off temperature of 90°C (2.00 mPa·s) than at the mash-off temperature of 78°C (1.72 mPa·s). When the thermostable α-amylase was added at 63°C (program E), a starch-negative mash was achieved with just a 10-min stand at 90°C. By applying the heat-stable α-amylase over the temperature increase period from 63 to 90°C, the enzyme has longer to act. In addition, the enzyme has the chance to act over the temperature

Table 12. The sugar profiles (g/100 mL) of worts separated from mashes (100% unmalted barley) composed of different levels of commercial α-amylase[a]

	α-Amylase level						
Sugar[b]	**0 U/g**	**57 U/g**	**143 U/g**	**570 U/g**	**855 U/g**	**1,140 U/g**	**2,850 U/g**
Fructose	0.19 (1.80)	0.15 (1.35)	0.19 (1.44)	0.14 (1.12)	0.13 (1.03)	0.13 (1.03)	0.12 (.05)
Glucose	0.58 (5.51)	0.64 (5.76)	0.62 (4.71)	0.53 (4.22)	0.59 (4.67)	0.58 (4.60)	1.1 (9.66)
Maltose	5.06 (48.10)	6.31 (56.74)	7.09 (53.92)	6.71 (53.47)	6.74 (53.37)	6.58 (52.18)	6.73(59.09)
Maltotriose	0.22 (2.09)	0.52 (4.68)	1 (7.6)	1.32 (10.52)	1.37 (10.85)	1.57 (12.45)	1.47 (12.91)
DP1–DP3	5.85 (55.56)	7.47 (67.18)	8.71 (66.24)	8.55 (68.13)	8.71 (68.96)	8.73 (69.23)	9.3 (81.65)
DP4–DP7	0.56 (5.32)	0.44 (3.96)	0.94 (7.15)	0.97 (7.73)	1.02 (8.08)	0.67 (5.31)	0.72 (6.32)
DP8–DP10+	3.92 (37.23)	3.06 (27.52)	3.31 (25.17)	2.88 (22.95)	2.78 (22.01)	3.08 (24.43)	1.25 (10.97)
DP4–DP10+	4.48 (42.55)	3.5 (31.47)	4.25 (32.32)	3.85 (30.68)	3.8 (30.09)	3.75 (29.74)	1.97 (17.30)
DP10+	3.82 (36.28)	2.68 (24.1)	2.65 (20.15)	2.21 (17.61)	2.1 (16.63)	2.15 (17.05)	0.93 (8.17)

[a] The results represent the absolute mean value of one wort sample. The sugar fractions as a percentage of the total wort carbohydrate are represented in parentheses. Mean values are shown.
[b] DP = Degree of polymerization.

ranges immediately following primary gelatinization of the starch granules through to the temperature ranges incorporating the gelatinization of the smaller B-type granules. When the thermolabile α-amylase (*B. subtilis*) was excluded from the mashing systems, even at the highest levels of thermostable α-amylase addition, starch-negative mashes were not achieved. The thermolabile α-amylase (*B. subtilis*) has its optimum temperature at ~60°C, while the thermostable α-amylase (*B. licheniformis*) has its optimum temperature at ~90°C. Considering that the primary gelatinization temperature of the larger granules can occur at 59–62°C (34) and the secondary gelatinization temperature of the smaller granules can occur at ~71°C (34), then the thermolabile α-amylase is able to complete gelatinization of the large granules while the thermostable α-amylase completes gelatinization of the smaller starch granules.

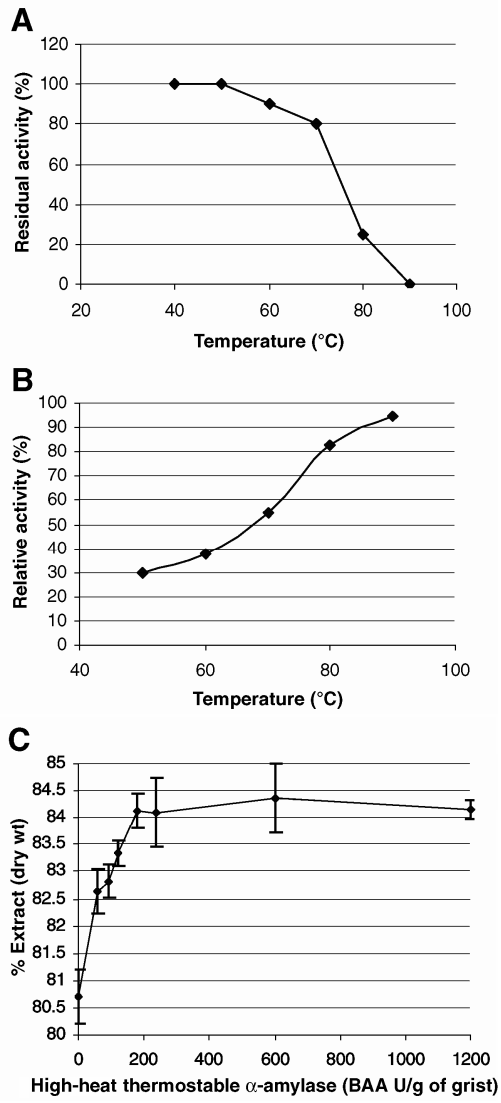

Figure 11. The **A,** residual activity and **B,** relative activity of the high-heat thermostable α-amylase (*B. licheniformis*) over a temperature range of 40 to 100°C and also **C,** the effect of its addition on mash extract recovery when mashing with raw barley (protease at 50 NPU U/g of grist, and β-glucanase at 1 BG U/g of grist). BAA = bacterial α-amylase, BG = β-glucanase, and NPU = neutral protease unit, with further details in text.

While this paper has concentrated on the hydrolyzing effects of commercial enzymes on unmalted barley substrate, it should also be mentioned that, when brewing with a combination of malted barley, unmalted barley, and commercial enzymes, the commercial enzymes have an impact on increasing the level of wort solubles by unmalted barley substrate degradation and also by further hydrolyzing the malted barley substrate. In previous work by the current authors (29,33) and others (21), it was shown that addition of enzymes to 100% malted barley mashes resulted in an increased extract recovery levels. Using optimized conditions for the added enzymes, a greater degree of nitrogen solubilization, together with FAN, was observed.

Conclusion

As shown in this paper, wort production from grists containing high levels of raw barley offers many challenges to the brewer. However, optimal levels of filterability, β-glucan breakdown, fermentable sugars, and α-amino nitrogen can be achieved with careful optimization of enzyme types, dosage levels, and mashing conditions of time, temperature, and application time with and without added malted grain.

ACKNOWLEDGMENTS

The authors acknowledge that this research was partly funded by the Irish Government under the National Development Plan, 2000–2006 (DAFF grant). We thank Charlotte Laarson, Dirk Simons, and Helge Ulmer, University College Cork, for their contributions to this work. We acknowledge Eoin Lalor of Kerry Bio-Science, Cork, Ireland, for providing us with enzyme samples and sugar analysis. We acknowledge the helpful and constructive comments of the referees.

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