

# The Relevance of Different Enzymes for the Hydrolysis of $\beta$ -glucans in Malting and Mashing

Makoto Kanauchi<sup>1</sup> and Charles W Bamforth<sup>2,3</sup>

## ABSTRACT

J. Inst. Brew. 114(3), 224–229, 2008

$\beta$ -Glucosidase and to a lesser extent endo- $\beta$ -1-4-glucanase are present in significant quantity in raw barley. These enzymes, as well as endo- $\beta$ -1-3, 1-4-glucanase, endo- $\beta$ -1-3-glucanase and exo- $\beta$ -1-3-glucanase, increase in activity during steeping and germination. Exo- $\beta$ -1-3-glucanase stands apart through its very late development during germination and it may be a limiting enzyme in malts that have not received substantial modification. Three separate exo-glucanases were located in malt and each of them displayed a preference for  $\beta$ 1-3 linkages. As the principle product of endo- $\beta$ -1-3, 1-4-glucanase is oligosaccharides with a  $\beta$ 1-4 linkage at the non-reducing end (from which end exo enzymes approach the substrate), this is likely to be a second reason (alongside the late development of the enzyme) why such oligosaccharides survive in significant quantities into wort. A third contributing factor may be the sensitivity of the exo-glucanases to ions such as potassium, sodium and magnesium.

**Key words:** Barley, endo, exo, glucanase.

## INTRODUCTION

The major component of the cell walls from the starchy endosperm of barley is a  $\beta$ 1-3, 1-4- glucan<sup>12</sup>. If this polymer is not solubilised and hydrolyzed a number of problems may arise in the brewing process, notably a decrease in the yield of extractable material, a reduction in rates of solid-liquid separation and the risk of poor clarity in the beer<sup>1,2</sup>. A fundamental role of the malting process is the development of the enzymes that will digest these walls and obviate these problems. The bulk of this enzymolysis occurs during the malting process *per se*, but, if incomplete, it can be continued in low temperature mash stands in the brewhouse where the digestion of  $\beta$ -glucans from any adjunct rich in this polymer must also occur.

That the bulk of glucan hydrolysis occurs during malting means that this polymer, which can represent as much as 10% of the dry weight of barley<sup>20</sup>, does not contribute of itself to fermentable extract. Indeed,  $\beta$ -linked glucan

must be completely degraded to glucose before it is rendered fermentable. Were it feasible to effect complete digestion of the polymer in raw barley to glucose within the brewhouse, then this would represent a substantial fermentable extract source. However, although brewing based on raw barley has long been experimented with and occasionally has achieved industrial significance<sup>11</sup>, there remains a reluctance by brewers to contemplate such “barley brewing”, not least for quality reasons and because it would demand the use of exogenous enzymes.

There is also a realization that the limited amounts of  $\beta$ -glucan that survive into beer, as well as  $\beta$ -linked oligosaccharide degradation products, constitute soluble fibre and putative prebiotics<sup>4</sup>. Developing that theme, it could be argued that avoidance of any glucose production whatsoever during glucan degradation, coupled with depolymerisation to a degree sufficient to avoid viscosity problems, would be the best compromise in producing beers of quality that also could offer health benefits additional to those already understood to attend the consumption of beer<sup>3</sup>.

Whatever the requirements or approaches, it is unequivocally the case that we need a clear understanding of the enzymology of  $\beta$ -glucan degradation. This can be considered, referencing the model of Bamforth and Kanauchi<sup>5</sup>, in two stages: an initial solubilisation or “increased-accessibility” of the glucan followed by its degradation. A range of “solubilases” exist<sup>6,7,18</sup>, but the focus of the present paper is on  $\beta$ -glucan hydrolysis rather than solubilisation. In this respect, the most important enzyme has long been realized to be an endo- $\beta$ -1-3,1-4-glucanase (EC 3.2.1.73), which hydrolyzes  $\beta$ 1-4 linkages on the reducing side of  $\beta$ 1-3 linkages, with the attendant production primarily of oligosaccharides containing 3 or 4 glucosyl linkages<sup>23</sup>. The principle products of this enzyme therefore have glucosyl residues at the reducing ends that are linked  $\beta$ 1-3 to the adjacent residue. A further product is longer oligosaccharides containing contiguous  $\beta$ 1-4 linkages and which derive from cellulosic regions in the polymer<sup>24</sup>. Conceptually these are substrates for an endo- $\beta$ -1-4-glucanase (EC 3.2.1.4)<sup>25</sup>. Other enzymes described in the glucanase complex are an endo- $\beta$ -1-3-glucanase (EC 3.2.1.39)<sup>8</sup>, suggested by some to be a solubilase<sup>9</sup>, broad specificity exo- $\beta$ -glucanases (EC 3.2.1.91)<sup>15</sup> and  $\beta$ -glucosidase/exo- $\beta$ -1-4-glucan hydrolases (EC 3.2.1.21)<sup>16</sup>.

In this paper, we make a side-by-side comparison of the development of certain of these enzymes and study some of their salient properties, with the aim of gaining a better understanding of their relative significance and how their activity may be manipulated.

<sup>1</sup>Department of Food Management, Miyagi University, 2-2-1 Hatatate Taihaku-ku Sendai Miyagi, 982-0215 Japan.

<sup>2</sup>Department of Food Science & Technology, University of California, Davis, CA 95616-8598, USA.

<sup>3</sup>Corresponding author. E-mail: cwbamforth@ucdavis.edu

## EXPERIMENTAL METHODS

### Germination of barley

Barley (30 g, Metcalf, 2004 harvest) was germinated according to Hoy et al.<sup>14</sup>. The barley was sterilized using a 1% calcium hypochlorite solution, and washed with water before steeping in water at 16°C for 8 h, air-resting at 16°C for 16 h and steeping again at 16°C for 24 h. The grain was germinated for 72 h at 16°C and samples were removed at indicated times for freezing at -30°C overnight and drying under vacuum.

### Extraction of germinating barley

Grain (30 g) was homogenized for 4 min in 50 mL of 50 mM sodium acetate buffer, pH 5.0 (containing 0.1 M NaCl) using a domestic blender. Insoluble material was removed by centrifugation at 16,000 g for 20 min at 4°C.

### Measurement of protein

Protein was measured by the method of Bradford<sup>10</sup>.

### Enzyme assays

Endo-( $\beta$ 1,3)( $\beta$ 1,4)-glucanase was assayed by a radial diffusion method<sup>21</sup>. Viscozyme (Novozymes) was used as a standard for establishing the calibration curve from which diffusion diameters were converted to reducing sugar production as measured by the Nelson-Somogyi method<sup>22</sup>.

Exo-( $\beta$ 1,3)-glucanase and exo-( $\beta$ 1,4)-glucanase were assayed by the production of glucose from laminarin and cellulose, respectively. Laminarin (5 mg, Nacalai Tesque, Cat# 20047-74, Kyoto, Japan) or cellulose (5 mg, Sigmacell type 20, Cat# C2258 Sigma) was suspended in 1 mL of 50 mM phosphate buffer (pH 6.0) and enzyme solution (0.05 mL) added. The mixture was incubated at 40°C for 60 min and the reaction was terminated by the successive addition of 0.1 mL each of 1 M HCl and 1 M NaOH. Glucose produced was assayed using a glucose assay kit (Cat# 439-90901, Wako Pure Chemical Industries, Ltd. Osaka).

$\beta$ -Glucosidase was assayed by measuring the release of glucose from p-nitrophenyl- $\beta$ -D-glucoside. Enzyme solution (0.05 mL) and 0.05 mL of 10 mM p-nitrophenyl- $\beta$ -D-glucoside (0.05 mL) were added to 1 mL of 0.1 M phosphate buffer (pH 4.0) and the mixture was incubated at 40°C for 20 min. The reaction was terminated by the addition of 3.0 mL of 3% sodium carbonate solution and absorbance was measured at 400 nm.

Endo-( $\beta$ 1,3)-glucanase and endo-( $\beta$ 1,4)-glucanase were assayed by adding enzyme solution (0.05 mL) to either 0.25 mL of laminarin or cellulose (5 mg/mL) in 1 mL of 50 mM phosphate buffer (pH 6.0). The mixture was incubated at 40°C for 60 min, when the reaction was terminated by the addition of 0.1 mL, first of 1 M HCl and then 1 M NaOH. The level of reducing sugars produced was measured by the Nelson-Somogyi method<sup>22</sup>. For each enzyme, one unit is defined as the amount of enzymes hydrolyzing 1 mM glucosidic linkages per min from the substrate.

### Partial purification of enzymes

Barley, germinated for 4 days and then freeze-dried, was the starting material, with extraction as described

above. Extracts were precipitated at between 40% and 70% of saturation with ammonium sulphate at 4°C and the precipitate, recovered by centrifugation, was re-dissolved in 5 mL of 50 mM phosphate buffer, pH 6.5, containing 2 mM ethylenediaminetetraacetic acid, disodium (EDTA, Cat# E5134; Sigma, St. Louis, MO, USA) and 2 mM dithiothreitol (Cat#D0632; Sigma, St. Louis, MO, USA). The solutions were dialyzed against 1 L of 50 mM phosphate buffer, pH 6.5 at 4°C. The dialyzed material was applied to a column (25 mm  $\times$  300 mm) of DEAE resin, (Macro-Prep support, Bio-Rad Ca USA) to fractionate exo-glucanases and to a column (25 mm  $\times$  300 mm) of CM resin, (Macro-Prep support, Bio-Rad Ca USA) to fractionate endo-glucanases. In each case, protein was eluted using a 0–1 M linear gradient of sodium chloride. The eluent flowed at 2 mL per min and 5 mL fractions were collected. Fractions containing glucanase activity were collected from the ion-exchange stage and re-precipitated using 80% saturation with ammonium sulphate. The precipitate was dissolved in 2 mL of 50 mM phosphate buffer, pH 6.5, then applied to a size-exclusion column (10 mm  $\times$  350 mm, P-100 gel, Bio-Rad Ca USA). Phosphate buffer (50 mM, pH 6.5) was flowed at 0.5 mL min<sup>-1</sup> as eluent and 3 mL fractions were collected. The protein content of the eluent was monitored at A<sub>280</sub> and the fractions were assayed for activity. Fractions containing the highest levels of activity were pooled.

### Heat tolerance of enzyme stability

Purified enzyme solutions were incubated at 20, 30, 40, 50 or 60°C for up to 30 min in tubes covered by tear drop condensers. Samples were immediately cooled down in ice prior to assaying for activity.

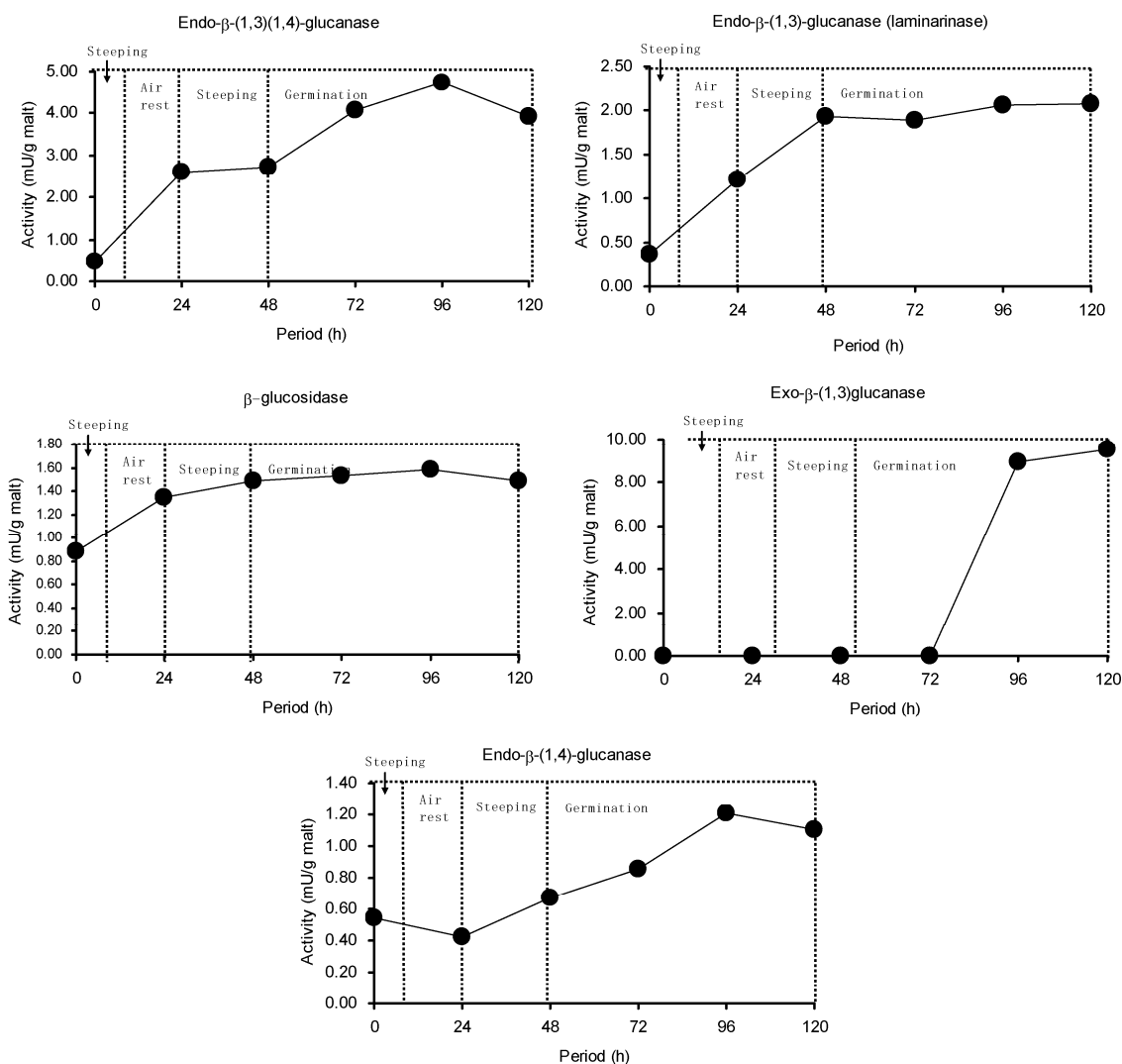
## RESULTS AND DISCUSSION

### Development of enzymes during malting

Figure 1 confirmed the findings of previous workers (see <sup>19</sup> for a first report) that there is very little endo- $\beta$ 1-3,1-4-glucanase in unmalted grain but rather it increases during steeping and germination with a late-germination decline.

Of the enzymes investigated,  $\beta$ -glucosidase alone was present in substantial quantity in raw grain and approximately doubled in activity with most of this increase occurring during steeping. This enzyme is understood to be primarily an exo- $\beta$ -1-4-glucanase that removes glucose from the non-reducing ends of  $\beta$ 1-4-linked glucans<sup>17</sup>. The longer the glucan chain, the greater the affinity the enzyme has for it<sup>16</sup>. Quite why this enzyme should be present in substantial quantity in raw grain is unclear.

Endo- $\beta$ 1-3-glucanase was marginally present in raw grain and increased during steeping, reaching a maximum before endo- $\beta$ 1-3,1-4-glucanase. This fits with the purported role of this enzyme in rendering the native glucan in a form suitable for digestion by endo- $\beta$ 1-3,1-4-glucanase<sup>9</sup>, but would also be consistent with a role for the enzyme in digesting the  $\beta$ 1-3 glucan rich walls said to be located immediately beneath the aleurone<sup>13</sup>. Endo- $\beta$ 1-4-glucanase developed later than endo- $\beta$ 1-3,1-4-glucanase during steeping. Exo- $\beta$ 1-3-glucanase stood apart with its



**Fig 1.** The development of various glucanases and glucosidase during malting.

pattern during malting. No activity was detectable in raw barley, indeed the enzyme did not increase in activity until after 24 h of germination and within the next day the bulk of the enzyme activity had been developed. This would suggest that the enzyme is not needed for early mobilization of the starchy endosperm and it might be inferred that shorter-grown grain and the beer derived from it would contain proportionately higher levels of beta-linked oligosaccharides than would malt germinated for longer periods.

### Partial purification of exo-β1-3-glucanase and exo-β1-4-glucanase

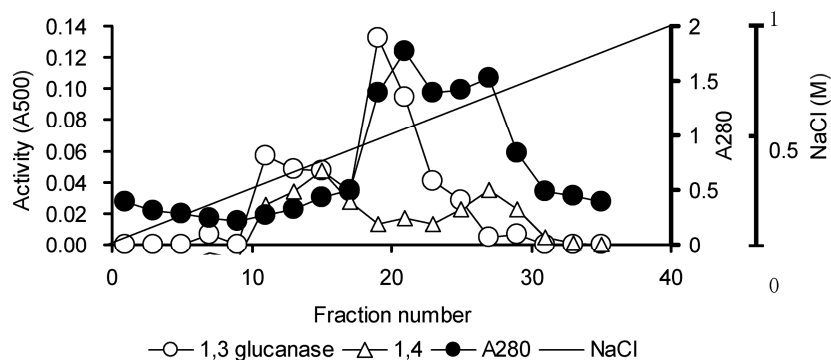
In anion exchange chromatography, three peaks of activity were obtained in assays measuring exo-β1-3-glucanase and exo-β1-4-glucanase (Fig. 2). Peak II represents solely an exo-β1-3-glucanase and peak III was predominately exo-β1-3-glucanase. In the low intensity peak I, both activities were present. Their distribution within this peak did not coincide exactly, but it is not possible, on the basis of this experiment, to say whether

or not they were due to the same enzyme. Previous reports hold that β-glucan exohydrolases display broad specificity for β1-4 and β1-3 linked glucosidic linkages (as well as β1-6 and β1-2 linked residues, which are not found in barley), with the preference being for β1-3<sup>15</sup>.

The three fractions from the ion exchange chromatography were subjected separately to gel permeation chromatography (data not shown). It was confirmed that peak II was devoid of exo-β1-4-glucanase activity. However, the maxima for 1-3 and 1-4 glucanases coincided for the other two fractions, suggesting that both bonds are substrates for a single enzyme. There was also substantial smearing of activity in both instances for reasons that have yet to be elucidated. In the case of fraction I, in which the enzyme was clearly of high molecular weight and thus eluted early, the smearing may result from adsorption of the enzyme on the Bio-gel.

It was found that peak II had a small amount of endo-β1-3, 1-4-glucanase activity (0.52 units/mL) but the other two fractions did not.

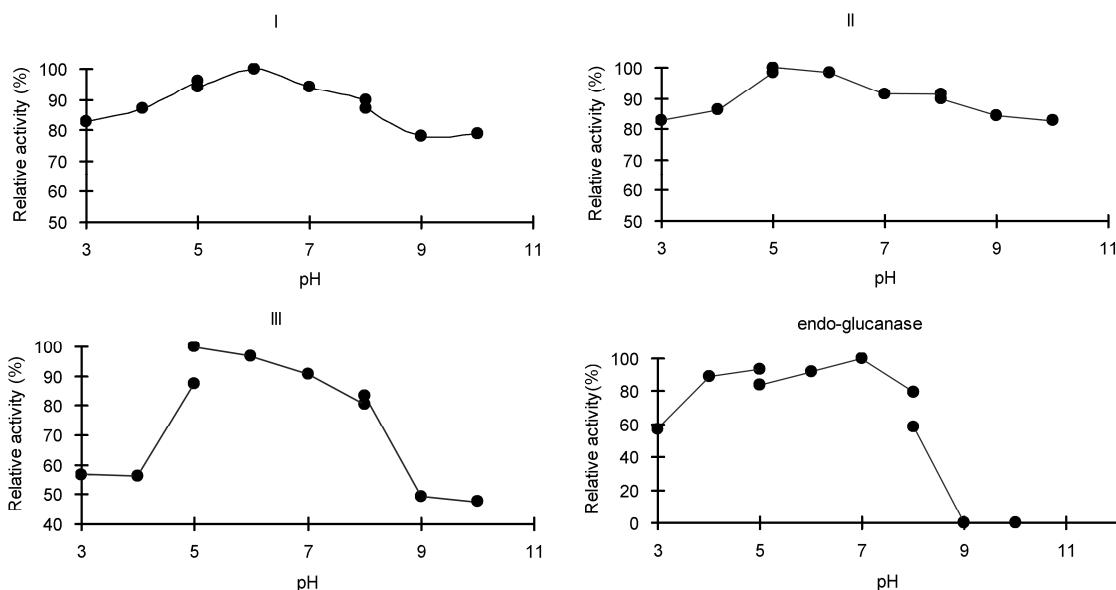
A summary of the purifications is given in Table I.



**Fig 2.** Ion-exchange chromatography of exo-gluconases. ○:  $\beta$ 1-3 glucanase, △:  $\beta$ 1-4 glucanase, ●: protein as  $A_{280}$ .

**Table I.** Purification of exo-gluconases.

		Protein ( $\mu$ g/mL)	Volume (mL)	Total protein (mg)	Activity (cellulose)	Activity (laminarin)	Total activity (cellulose)	Total activity (laminarin)	Specific activity (cellulose)	Specific activity (laminarin)	-fold purification (cellulose)	-fold purification (laminarin)	Yield (cellulose) (%)	Yield (laminarin) (%)
Extract		3933.3	280.0	1101.3	1.2	1.9	348.8	523.2	1.1	475.1	1.0	1.0	100.0	100.0
Ammonium Sulfate		4116.0	90.0	370.4	0.5	8.8	43.7	791.4	1.3	2136.3	1.2	4.5	12.5	151.2
Ion Exchange chromatography	I	223.9	45.0	10.1	0.1	-	3.2	-	7.0	-	6.2	-	0.9	-
	II	234.9	42.0	9.9	-	1.5	-	63.5	-	6440.2	-	13.6	-	12.1
	III	68.6	45.0	3.1	-	0.3	-	12.3	-	3970.2	-	8.4	-	2.3
Size exclusion chromatography	I	103.2	11.8	1.2	0.1	-	1.0	-	72.0	-	63.6	-	0.3	-
	II	157.4	7.4	1.2	-	3.8	-	27.9	-	23947.2	-	50.4	-	5.3
	III	94.0	7.6	0.7	-	1.7	-	12.6	-	17599.1	-	37.0	-	2.4



**Fig 3.** The pH optimum for the three exo-gluconases (I, II and III) and endo- $\beta$ 1-3,1-4-gluconase.

### pH optima

All of the isolated fractions displayed broad pH optima (Fig. 3 in which replicate values in the pH curves are due to buffer changes). Only at pH 9 and higher was endo- $\beta$ 1-3,1-4-gluconase activity absent, whereas even at this pH there was exo- $\beta$ 1-3-gluconase and exo- $\beta$ 1-4-gluconase activity. Regarding pH values that are commercially relevant in mashing, there would be very little impact on any gluconase activity due to any pH change over the usual range of 5 to 5.5.

### Impact of temperature

Endo- $\beta$ 1-3,1-4-gluconase has long been known to be a heat-labile enzyme and this has been confirmed (Fig. 4). By contrast, each of the exo-gluconase fractions displayed a biphasic pattern during heat treatment. In each instance there was an initial activity loss that was even more precipitous than that for the endo-enzyme, but the activity subsided to a finite residual level, which was lower as the temperature increased. Some activity could be detected after treatment at temperatures as high as 80°C. These

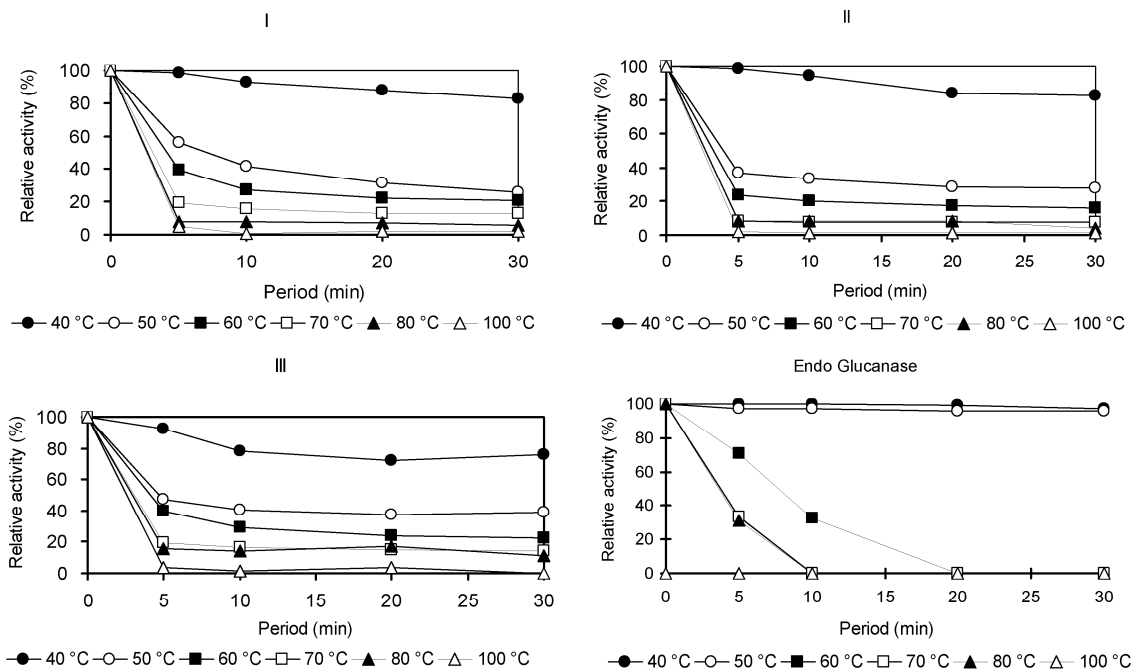


Fig 4. The heat sensitivity of the three exo-glucanases (I, II and III) and endo- $\beta$ 1-3,1-4-glucanase.

Table II. Kinetic constants for the exo-glucanases.

	Km cellulose (mg/mL)	Km laminarin (mg/mL)	Vmax cellulose (mg/min)	Vmax laminarin (mg/min)
I	162.3	0.78	0.012	0.014
II	3.01	1.03	0.0045	0.061
III	36.64	1.10	0.005	0.0157

observations suggest that there are both heat-stable and heat-labile components within these fractions. Non-denaturing polyacrylamide gel electrophoresis of the various purified fractions (not shown) revealed a solitary protein band for endo- $\beta$ 1-3,1-4-glucanase (purification procedure not shown) but multiple bands for each of the exo-glucanases. It is presumed that certain of these bands represent heat-stable isozymes whereas others are heat-labile forms. This was not pursued further, however, because, just as for pH, it does not appear that the temperatures involved in mashing would preclude some exo-glucanase activity.

### Kinetic analysis of the exo-acting enzymes

Table II presents the Michaelis constant and maximum velocity data for the three partially purified exo-enzymes. Despite the inability to detect exo- $\beta$ 1-4-glucanase activity in peak II (Fig. 2) it was found that the fraction concerned did display activity with cellulose although the Vmax was relatively low. There was a higher affinity and Vmax in each fraction for laminarin, confirming previous reports<sup>15</sup> that the preferred glycosidic linkage for the enzyme to attack is  $\beta$ 1-3. Hrmova et al.<sup>16</sup> showed that for  $\beta$ 1-3 and  $\beta$ 1-4 linked oligosaccharides, respectively, the activity of the enzymes was greater if the degree of polymerization was 3 or 4 or greater. They showed that cellobiose had the lowest activity of all with the enzyme. The enzyme during malting and brewing is confronted with primarily G4G3G and G4G4G3G (the main products of attack on mixed linkage  $\beta$ -glucan by endo- $\beta$ 1-3,1-4-glucanase) and it must

attack these from the non-reducing end (i.e. a  $\beta$ 1-4 linkage first). Such mixed linkage oligosaccharides were shown to be substrates for the exohydrolases and  $\beta$ -glucosidase<sup>16</sup>, though it was observed that the former preferred longer  $\beta$ -linked dextrans and oligosaccharides containing only  $\beta$ 1-3 linkages whereas the latter preferred longer oligosaccharides containing only  $\beta$ 1-4 linkages. It seems that an enzyme(s) preferring  $\beta$ 1-3 links is confronted with a  $\beta$ 1-4 linkage, which may explain the tendency of  $\beta$ -linked oligosaccharides to survive mashing and emerge into beer.

Exo-glucanases in peaks I and III were substantially more sensitive to the presence of a range of ions and other inhibitors than was exo-glucanase in peak II and, especially, the endo-glucanase (except mercuric chloride in the latter instance) (Table III). It would be worthwhile exploring whether the levels of ions such as potassium, sodium, magnesium are sufficient to impede the action of exo-glucanases in the mash.

## CONCLUSIONS

$\beta$ -Glucosidase and endo- $\beta$ 1-4-glucanase are present in raw barley and, like endo- $\beta$ 1-3,1-4-glucanase, endo- $\beta$ 1-3-glucanase and exo- $\beta$ 1-3-glucanase, increase in activity during malting. Exo- $\beta$ 1-3-glucanase develops very late during germination and may be in limiting quantity in insufficiently modified malts. There are three separate exo-glucanases in malt, each displaying a preference for

**Table III.** Susceptibility of glucanases to inhibitors and activators.

Inhibitor	Concentration	Exo-glucanase I	Exo-glucanase II	Exo-glucanase III	Endo- $\beta$ -glucanase
KCl	1 mM	51.1	74.3	58.3	87.3
NaCl	1 mM	50.9	77.9	58.3	84.8
MgSO <sub>4</sub>	1 mM	48.7	77.9	59.2	82.4
ZnSO <sub>4</sub>	1 mM	51.7	76.2	56.8	79.9
MnSO <sub>4</sub>	1 mM	96.7	102.8	101.2	100.5
CuSO <sub>4</sub>	1 mM	53.4	69.8	55.8	76.3
CoSO <sub>4</sub>	1 mM	50.4	75.7	61.2	78.9
FeCl <sub>2</sub>	1 mM	44.0	77.7	40.3	82.7
Iodoacetamide	1 mM	50.9	79.8	56.3	101.8
HgCl <sub>2</sub>	1 mM	55.6	80.5	58.3	45.3
CaCl <sub>2</sub>	1 mM	55.3	80.2	59.2	101.7
EDTA	1 mM	57.1	79.2	61.7	96.3
Azide	1 mM	58.1	79.2	57.8	95.3
NBS	1 mM	21.1	35.8	43.7	-
NEM	1 mM	111.1	135.1	112.1	98.3
EGTA	1 mM	113.9	137.8	111.7	-
DTT	1 mM	17.1	80.7	45.6	101.1

$\beta$ 1-3 linkages. As the principle product of endo- $\beta$ 1-3,1-4-glucanase is oligosaccharides with a  $\beta$ 1-4 linkage at the non-reducing end (from which end exo enzymes approach the substrate), this is likely to be a further reason why such oligosaccharides survive in significant quantities into wort. The exo-glucanases are also sensitive to ions such as potassium, sodium and magnesium.

#### REFERENCES

- Bamforth, C. W., Barley  $\beta$ -glucans: their role in malting and brewing. *Brew. Dig.*, 1982, **57** (6), 22-27, 35.
- Bamforth, C. W.,  $\beta$ -Glucan and  $\beta$ -glucanases in malting and brewing: practical aspects. *Brew. Dig.*, 1994, **69** (5), 12-16, 21.
- Bamforth, C. W., Beer: Health and Nutrition, 2004, Blackwell: Oxford.
- Bamforth, C. W. and Gambill, S. C., Fiber and putative prebiotics in beer. *J. Am. Soc. Brew. Chem.*, 2007, **65**, 67-69.
- Bamforth, C. W. and Kanauchi, M., A simple model for the cell wall of the starchy endosperm in barley. *J. Inst. Brew.*, 2001, **107**, 235-240.
- Bamforth, C. W., Martin, H. L. and Wainwright, T., A role for carboxypeptidase in the solubilization of barley  $\beta$ -glucan. *J. Inst. Brew.* 1979, **85**, 334-338.
- Bamforth, C. W., Moore, J., McKillop, D., Williamson, G. and Kroon, P. A., Enzymes from barley which solubilize  $\beta$ -glucan. Proceedings of the European Brewery Convention Congress, Maastricht, IRL Press : Oxford, 1997, pp. 75-82.
- Bathgate, G. N., Palmer, G. H. and Wilson, G., The action of endo- $\beta$ -glucanases on barley and malt  $\beta$ -glucans. *J. Inst. Brew.* 1974, **80**, 278-285.
- Bathgate, G. N. and Dalgleish, C. E., The diversity of barley and malt  $\beta$ -glucans. Proceedings of the American Society of Brewing Chemists, 1974, pp. 32-36.
- Bradford, M. M., A rapid and sensitive method for the quantitation or microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 1976, **72**, 248-254
- Brenner, M. W., Brewing with barley. *Tech. Q. Master Brew. Assoc. Am.*, 1972, **9**, 12-17.
- Fincher, G. B., Morphology and chemical composition of barley endosperm cell walls. *J. Inst. Brew.*, 1975, **81**, 116-122.
- Fulcher, R. G., Setterfield, G., McCully, M. E. and Wood, P. J., Observations on the aleurone layer. II. Fluorescence microscopy of the aleurone-sub-aleurone junction with emphasis on possible  $\beta$ -1,3-glucan deposits in barley. *Aust. J. Plant Physiol.* 1977, **4**, 917-928.
- Hoy, J. L., Macauley, B. J. and Fincher, G. B., Cellulases of plant and microbial origin in germinating barley. *J. Inst. Brew.*, 1981, **87**, 77-80.
- Hrmova, M. and Fincher, G. B., Barley  $\beta$ -D-glucan exohydrolases. Substrate specificity and kinetic properties. *Carbohydrate Research*, 1998, **305**, 209-221.
- Hrmova, M., Harvey, A. J., Wang, J., Shirley, N. J., Jones, G. P., Stone, B. A., Høj, P. B. and Fincher, G. B., Barley- $\beta$ -D-glucan exohydrolases with  $\beta$ -glucosidase activity. Purification, characterization and determination of primary structure from a cDNA clone. *J. Biol. Chem.*, 1996, **271**, 5277-5286.
- Hrmova, M., MacGregor, E. A., Biely, P., Stewart, R. J. and Fincher, G. B., Substrate binding and catalytic mechanism of a barley  $\beta$ -D-glucosidase/(1,4)- $\beta$ -D-glucan exohydrolase. *J. Biol. Chem.*, 1998, **273**, 11134-11143.
- Kanauchi, M. and Bamforth, C. W., Enzymic digestion of walls purified from the starchy endosperm of barley. *J. Inst. Brew.* 2002, **108**, 73-77.
- Luchsinger, W. W., English, H. and Kneen, E., Autolytic action of gumases in malting and brewing. Influence of malting and brewing on barley gums. Proceedings of the American Society of Brewing Chemists, 1958, pp. 40-46.
- Martin, H. L. and Bamforth, C. W., An enzymic method for the measurement of total and water-soluble  $\beta$ -glucan in barley. *J. Inst. Brew.*, 1981, **87**, 88-91.
- Martin, H. L. and Bamforth, C. W., Application of a radial diffusion assay for the measurement of  $\beta$ -glucanase in malt. *J. Inst. Brew.*, 1983, **89**, 34-37.
- Somogyi, M., Notes on sugar determination. *J. Biol. Chem.* 1952, **195**, 19-23.
- Varghese, J. N., Garrett, T. P., Colman, P. M., Chen, L., Høj, P. B. and Fincher, G. B., Three-dimensional structures of two plant beta-glucan endohydrolases with distinct substrate specificities. *Proc. Natl. Acad. Sci.*, 1994, **91**, 2785-2789.
- Woodward, J. R., Fincher, G. B. and Stone, B. A., Water soluble (1 $\rightarrow$ 3), (1 $\rightarrow$ 4)- $\beta$ -D-glucans from barley (*Hordeum vulgare*) endosperm. II. Fine structure. *Carb. Polym.* 1983, **3**, 207-225.
- Yamashita, H., Uehara, H., Tsumura, Y., Hayase, F. and Kato, H., Precipitate forming reaction of  $\beta$ 1-4-glucanase in malt. *Agric. Biol. Chem.* 1987, **51**, 655-664.

(Manuscript accepted for publication July 2008)