MEASUREMENT OF MALT BETA-GLUCANASE

B. V. McCLEARY

(Biological and Chemical Research Institute, N.S.W. Department of Agriculture, Rydalmere, Australia)

A procedure has been developed for the assay of malt β-glucanase [a(1→3)(1→4)—β—D—glucanase] which employs as substrate, barley β-glucan dyed with Remazolbrilliant Blue and chemically modified with carboxymethyl groups to increase solubility. The described assay procedure together with a modified extraction format allows analysis of up to ten malt samples in less than 80 min. Also, the procedure is specific for enzymes active on barley β-glucan, is accurate and reliable, and can be readily applied to the analysis of β-glucanase in malt, green malt and wort.

KEYWORDS: malt β-glucanase, malt, Azo-Barley glucan, wort, assay.

INTRODUCTION

Barley-endosperm, cell-wall β-glucan [(1→3)(1→4)—β—D—glucan] can seriously affect the malting quality of barley grain and the brewhouse performance of the derived malt and of unmalted barley used as adjunct.[1-3] Consequently, there has been considerable interest in the changes in amount and properties of this polymer during the malting and brewing processes. On malting of barley, there is a preliminary lag-phase followed by a rapid depletion of barley β-glucan. This is paralleled by an increase in specific *endo* (1→3)(1→4)—β—D—glucanase [EC 3.2.1.73] (malt β-glucanase).[3-6] Hydrolysis by malt β-glucanase has a dramatic effect on the viscosity of barley β-glucan solutions; thus even limited depolymerization by this enzyme is likely to alleviate the filtration problems caused by high viscosity (high molecular weight) barley β-glucans.[2,7,8] A second enzyme, termed "β-glucan solubilase" (thought to be a carboxypeptidase) has been implicated in the release of the β-glucan into solution.[9,10]

With the confirmed central role played by malt β-glucanase in the depolymerisation of barley β-glucan during malting and most probably also during mashing[7] (particularly if mashing-in temperatures below 50°C are employed), there has been considerable interest in the availability of a simple and reliable method for the estimation of the β-glucanase content of malt. Assays based on the measurement of the decrease in viscosity of barley β-glucan solutions have been used most widely,[2,11,12] but other assays based on the increase in reducing power when β-glucan is hydrolysed,[13] or based on the rate of diffusion of malt β-glucanase through an agar gel containing a β-glucan/Congo red complex[14] have also been proposed. The assay based on the increase in reducing power on incubation of malt extract with either barley β-glucan or lichenan[13] is of no use in the routine assay of activity in malt extracts because these extracts contain high concentrations of reducing sugars. Dialysis, precipitation or ultrafiltration[13] of malt extracts to remove these reducing sugars is not feasible on a routine basis because these procedures are time consuming and also because the malt β-glucanase is so labile[7] that activity in the final preparation may not accurately reflect that present in the malt. The limited accuracy and reliability of the previously recommended viscometric method[15] has been known for some time, but the method persisted due to the unavailability of a more reliable alternative. Recently, Martin and Bamforth[14] proposed an assay for malt β-glucanase based on the rate of radial diffusion of the enzyme through agar plates containing a barley β-glucan/Congo red complex. Wood[16] had previously used such an assay for the measurement of trace quantities of *endo*-β-glucanases in various commercial enzyme preparations. Although this radial diffusion assay is simple in operation and readily demonstrates the presence or absence of *endo*—(1→3)(1→4)—β—D—glucanase, a recent inter-laboratory evaluation of this method and the IRVU viscometric method demonstrated that neither was sufficiently precise to warrant its inclusion in the Recommended Methods of the Institute of Brewing.[15] An altenative approach to the analysis of polysaccharide *endo*-hydrolases is the use of dye-labelled substrates, i.e., carbohydrate polymers to which a dye is covalently attached. Such substrates have found widespread use in the measurement of α-amylase,[17-19] cellulase [*endo*—(1→4)—β—D—glucanase][19], (1—4)—β—D—xylanase[20] and (1—4)—β—D—mannanase.[21] In the current paper the application of a soluble dye-labelled substrate to the analysis of malt β-glucanase activity will be described.

EXPERIMENTAL

Enzymes

Cellulases [*endo*—(1→4)—β—glucanase] [EC 3.2.1.4] were purified from the commercial preparations, Celluclast (*Trichoderma reesei*) and Finizym (*Aspergillus niger*) from Novo Industrias, Denmark and from a *Penicillium emersonii* preparation (BGHS) from Biocon (Australia) Pty. Ltd. The major cellulase in the Finizym preparation was prepared as previously described.[22] For the purification of cellulases from the Celluclast preparation, an aliquot (50 mL) of the preparation was diluted to 250 mL with distilled water, the pH adjusted to 8.0 and the solution chilled and dialyzed against ice-cold tris/HCl buffer (20 mM, 5L) overnight. The solution was chromatographed on

DEAE-Sepharose CL-6B (bed volume, 3.2 x 23 cm), using a KCl gradient (0-0.5M, total volume 2 L) in tris HCl buffer (20 mM, pH 8). The major peak of cellulase activity, which eluted at a KCl concentration of 0.15 M, was concentrated by ammonium sulphate precipitation (50 g/100 mL) and collected by centrifugation (3,000g, 20 min). On dissolution in a minimum volume of distilled water it was applied to a column (2.8 x 90 cm) of Ultrogel AcA 44 and eluted with acetate buffer (10 mM pH 4.5). The active fraction, which eluted as a single, sharp peak, was treated with citrate buffer to give a final concentration of 20 mM and a pH of 3.0. This enzyme was then applied to SP-Tris Acrylamide (bed volume 3 x 10 cm) and eluted with a linear KCl gradient (0-0.5 M, total volume 500 mL) in citrate buffer (20 mM pH 3.0). Two peaks of activity eluted (I and II); I did not bind to the column, while II eluted at a KCl concentration of 0.08 M. Each fraction appeared as several bands on isoelectric focusing with pI values of 3.5-4.5 (II) and 4.3-4.8 (I). The fractions showed very similar relative rates of hydrolysis of a range of substrates including CMC 4M, CMC 7M, barley β-glucan, tamarind amyloid and hydrocellulose.[23]

Penicillium emersonii cellulases were purified by essentially the same format as employed for the *Trichoderma reesei* cellulases. Two peaks of activity eluted from DEAE-Sepharose CL-6B, at KCl concentrations of 0.15 M (I) and 0.20 M (II). The enzymes did not bind to SP-Tris acrylamide at pH 3 (20 mM citrate buffer). They varied markedly in their specificity.[23] Fraction I appeared as a single band next to the anode wick (pI~3) on isoelectric focusing, whereas Fraction II appeared as several bands (pI 3-3.6).

Malt β-glucanase was prepared by suspending freeze-dried and milled green malt (collected just before kilning, 400g) in sodium acetate buffer (0.1 M, pH 4.6, 1L). The suspension was stirred at room temperature for 30 min, centrifuged (3,000 g, 20 min) and the supernatant filtered through muslin (to remove floating debri) and treated with ammonium sulphate (50g/100 mL). The precipitate which formed on storing the solution at 4° C for 4 h was collected by centrifugation (3,000 g, 20 min) dissolved in a minimum volume of demineralized water and dialyzed against ice-cold sodium acetate buffer (20 mM, pH 4.5, 10L) for 20 h. This solution was centrifuged (3,000 g, 20 min) and the supernatant applied to a column of CM-Sepharose (3 x 21 cm). The column was eluted with a linear potassium chloride gradient (0-0.4M) in sodium acetate (20 mM, pH 4.5). Two peaks of activity were recovered, I and II. Fraction I was devoid of β-glucosidase activity and was thus used to standardise the dye-labelled substrate into aliquots and stored frozen.

Preparation of substrates

Low-viscosity (~ 2 dL/g) barley β-glucan, RBB-barley β-glucan and RBB-CM-barley β-glucan (Azo-Barley glucan) were prepared as described elsewhere.[24] Carboxymethyl Cellulose 4M6F was obtained from Hercules Inc., Wilmington, Delaware, U.S.A., and was used without further purification. Remazol Black B dyed barley β-glucan was prepared as for the RBB-barley β-glucan except that the dye to barley β-glucan ratio used was 1:10.

Routine extraction of malt β-glucanase

Malt samples (at least 20 g) were milled to pass a 0.5 mm screen. Samples (0.5 g) were accurately weighed into centrifuge tubes (12 mL capacity), sodium acetate buffer (6.0 mL, 0.3M pH 4.6) added and the contents occasionally stirred on a vortex mixer over 15 min at room temperature. The tubes were centrifuged (1,000 g, 10 min), incubated at 30° C for 5 min and aliquots removed for the assay of malt β-glucanase using Azo-Barley glucan substrate.

Assay of malt β-glucanase using Azo-Barley glucan substrate.

Aliquots (0.5 mL) of Azo-Barley glucan (1% w/v, salt-free) were dispensed with an Eppindorf Multipette positive displacement pippettor into centrifuge tubes (12 mL capacity) and the tubes preincubated at 30° C for 5 min. To each tube an aliquot (0.5 mL) of malt extract, prepared as described and preincubated at 30° C for 5 min, was added. The tubes were stirred vigorously on a vortex mixer and incubated at 30° C for exactly 10 min. The reaction was terminated by the addition of precipitant solution (3.0 mL) and the tube contents stirred vigorously.

After standing at room temperature for 5 min, the tubes were centrifuged (1,000 g) for 10 min and the absorbance (590 nm) of the clear supernatant solution read against a reaction blank. The reaction blank was prepared by adding precipitant solution to the Azo-Barley glucan before addition of malt extract. Malt β-glucanase activity was determined by referring the absorbance values to an Azo-Barley glucan calibration curve prepared using dialyzed malt β-glucanase. The activity per kilogram of malt on an "as is" basis was determined using the equation:

U/kg malt flour = U/0.5 mL extract x 12 x 2,000

U . . . International Unit of enzyme activity; equals one micromole of reducing sugar equivalent released per minute at 30° C and pH 4.6

12 . . . 0.5 mL of extract out of 6.0 mL was analysed

2,000 . . . weight adjustment factor (0.5 g of malt was extracted but the results are presented per kg of malt).

Precipitant solution

The precipitant solution was prepared by dissolving NaCl (40 g), $NaWO_4.2H_2O$ (40 g), $NaH_2PO_4.2H_2O$ (40 g) and phenol (4 g) in water (1.8 L). The pH was adjusted to 2.6 with concentrated hydrochloric acid and the volume adjusted to 2 L.

Reducing sugar assay

Suitably diluted, dialyzed malt extract (50μL) was incubated with a solution of barley β-glucan (0.5 mL, 5 mg/mL) in sodium acetate buffer (100 mM, pH 4.6) for up to 10 min at 30° C. The reaction was terminated and the colour developed using Nelson/Somogyi

reagent solutions[25] as previously described[22] except that 0.5 mL of reagent D was used to stop the reaction and reagent E was prepared by diluting reagent C 5-fold. One Unit of enzyme activity releases one μ-mole of reducing-sugar equivalent (as glucose) per min at 30°C and pH 4.6.

Preparation of Azo-Barley glucan calibration curve

The calibration curve relating the increase in absorbance (590 nm) of the supernatant solution on incubation of Azo-Barley glucan with malt β-glucanase was prepared using malt β-glucanase I recovered from CM-Sepharose chromatography and was standardized using the Nelson/Somogyi reducing-sugar assay[25] with barley β-glucan as substrate. Aliquots (0.5 mL) of malt β-glucanase I (0-30 mU/0.5 mL) in acetate buffer (0.3M, H 4.6) were incubated with Azo-Barleyglucan substrate (0.5 mL, 1% w/v) at 30°C for exactly 10 min, the reaction terminated and the absorbance recorded as for crude malt extracts.

Variations on the standard extraction and β-glucanase assay procedure

The effect of sodium acetate buffer concentration and the pH of the precipitant solution on the measured absorbance values were determined simply by varying the former between 50 mM and 1.0 M, and the latter between pH 2.0 and 3.0. Effectiveness of enzyme extraction was determined by re-extracting the pellets obtained on centrifugation of the initial extraction mixture. Likely effects of maltose in the malt extract on colour release from Azo-Barley glucan were studied using malt β-glucanase I in sodium acetate (0.3M) buffer plus maltose (0-5% w/v).

Properties of malt β-glucanase

pH activity was determined by incubating Azo-Barley glucan (0.5 mL, 1% w/v) with malt extract in sodium acetate buffer (0.3M) at pH 3.5-5.5. The stability of the enzyme at different temperatures under simulated mashing conditions was determined by adding an aliquot of water pre-equilibrated at 40-70°C to a sample of malt flour (0.5 g). The tubes were vigorously stirred and incubation at the respective temperature continued up to 20 min. After various time intervals tubes were removed and immediately cooled in an ice-water bath. The contents were diluted by adding sodium acetate buffer (3 mL, 0.5M, pH 4.6). The tubes were centrifuged (1,000 g, 10 min) and the supernatant solutions assayed for malt β-glucanase by the standard procedure. The changes in malt β-glucanase activity during the malting of barley (Weeah *var.*) was studied by removing samples of green malt from a commercial maltings. The samples were immediately frozen and then freeze-dried and milled to pass a 0.5 mm screen. Malt β-glucanase was assayed using the standard procedure except that extracts containing high enzyme activity had to be diluted 2-3 fold before assay.

Hydrolysis of Azo-Barley glucan by cellulases

Assays were performed exactly as for the malt β-glucanase assay. Enzymes employed were *Aspergillus niger* cellulase, *Penicillium emersonii* cellulase II and *Trichoderma reesei* cellulase I, each at 40-50 mU assay. Incubations were performed at 30°C and assays terminated by the addition of precipitant solution. However, the precipitant did not completely terminate the reaction, so, after addition of precipitant solution, the reaction mixtures and blanks (containing enzyme) were centrifuged immediately (1,000 g, 5 min) and the supernatant solution separated from the pellet. The cellulase enzymes were standardized against barley β-glucan (5 mg/mL) at pH 4.5.

Measurement of barley β-glucan

(1→3)(1→4)—β—D—Glucan in barley and malt was measured as previously described.[22,26]

RESULTS AND DISCUSSION

Direct dyeing of barley β-glucan with Remazol-brilliant Blue R (RBB) or Remazolbrilliant Black B (R-Black), to a dye content of approximately one dye molecule per 20-25 anhydro-hexose units, yields an insoluble product. The latter substrate remains insoluble even on heating, but the former can be dissolved by heating to 90-100°C together with vigorous shaking or mixing of the tube contents. The substrate remains soluble on cooling to 50°C but then forms a colloidal suspension which eventually settles from solution. This RBB-barley β-glucan substrate gives excellent reaction kinetics on incubation with malt β-glucanase, but because of its insoluble nature it is inconvenient to use on a routine basis. Both this and R-Black barley β-glucan could find considerable use in locating (1→3)(1→4)—β—D—glucanases and cellulases in electrophoretograms and also for identifying micro-organisms producing these enzymes.

The solubility problem with dye-labelled barley β-glucan was overcome by chemically modifying the glucan before dyeing. This was achieved by treatment with chloroacetic acid to introduce carboxymethyl groups. The degree of carboxymethylation was critical. The optimal degree of substitution (d.s.) to impart solubility without seriously affecting the susceptibility of the polysaccharide to hydrolysis by malt β-glucanase was between 0.08 and 0.12. The optimal degree of dyeing of this polysaccharide was one dye molecule per 25 anhydro-hexose units.

The precipitant solution employed in assays using RBB-CM-Barley β-glucan (Azo-Barley glucan) is based on a precipitant commonly used for removing protein from solution. This precipitant also effectively removed high degree of polymerization (d.p.) substrate molecules from solution. On incubation of Azo-Barley glucan with malt β-glucanase under standard assay conditions, treatment with the precipitant followed by centrifugation yielded a stable, crystal-clear, supernatant solution suitable for spectrophotometric measurement of absorbance values. The concentration of the acetate buffer in the malt extract and the pH and balance of salts in the precipitant solution were selected empirically to give the minimum possible blank value consistent with a useful reaction curve. The effect of precipitant pH on absorbance values when Azo-Barley glucan is icubated with malt extract is shown in Fig 1. Over the pH range 2.5-3.0 the effect

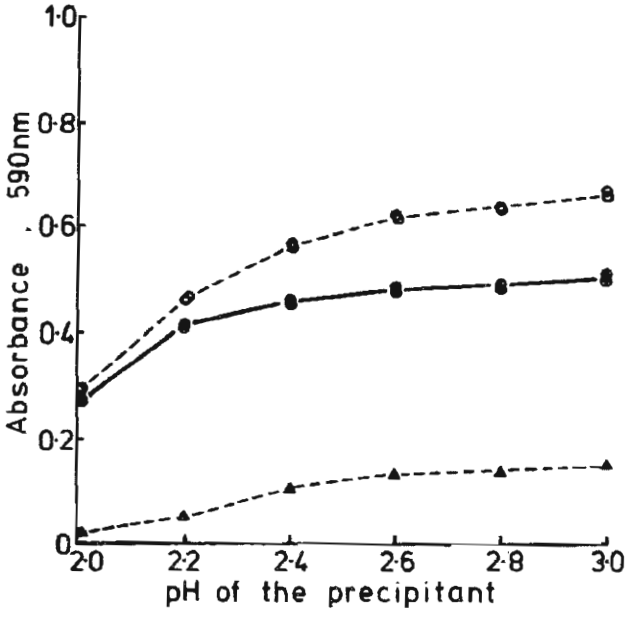

FIGURE 1

Effect of precipitant pH on the level of soluble dye-labelled fragments after centrifugation of the reaction mixtures. Enzyme/substrate mixtures were incubated at 30°C for 10 min; (▲) reaction blank; (O) reaction; (●) reaction minus blank.

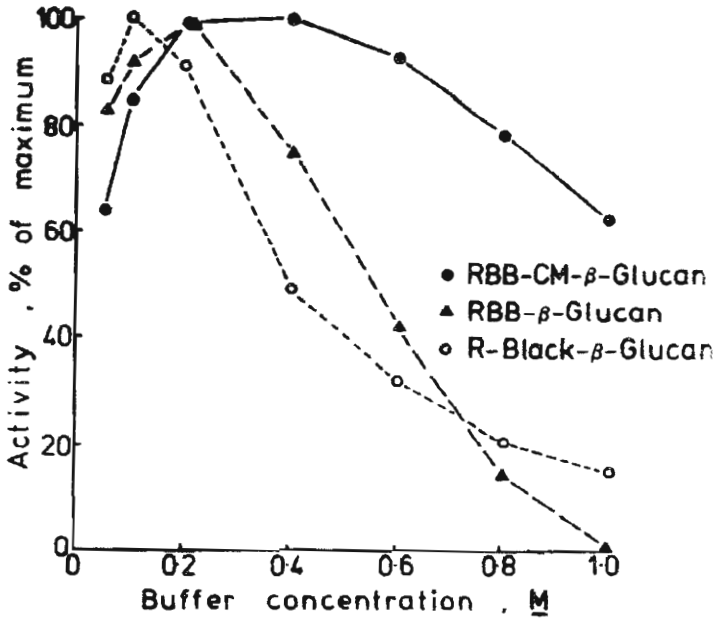

FIGURE 2

Effect of the concentration of extractant buffer (sodium acetate, pH 4.6) on absorbance (590 nm) on incubation of malt extract with dye-labelled barley β-glucan; (●) RBB-CM-β-glucan (Azo-Barley glucan); (▲) RBB-β-glucan; (O) R-Black-β-glucan.

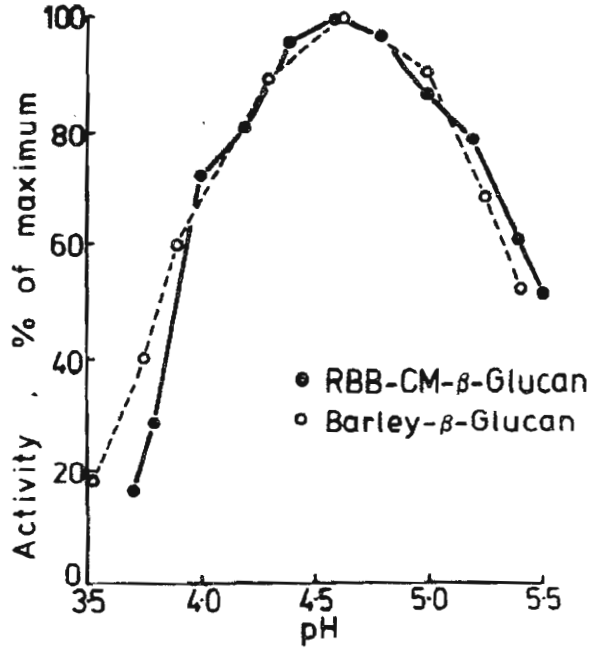

FIGURE 3

pH-Activity curves for dialysed malt β-glucanase on (●) Azo-Barley glucan and (O) barley β-glucan (Nelson/Somogyi assay procedure). The dialyzed enzyme was adjusted to 0.3 M acetate buffer (pH 3.5-5.5), before assay.

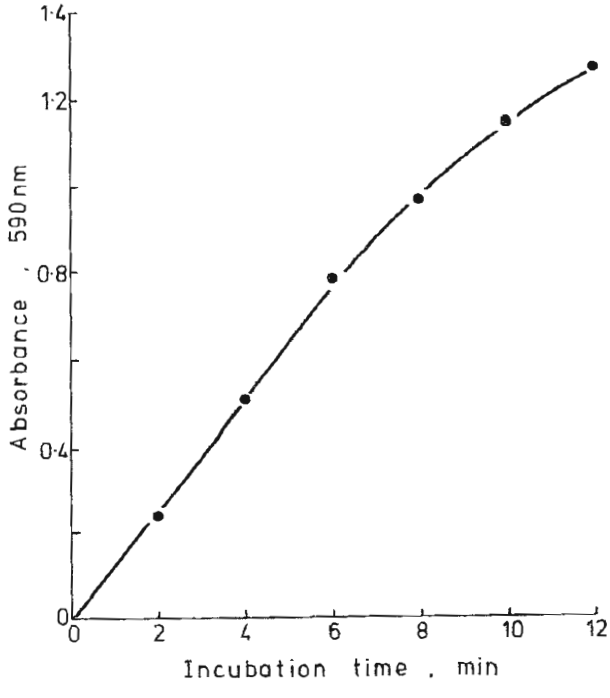

FIGURE 4

Rate of increase in absorbance (590 nm) of the supernatant solution (on centrifugation) on incubation of Azo-Barley glucan with malt β-glucanase (25 mU/assay) under standard assay conditions.

of precipitant pH is minimal but still significant. The concentration of the sodium acetate buffer (pH 4.6) used to extract the malt β-glucanase is also critical. In the described Azo-Barley glucan assay procedure, buffer concentrations in the range of 200-400 mM gave the same and maximal absorbance values (Fig. 2). Since the amount of dye-labelled polysaccharide precipitating from solution is a consequence of the final concentration of salt and the pH of the reaction mixture after addition of the precipitant, then alterations to the extraction buffer would require changes in the precipitant. The optimal pH for activity of malt β-glucanase on both barley β-glucan and Azo-Barley glucan is 4.6 (Fig. 3); thus the precipitant pH is essentially dictated by this and was optimal at 2.6.

Malt β-glucanase is very heat-labile and, on extraction, is possibly also susceptible to protease attack. In the current studies, malt was extracted at 4°C, 30°C and room temperature (23°C), for up to 60 min. Results indicated that the enzyme could be effectively extracted in 15 min with occasional stirring on a vortex mixer, when a malt to buffer ratio of 1:12 was employed. Re-extraction of the pellet showed that more than 95% of the enzyme had been recovered in the first extract. The remaining activity may simply have been in the liquid remaining in the pellet. Further, the extracted enzyme was quite stable at room temperature and at 30°C for several hours, indicating that, under these conditions at least, the enzyme was resistent to proteolysis.

The time course of hydrolysis of Azo-Barley glucan by malt β-glucanase is shown in Fig. 4 and the standard curve relating absorbance at 590 nm to activity of malt β-glucanase under standard assay conditions (10 min, 30°C) is shown in Fig. 5. The standard curve is essentially linear over the range of 0.1-1.0 absorbance units. Since this curve was prepared using a partially purified malt β-glucanase it was essential to demonstrate that other malt components (particularly maltosaccharides) do not affect this standard curve. To do this, the rate of hydrolysis of Azo-Barley glucan by malt β-glucanase I containing maltose at levels of 0-50 mg/mL, was studied. The added maltose had no detectible effect on the reaction curve.

In the present study, the activity in all the malts examined, (excluding green malts) gave absorbance values that fell within the linear range of the standard curve. Green malts contained up to three times the activity of kilned malt and thus these extracts had to be diluted 2-3 fold before assay. The accuracy and reliability of the extraction and assay procedure is shown in Table I where the results obtained on analysing five selected samples in triplicate on two different days is shown. The values are expressed as U/kg malt.

The described assay procedure is specific for enzymes hydrolyzing barley β-glucan, which includes both malt β-glucanases and cellulases. Similar relative initial rates of hydrolysis of Azo-Barley glucan and barley β-glucan (by the Nelson/Somogyi reducing sugar assay) by malt β-glucanase and by *Aspergillus niger*, *Trichoderma reesei* and *Penicillium emersonii* (II) cellulases were observed. However, in well modified malt, the concentration of cellulase [*endo*-(1→4)—β—D—glucanase] is far outweighed by that of malt β-glucanase [specific *endo*-(1→3)(1→4)—β—D-glucanase]. Actually, the assay procedure as described is not ideally suited for the measurement of cellulase activity because the precipitant solution used is not completely effective in inactivating these enzymes.

The malt β-glucanase assay procedure currently described could potentially find widespread use in the malting and brewing industries. It offers the malster a simple method for monitoring changes in this activity during both malting and kilning, helping him to optimise both of these operations. This is demonstrated in Fig. 6 where changes in activity of malt β-glucanase and of barley β-glucan during the malting of barley (*var.* Weeah), is shown. After initiation of the steep there is no significant increase in malt β-glucanase for 18 hours. There is then a steady increase in activity over the next 35 hours, followed by a rapid increase. These changes parallel the pattern of barley β-glucan depletion and are consistent with previously reported results.[2,6] Kilning of the malt led to a greater than 50% decrease in malt β-glucanase activity.

To the brewer, a knowledge of malt β-glucanase levels is of value in predicting the ability of the malt to depolymerise endogenous barley β-glucan as well as that which might be added exogenously, particularly if the adjunct is unmalted barley. Mashing-in temperatures can be modified to account for the temperature lability of malt β-glucanase, and the level of this enzyme in the mash can be readily assayed with Azo-Barley glucan. The temperature stability of malt β-glucanase in simulated mashes at a range of temperatures is shown in Fig. 7. It is apparent that the enzyme is rapidly inactivated at 65°C, but at 50°C, about 35% of the activity still remains after 20 min. This could contribute significantly to barley β-glucan degradation, suggesting that the incorporation of a low temperature (50°C) mashing-in step may well alleviate the problems caused by this glucan.

ACKNOWLEDGEMENTS

The author thanks Ms. I. Shameer for expert technical assistance and Ms M. Daniels for assistance in the preparation of this manuscript. Malt samples were kindly provided by Mr. W. Tremayne, Barrett Burston Pty. Ltd., Thornleigh, N.S.W. This research was funded by a grant from the Australian Barley Research Council and the author thanks Biocon (Australia) for financially supporting the presentation of this paper.

REFERENCES

1. Erdal, K. & Gjertsen, P., *Proceedings of the 11th Congress of the European Brewery Convention, Madrid,* 295 (1967).
2. Bourne, D. T. & Pierce, J. S., *Journal of the Institute of Brewing, 76,* 328 (1970).
3. Bamforth, C. W., *Brewers Digest, 57,* (6) 22 (1982).
4. Preece, I. A. & Hoggan, J., *Journal of the Institute of Brewing, 62,* 486 (1956)

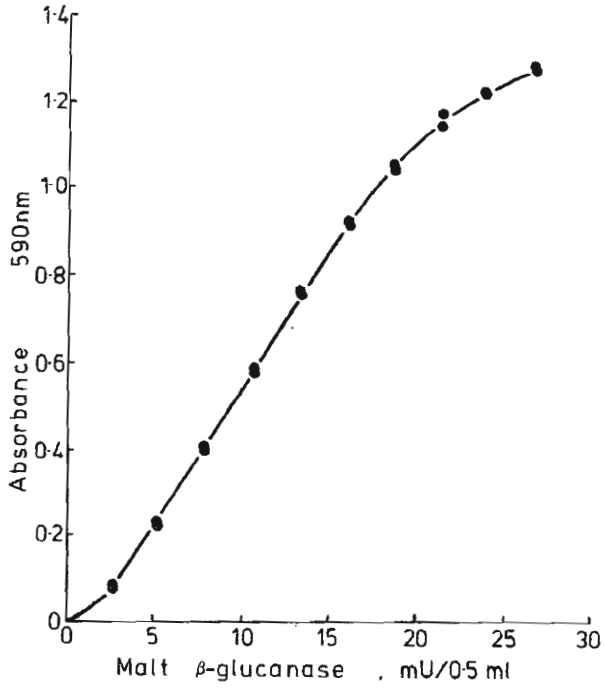

FIGURE 5
Standard curve relating activity of malt β-glucanase (mU/assay) on barley β-glucan (5 mg/mL) to absorbance increase (590 nm) on hydrolysis of Azo-Barley glucan for 10 min at 30°C.

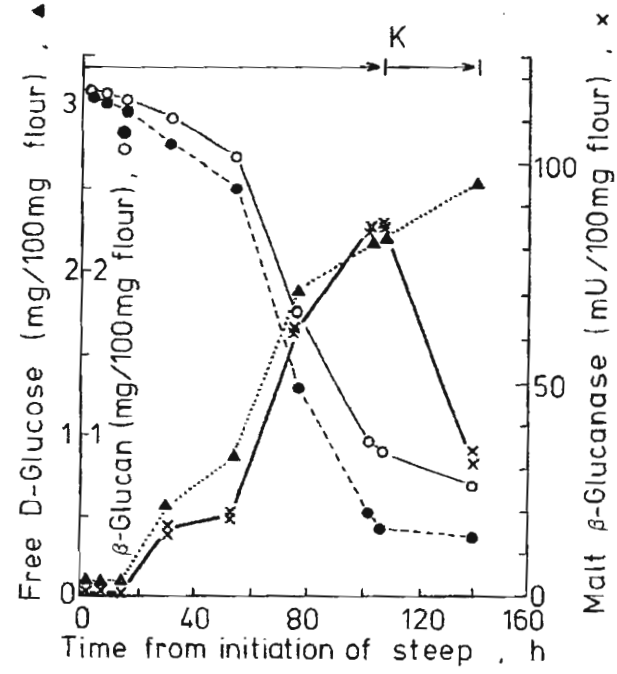

FIGURE 6
Changes in malt β-glucanase and barley β-glucan and glucose levels during the malting of barley (*var.* Weeah). K, initiation of kilning; (●) alcohol washed malt flour; (○) unwashed malt flour.

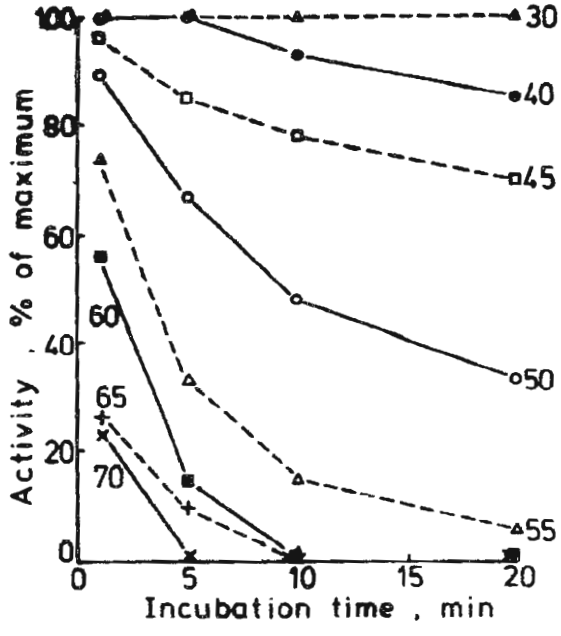

FIGURE 7
Temperature stability of malt β-glucanase under simulated mashing conditions. Experimental details are given in the text.

TABLE I
DETERMINATION OF β-GLUCANASE CONTENT OF MALT USING THE AZO-BARLEYGLUCAN ASSAY PROCEDURE

Malt Sample	Malt β-Glucanase (U/kg)			
	Day 1		Day 2	
	Replicate 1	Replicate 2	Replicate 1	Replicate 2
A	345*	350	350	360
	350*	355	355	360
B	190	190	195	193
	185	190	195	195
C	275	275	280	280
	270	275	280	280
D	150	150	150	148
	145	150	145	148
E	420	415	430	445
	420	415	435	440

*Duplicate determinations on a given extract.

5. Luchsinger, W. W., English, H., Cochrane, D. G. & Kneen, E., *Proceedings of the American Society of Brewing Chemists,* 40 (1958).
6. Bamforth, C. W., *Journal of the Institute of Brewing, 91,* 154 (1985).
7. Bourne, D. T., Jones, M. & Pierce, J. S., *Technical Quarterly of the Master Brewers Association of the Americas, 13,* 3 (1976).
8. Crabb, D. & Bathgate, G. N., *Journal of the Institute of Brewing, 79,* 519 (1973).
9. Bamforth, C. W., Martin, H. L. & Wainwright, T., *Journal of the Institute of Brewing, 85,* 334 (1979).
10. Bamforth, C. W., *Proceedings of the 18th Congress of the European Brewing Convention, Copenhagen,* 335 (1981).
11. Bathgate, G. N., *Journal of the Institute of Brewing, 85,* 92 (1979).
12. Woodward, J. R. & Fincher, G. B., *European Journal of Biochemistry, 121,* 663 (1982).
13. Denault, L. J., Allen, W. G., Boyer, E. W., Collins, D., Kramme, D. & Spradlin, J. E., *Journal of the American Society of Brewing Chemists, 36,* 18 (1978).
14. Martin, H. L. & Bamforth, C. W., *Journal of the Institute of Brewing, 89,* 34 (1983).
15. Buckee, G. K., *Journal of the Institute of Brewing, 91,* 264 (1985).
16. Wood, P. J., *Carbohydrate Research, 94,* C19 (1981).
17. Ceska, M., Birath, K. & Brown, B., *Clinical Chemica Acta, 26,* 437 (1969).
18. Babson, A. L., Tenney, S. A. & Megrew, R. E., *Clinical Chemistry, 16,* 39 (1970).
19. McCleary, B. V., *Carbohydrate Research, 86,* 97 (1980).
20. Biely, P., Mislovicova, D. & Toman, R., *Analytical Biochemistry, 144,* 142 (1985).
21. McCleary, B. V., *Carbohydrate Research, 67,* 213 (1978).
22. McCleary, B. V. & Glennie-Holmes, M., *Journal of the Institute of Brewing, 91,* 285 (1985).
23. McCleary, B. V., *Gums and Stabilizers for the Food Industry, Proceedings of the 3rd International Conference, Wrexham,* (1985) *in press.*
24. McCleary, B. V., *Carbohydrate Polymers, in press.*
25. Somogyi, M., *Journal of Biological Chemistry, 195,* 19 (1952).
26. McCleary, B. V. & Nurthen, E., *Journal of the Institute of Brewing, in press.*