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A Soluble Chromogenic Substrate for the Assay of (1 → 3)(1 → 4)-β-D-Glucanase (Lichenase)

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SUMMARY

A simple procedure for the assay of (1 → 3)(1 → 4)-β-D-glucanase (lichenase) has been developed. This assay employs as substrate barley (1 → 3)(1 → 4)-β-D-glucan dyed with Remazolbrilliant Blue R and chemically modified with carboxymethyl groups to increase solubility. Preparation of this substrate required the development of an improved procedure for the extraction and purification of barley β-glucan. Assays based on the use of the described chromogenic substrate at pH 6·5 are sensitive and specific for enzymes active on barley β-glucan.

INTRODUCTION

The major carbohydrate component of barley endosperm cell walls is a mixed-linkage (1 → 3)(1 → 4)-β-D-glucan (barley β-glucan; Fincher (1975)) which represents from 2·8 to 5·5% of the total grain weight. During the malting process it is essential that this glucan is effectively depolymerised by the endogenous *endo*-(1 → 3)(1 → 4)-β-D-glucanases [EC 3.2.1.73]. Barley β-glucan remaining in poorly modified malts can, through its effect upon viscosity, lead to reduced rates of wort filtration, to haze, precipitate and gel formation in beer, and possibly also to a reduced efficiency of extraction of sugars from the mashed grain (Bamforth, 1982). These problems can be reduced or eliminated by the addition of microbial cellulase [EC 3.2.1.4] or (1 → 3)(1 → 4)-

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β-D-glucanase preparations during mashing (usually performed at about 65–70°C), or by performing an initial low-temperature mash to prevent inactivation of the temperature-sensitive malt β-glucanases (Bourne *et al.*, 1976).

Enzymes used for the degradation of barley β-glucan, whether of microbial origin or naturally present in malt, are usually assayed using barley β-glucan as substrate. In some cases it is possible to employ assays based on the measurement of the increase in reducing end-groups on hydrolysis of this substrate (Denault *et al.*, 1978) but, generally, high levels of reducing sugars in the enzyme preparations preclude the use of such assay procedures. For the measurement of β-glucanase in malt extracts it has been necessary to resort to more tedious and less accurate assays (Buckee, 1985) based on the decrease in viscosity of barley β-glucan solutions (Bourne & Pierce, 1970) or on the rates of diffusion of malt β-glucanase through agar containing a β-glucan/Congo-red complex (Martin & Bamforth, 1983).

An alternative approach, not yet employed for the measurement of (1→3)(1→4)-β-D-glucanase, is the use of dye-labelled substrates (carbohydrate polymers to which a dye is covalently attached). Such substrates have been widely employed for the assay of several polysaccharide *endo*-hydrolases including α-amylase (Ceska *et al.*, 1969), (1→4)-β-D-glucanase (Rinderknecht, 1967), (1→4)-β-D-mannanase (McCleary, 1978) and (1→4)-β-D-xylanase (McCleary, 1982; Biely *et al.*, 1985). Generally, the dye-labelled substrates are insoluble and thus are relatively resistant to enzyme attack and are tedious to dispense on a routine basis. Susceptibility to enzyme attack can be greatly increased by crosslinking the polysaccharide, which prevents polymer alignment (e.g. Phadebas® substrate for α-amylase, Pharmacia Fine Chemicals AB, Sweden). Another approach is to use polysaccharides which will yield soluble substrates, e.g. amylopectin (Babson *et al.*, 1970) in the preparation of Dyamyl L® (General Diagnostics, Division of Warner-Lambert US Inc., USA) or carob galactomannan (McCleary, 1978) in the preparation of Remazolbrilliant Blue R (RBB)–carob galactomannan. Soluble substrates can also be prepared by chemically modifying native polysaccharides before dyeing, e.g. RBB-dyed carboxymethylamylose, -cellulose or -xylan (McCleary, 1980, 1982). Hydroxyethyl cellulose derivatives have also been used for this purpose (Biely *et al.*, 1985). Soluble, dyed substrates have the advantage that they are easily dispensed on a routine

basis, require less to give optimal reaction kinetics and they yield a more sensitive assay than do insoluble substrates. This paper describes a method for the preparation and use of carboxymethyl-barley β-glucan dyed with Remazolbrilliant Blue, for the assay of (1 → 3)(1 → 4)-β-D-glucanase. An improved procedure for the preparation of (1 → 3)(1 → 4)-β-D-glucan from barley flour is also described.

MATERIALS AND METHODS

Enzymes

Lichenase (*endo*(1 → 3)(1 → 4)-β-D-glucanase) was purified to electrophoretic homogeneity by substrate affinity chromatography on DEAE-cellulose as previously described (McCleary & Glennie-Holmes, 1985) and had a specific activity of 115 U mg^{-1} protein on barley β-glucan (5 mg ml^{-1}) at 40°C and pH 6·5. One unit (U) of activity is the amount of enzyme which releases 1 μmol of reducing-sugar equivalents per minute at pH 6·5 and at the temperature employed in the assay. Temperature stable α-amylase [EC 3.2.1.1] (Hitempase) (Biocon Australia Pty Ltd, Boronia, Victoria) was incubated at 85°C for 80 min to remove trace levels of *endo*-β-glucanase. Cellulases (*endo*-(1 → 4)-β-D-glucanases) were purified from the commercial preparations Celluclast (*Trichoderma reesei*) and Finizym (*Aspergillus niger*) (Novo Industrias, Denmark) and from a *Penicillium emersonii* preparation (Biocon Australia Pty Ltd). The major cellulase in Finizym was purified as described previously (McCleary & Glennie-Holmes, 1985). Purification of the Celluclast and *Penicillium emersonii* cellulases will be described separately (McCleary, 1986).

Preparation of low viscosity barley β-glucan

Barley (var. *bandulla*) was milled to pass a 1 mm screen and 1 kg was suspended in aqueous ethanol (90% v/v, 2 litres) and heated in a steam bath for 10 min after the alcohol began to boil. The slurry was poured onto nylon mesh and excess liquid removed by squeezing. The solid residue was resuspended in aqueous ethanol (90% v/v, 2 litres) at room temperature and free liquid removed as above. The residue was then suspended in demineralised water (4 litres) and stirred to

give a homogeneous slurry. Heat-treated (85°C, 80 min) Hitempase (40 ml) was added and the slurry incubated in a boiling water bath until all the starch had been hydrolysed (approximately 90 min) as determined by an iodine/potassium iodide reagent. After 45 min, the slurry was homogenised to remove lumps. The temperature was then equilibrated to 60°C and lichenase (3U) (available from Biocon Australia Pty Ltd) in 100 ml of water was added. The slurry was mixed well, incubated at 60°C for 60 min and then reheated to 90°C (for 20 min) to inactivate lichenase. The slurry was cooled to 60°C by the addition of water (1 litre), homogenised with a Waring blender and then squeezed through nylon mesh to recover most of the liquid. The filtrate was centrifuged (3000 *g*, 20 min) and the clear, yellow supernatant solution was treated with ammonium sulphate (200 g litre^{-1}) and stored at 4°C overnight. Barley β-glucan, which precipitated from solution, was recovered by centrifugation (3000 *g*, 15 min), suspended in aqueous ethanol (20% v/v, 2 litres) and stirred for 15 min. The glucan was recovered by centrifugation (3000 *g*, 15 min) and the washing step repeated. The recovered glucan was then washed twice by suspension in ethanol (95% v/v, 1 litre), recovered on nylon mesh and redissolved in vigorously stirred hot water (4 litre, 80°C) over 30 min. The solution was homogenised in a Waring blender and centrifuged (3000 *g*, 20 min). The clear, colourless supernatant was added to an equal volume of ethanol and the precipitated β-glucan collected on nylon mesh, washed twice with alcohol, once with acetone and dried *in vacuo*.

Preparation of RBB-CM-barley β-glucan (Azo-Betaglucan)

To a Quickfit flask (1 litre) containing low viscosity barley β-glucan (30·0 g) was added chloroacetic acid (3·0 g) dissolved in ethanol (420 ml, 95% v/v). The slurry was heated under reflux with rapid stirring on a hot-plate magnetic stirrer. On reaching reflux temperature, a solution of sodium hydroxide (3·9 g) in an ethanol/water mixture (5:2, 84 ml) was added slowly over a period of 10 min and the suspension stirred a further 20 min under vigorous reflux conditions. The slurry was poured onto a sintered glass funnel, the residue washed under vacuum with aqueous ethanol (80% v/v, 2 litres) and then dissolved in water (1·5 litres at 70°C) with blending if necessary. While stirring and maintaining the temperature at 70–73°C, anhydrous sodium sulphate (150 g) and Remazolbrilliant Blue R (6 g)

were added. After 5 min, 30 ml of a 25% aqueous solution of trisodium phosphate was added and stirring was continued for a further 55 min. While still hot, the reaction mixture was centrifuged (3000 *g*, 5 min) and the supernatant discarded. The pellet was dissolved in hot water (80°C) by stirring on a hot-plate magnetic stirrer for 30 min. If necessary, the suspension was homogenised with a Waring blender to achieve solution. The β-glucan was reprecipitated by the addition of ethanol (1·5 vol) followed by a sufficient volume of saturated sodium chloride solution to produce a colloidal precipitate. After 10 min the precipitate had settled to form a rubbery mass which was recovered on a nylon mesh and excess liquid was removed by squeezing. The dyed glucan was redissolved in water as above and the precipitation and redissolution steps were repeated (about three times) until all free dye was removed. The glucan was redissolved in water (1 litre) and dialysed against slowly flowing demineralised water overnight. The concentration of the substrate and the dye/carbohydrate ratio were determined after acid hydrolysis of the substrate. For acid hydrolysis, an aliquot (0·5 ml) of dyed substrate was incubated with 1·5 M H_2SO_4 (0·5 ml) in a steam bath for 1 h. The hydrolysate was diluted to 10 ml and aliquots were removed for the determination of total carbohydrate (phenol/sulphuric acid method) (Dubois *et al.*, 1956), and for the measurement of absorbance at 590 nm.

The substrate concentration was adjusted to 1·0% w/v (carbohydrate) with water, and sodium azide (to 0·02% w/v) was added to prevent microbial contamination.

Preparation of RBB-barley β-glucan

Low viscosity barley β-glucan (10 g) was dissolved in water (1 litre at 70°C). With the temperature maintained at 70–73°C, anhydrous sodium sulphate (100 g) and Remazolbrilliant Blue R (2 g) were added. After 5 min, trisodium phosphate (5 g) dissolved in water (20 ml) was added, and stirring continued for a further 55 min at 70°C. The dyed β-glucan was recovered, washed and reconstituted as described for the RBB-CM-barley β-glucan.

Precipitant solution

The precipitant solution was prepared by dissolving NaCl (40 g), sodium tungstate dihydrate ($NaWO_4.2H_2O$, 40 g), $NaH_2PO_4.2H_2O$

(30 g), $CH_3COONa.2H_2O$ (40 g) and phenol (4 g) in water (1·8 litres). The pH was adjusted to 4·0 with conc. HCl and the volume adjusted to 2 litres.

Assay of (1 → 3)(1 → 4)-β-D-glucanase (lichenase)

Reducing-sugar assay
Enzyme preparation (50 μl) was incubated with a solution of barley β-glucan (0·5 ml, 5 mg ml^{-1}) in phosphate buffer (50 mM, pH 6·5) for up to 10 min at 30°C. The reaction was terminated and the colour developed using Nelson/Somogyi reagent solutions as previously described (McCleary & Glennie-Holmes, 1985), except that 0·5 ml of reagent D was used to stop the reaction and reagent E was prepared by diluting reagent C five-fold. One unit (U) of enzyme activity releases 1 μmol of reducing-sugar equivalent (as glucose) per minute at 30°C and pH 6·5.

Chromogenic assay
Enzyme preparation (50 μl) was incubated with dyed CM-barley β-glucan (0·5 ml, 10 mg ml^{-1}) plus phosphate buffer (0·5 ml, pH 6·5, 50 mM) for up to 10 min at 30°C and the reaction was terminated by the addition of precipitant solution (3·0 ml). The solution was vortexed for 5 s and centrifuged (1000 *g*, 10 min), and the absorbance of the supernatant was measured at 590 nm.

RESULTS AND DISCUSSION

Preparation and purity of (1 → 3)(1 → 4)-β-D-glucan from barley flour

In procedures previously reported for the preparation of (1 → 3)(1 → 4)-β-D-glucan (barley β-glucan) from barley flour, the flour, after heat inactivation of endogenous enzymes, was extracted with water at temperatures (40–60°C) below those which induced gelatinisation of starch granules. This resulted in a β-glucan extract with limited contamination by starch. However, this procedure gives low and variable yields of barley β-glucan. In the current procedure, yields of this glucan have been increased three- to four-fold by extraction at

high temperatures (~ 90°C) at which most of the barley β-glucan is solubilised. Under these conditions the starch gelatinises, but concurrent treatment with a temperature-stable α-amylase (devoid of *endo*-β-glucanase) removes this polymer effectively. The barley β-glucan extracted by the described procedure (if lichenase treatment is not included) is three to four times more viscous than that extracted at 40°C, an observation which effectively explains the variable ease of extraction of barley β-glucan fractions as reported by others (Martin & Bamforth, 1983). In the current assay procedure, a substrate concentration of 1% w/v is required to obtain optimal reaction kinetics. Although the high viscosity barley β-glucan can be dissolved to give such concentrations, a β-glucan of lower viscosity (~ 2 dl g^{-1}) produces a more readily dispensable substrate.

Barley β-glucan of the desired viscosity was prepared by controlled treatment of the extraction slurry with low levels (3 U kg^{-1} flour) of pure lichenase. This treatment reduced the intrinsic viscosity of the recovered barley β-glucan from 7 dl g^{-1} to about 2 dl g^{-1}, a value similar to that obtained by us for the 40°C soluble fraction. A further advantage of incorporating this lichenase treatment step is that by lowering the suspension viscosity it makes the purification considerably easier and results in a purer product (removal of insoluble denatured protein is more effective).

The recovery of barley β-glucan was approximately 3–3·5% of the dry weight of the flour extracted (i.e. more than 70% of the theoretical maximum). This β-glucan was devoid of starch as shown by a negative iodine test and also by the lack of release of glucose on treatment with glucoamylase. Treatment with lichenase plus β-glucosidase or *Aspergillus niger* cellulase plus β-glucosidase gave approximately 98% conversion to glucose.

Development of the assay procedure for lichenase

The development of procedures for the routine assay of polysaccharide *endo*-hydrolases based on the use of soluble, dye-labelled polysaccharide substrates requires the preparation of substrates which are soluble but are still highly susceptible to enzymic hydrolysis.

The direct dyeing of barley β-glucan with Remazolbrilliant Blue R produces a dyed polysaccharide (RBB: carbohydrate = 1:15) which is insoluble in water at room temperature. The material can be

solubilised by incubation in a boiling water bath for a few minutes, followed by vigorous blending. This substrate remains soluble at temperatures down to 50°C, but below this it rapidly coagulates and settles from solution. The substrate can be effectively used in the assay of (1→3)(1→4)-β-D-glucanase and reaction curves relating increase in absorbance (590 nm) with enzyme units or with time of reaction are excellent. However, the need to heat and blend the substrate before each use is unacceptable and could lead to inaccuracies due to an increased substrate concentration through loss of water on heating.

A water-soluble, dye-labelled barley β-glucan substrate was prepared by the introduction of carboxymethyl (CM) groups into the polysaccharide before dyeing. The extent of carboxymethylation was critical. The optimal degree of substitution (*DS*) to impart solubility without seriously affecting the susceptibility of the polysaccharide to lichenase hydrolysis was between 0·08 and 0·12. At *DS* values below 0·08 the dyed polysaccharide was partially insoluble in water and on storage at 4°C for extended periods a high proportion of the polymer settled from solution. At *DS* values approaching 0·2 the dyed polysaccharide was relatively resistant to enzyme attack. Also, dyed polysaccharides with this degree of carboxymethylation were hard to recover during preparation (they did not readily precipitate) and in the standard assay procedure they gave unacceptably high blank values.

Under the standard conditions for preparation of CM-barley β-glucan, the ratio of chloroacetic acid to β-glucan is 1:10. If this ratio is increased to 1:6 the extent of carboxymethylation almost doubles, resulting in less effective dyeing of the polysaccharide under standard conditions (i.e. the RBB: anhydro-glucose ratio is reduced from 1:25 to 1:40). Changing the ratio of RBB to β-glucan in the dyeing reaction from 1:5 to 1:3 increases the RBB: anhydro-glucose ratio in the product from 1:25 to 1:15, while reducing the RBB:β-glucan ratio to 1:7 produces a dyed β-glucan with a dye:anhydro-glucose ratio of 1:35. Increasing the dye content to 1:15 significantly decreases the susceptibility of the substrate to enzymic hydrolysis. The rate of increase in the absorbance at 590 nm under standard assay conditions is less than 60% of that with the substrate prepared under the recommended conditions (with a dye:anhydro-glucose ratio of ~1:25). Further, substrates containing less than one dye molecule per 25 sugar residues are hydrolysed at a similar rate to those with a dye:anhydro-glucose ratio of 1:25 and thus give a lesser rate of increase in absorb-

ance at 590 nm with a given level of enzyme (because the released fragments contain less dye).

The two major requirements of the precipitant employed with soluble chromogenic substrates are that it stops the enzymic reaction and that on centrifugation or filtration a clear solution suitable for the spectrophotometric measurement of absorbance values is obtained. Previously published methods have included a range of precipitants including ethanol and an ethylene glycol monomethyl ether/water/salt mixture (Babson *et al.*, 1970; McCleary, 1978). Both of these are useful, but have particular limitations. Ethanol does not give a clear supernatant solution for enzyme preparations containing high levels of protein. Also, the relatively rapid evaporation of ethanol from the supernatant solution could lead to inflated absorbance values. The precipitant based on ethylene glycol monomethyl ether is very effective but is toxic and needs to be handled carefully. Consequently, an alternative precipitant solution based on a mixture of buffer salts,

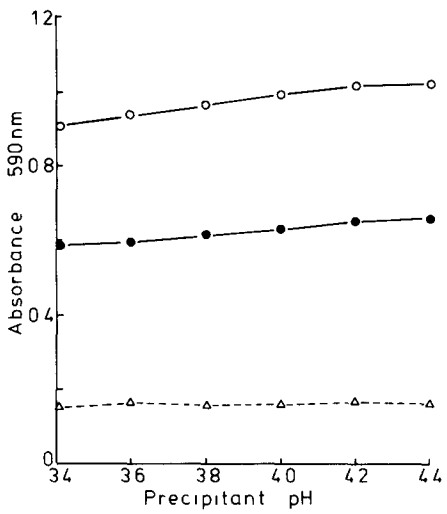

Fig. 1. Effect of precipitant pH on the level of soluble dye-labelled fragments after centrifugation of the reaction mixtures. Enzyme/substrate mixtures were incubated for (△) 0, (●) 3 or (○) 6 min before addition of precipitant solution.

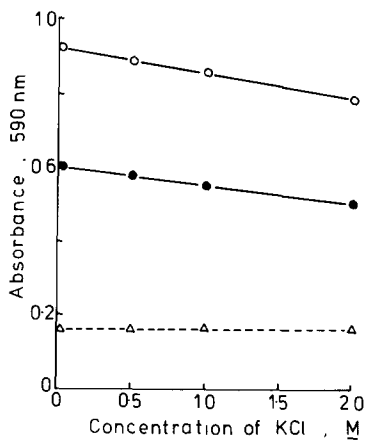

Fig 2. Effect of the concentration of KCl in the enzyme solution on the level of soluble dye-labelled fragments after centrifugation of the reaction mixtures. Enzyme/substrate mixtures were incubated for (△) 0, (●) 3 or (○) 6 min before addition of precipitant solution.

sodium tungstate and phenol has been developed. This solution is a modification of a mixture routinely employed for protein precipitation. Since the effectiveness of precipitation is likely to be a consequence of both precipitant pH and the final salt concentration in the assay mixture, these parameters were carefully evaluated. The effect of precipitant pH on blank absorbance values and on values after incubation of substrate with lichenase (28 mU per assay) for 3 or 6 min at 30°C and pH 6·5 is shown in Fig. 1. It is evident that precipitant pH does affect absorbance values in reaction mixtures but this effect is limited.

A proposed use for this assay is the screening of column chromatographic eluates and microbial culture preparations in which the buffer salt concentration is likely to vary significantly. The effect of the concentration of KCl in enzyme preparations on the measured absorbance values is shown in Fig. 2. Salt does have an effect, but not to such an extent that it limits the use of the assay.

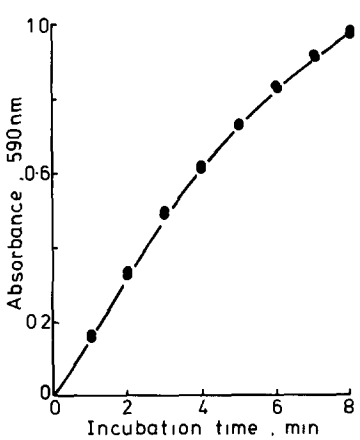

Fig. 3. Rate of release of soluble chromogenic material on hydrolysis of Azo-Betaglucan by lichenase (28 mU per assay) at 30°C.

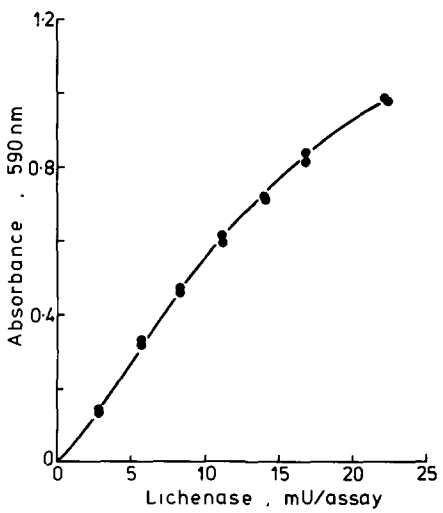

Fig. 4. Standard curve relating activity of lichenase (mU per assay) on barley β-glucan (5 mg ml^{-1}) to absorbance increase (at 590 nm) on hydrolysis of Azo-Betaglucan for 10 min at 30°C.

The time course of release of low degree of polymerisation chromogenic material from Azo-Betaglucan which was prepared under optimal conditions and using the recommended precipitant (pH 4·0) is shown in Fig. 3. A standard curve relating lichenase enzyme units (on barley β-glucan by the Nelson/Somogyi reducing sugar method (Somogyi, 1952)) to absorbance increase (at 590 nm) on hydrolysis of this substrate is shown in Fig. 4. The assay is very reproducible as shown by the similar duplicate assay values. Azo-Betaglucan is hydrolysed by cellulase enzymes at or near their optimal pH for activity (pH 4–4·5), but at the pH (pH 6·5) employed in the current assay procedure the cellulases from *Trichoderma reesei*, *Penicillium emersonii* and *Aspergillus niger* have limited activity.

In our laboratory, this assay procedure is routinely used to assay lichenase (from *Bacillus subtilis* culture preparations) and malt β-glucanase (a specific *endo*-(1 → 3)(1 → 4)-β-D-glucanase). Lichenase is of particular use in the assay of barley β-glucan in barley, malt, wort and beer. The level of (1 → 3)(1 → 4)-β-D-glucanase in malt is one parameter currently used commercially as an index of malt quality. The application of the currently described assay procedure for the analysis of malt β-glucanase is presented separately (McCleary, 1986).

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