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A SIMPLE ASSAY PROCEDURE FOR β-D-MANNANASE

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ABSTRACT

A simple assay procedure for β-D-mannanase enzyme has been developed which employs carob D-galacto-D-mannan dyed with Remazolbrilliant Blue Additionally, the procedure is quantitative, relatively sensitive, and highly specific for β-D-mannanase enzyme It can be readily used for the determination of β-D-mannanase activity in crude enzyme preparations and column-chromatography eluates

INTRODUCTION

The enzymes which hydrolyze D-galacto-D-mannan, β-D-mannanase[1] (EC 3 2 1 78) and α-D-galactosidase[2] (EC 3 2 1 22) are useful in the analysis of the fine structure of these polysaccharides But, whereas simple assay systems that use nitrophenyl α-D-galactopyranoside substrates are available to aid in the purification of α-D-galactosidase enzymes, at present the assay systems for β-D-mannanase enzymes are, in general, tedious and quite often employ nonspecific substrates

Perhaps the most specific assay for β-D-mannanase employs the substrate β-D-mannan extracted from palm seeds, and monitors the reaction by increased reducing power[3–5] However, β-D-mannan is insoluble, which can lead to significant variations in different assays, and incubations need to be performed in a shaking water-bath Also, this substrate is susceptible to hydrolysis by β-D-mannosidase Other assay procedures based on the decrease in viscosity of D-galacto-D-mannan or D-gluco-D-mannan solutions due to *endo*-depolymerisation by β-D-mannanase[6 7] are also quite specific, but do not readily lend themselves to the routine analysis of large numbers of samples

Some of the limitations of the just mentioned assay procedures can be overcome by use of either soluble D-galacto-D-mannans with low D-galactose contents (*e g*, carob D-galacto-D-mannan, 23% Gal)[8] or soluble D-gluco-D-mannans[9] as substrates, with determination of increased reducing sugar levels However, both of these substrates can be hydrolyzed by other glycosidase or glycanase activities that also increase reducing sugar levels In view of the present inadequacies in assay procedures for β-D-mannanase enzymes, the aim of the current work was to develop a simple and reliable alternative

EXPERIMENTAL

Extraction and purification of D-*galacto*-D-*mannans and* D-*mannan* — Lucerne D-galacto-D-mannan was extracted and purified from seed material as previously described[10] Guar and carob D-galacto-D-mannans were prepared from commercially available flours (Sigma Chemical Co , St Louis, MO 63178, U S A) The flours were suspended in water at 60° (20 g/l) and stirred vigorously with a Sorvall Omnimixer until the solutions became very viscous These solutions were centrifuged at 3 000 *g* for 30 min and the galactomannans precipitated from the supernatant with ethanol washed with ethanol, acetone, and ether, and dried

D-Mannan was extracted from seeds of *Livistona australis* by a method similar to that previously used for the extraction of Bangalow palm D-mannan[8] Some of the purified mannan was reduced with $NaBH_4$ (0 1 g/g mannan), after dissolution of the polymer in 10% NaOH, to decrease background color when this polysaccharide was used as substrate in assays where reducing sugar levels were determined

Preparation of soluble D-*mannan* — Reduced mannan (0 2 g) was dissolved in NaOH (2 ml, 10% w/v), and the solution was diluted five fold and neutralized with M HCl The volume was immediately adjusted with 0 1M acetate buffer (pH 5) to the desired concentration At a concentration of 0 1%, the polymer remained in solution for at least one week at 4°

Preparation of dried D-*galacto*-D-*mannans* — The method of Rinderknecht *et al*[11] was modified Galactomannan (20 g) was suspended in water (2 l) with vigorous stirring at 60° with a Sorvall Omnimixer Stirring was continued until the polymer dissolved (30 min) and then Remazolbrilliant Blue R (RBB) (4 or 10 g) and Na_2SO_4 (200 g) were added After 45 min, Na_3PO_4 (10 g) in water (20 ml) was added and stirring continued for a further 75 min The solution was then cooled and dialyzed overnight against flowing tap-water The gelatinous, dyed galactomannan was recovered by precipitation with ethanol, followed by either centrifugation at 1 500 *g* or filtration through cheesecloth Free dye was removed by washing the polymer with aq ethanol When devoid of free dye the polymer was washed with acetone and dried The dye content of the polymer was controlled by varying the amount of RBB used Under the conditions described, 4 g of RBB per 20 g of carob galactomannan gave a dyed polymer with an RBB to anhydrohexose ratio of ~1 50, and 10 g of RBB per 20 g of carob galactomannan gave a ratio of ~1 15 For simplicity, the shorthand notation, RBB–carob galactomannan (1 50) is employed to denote a dyed carob galactomannan sample having one RBB molecule per 50 anhydrohexose molecules

For use as substrates, the polysaccharides (0 86 g) were dissolved in water (100 ml) at 60° with vigorous stirring for 30 min, followed by blending with an Ultraturrax Before use, 2 3M acetate buffer (15 ml, pH 5) was added to give a final buffer concentration of 0 3M and a substrate concentration of 0 75% When this solution was stored at low temperatures, the polymers precipitated, but RBB–carob

galactomannan (1 50) could be redissolved by heating the substrate to 40° and blending

This substrate can be used directly or, alternatively, the viscosity can be greatly decreased by pre-incubation of the solution (115 ml) with the equivalent of 6 mg of Cellulase C-7502 (*i e* , ≈2 U of β-mannanase, Sigma) for 20 min at 40° with termination of the reaction by heating to 100° This treatment gives a dramatic decrease of the viscosity with an insignificant increase in the blank absorbance-value

Preparation of dyed D-*mannan* — D-Mannan (5 g) was dissolved in NaOH (50 ml, 10% w/v), neutralized with M HCl, adjusted to one litre with H_2O, and dyed as for galactomannans The dyed polymer was centrifuged off, washed with aq ethanol to remove free dye, and then washed with acetone and dried For use as substrate, dyed mannan (1 5 g) was homogenized in 0 1M acetate buffer (100 ml, pH 5) with an all-glass Tenn–Broeck homogenizer All incubations were performed at 40° in a shaking water-bath

RBB to anhydrohexose ratios of dyed polymers — Dyed polysaccharides were hydrolyzed by incubation for 3 h at 100° in the presence of 0 75M H_2SO_4 The solutions were diluted and centrifuged RBB was determined directly from the absorbance values (at 590 nm) by reference to RBB standards subjected to the same treatment The absorbance characteristics of RBB appeared to be unaffected by this acid hydrolysis step Carbohydrate concentration in the acid hydrolyzate was determined with the anthrone method[12] Complete hydrolysis of the dyed polysaccharide was confirmed by comparison with the results of the reducing sugar level by the PAHBAH method (see later) and of the anthrone total carbohydrate

*Preparation of β-*D-*mannanase B from lucerne* — β-D-Mannanase B of lucerne was prepared from 1 kg of germinated seed as previously described[8] by column chromatography on DEAE-cellulose, Sephadex G100, and CM-cellulose with the modification that the filtrate of the crude extract through muslin was incubated for 1 h at 40° (pH 5) to allow hydrolysis of residual galactomannan Also, a second DEAE-cellulose chromatography step was included after the first DEAE-cellulose step Recently, this enzyme has also been purified by substrate-affinity chromatography Details of this purification procedure will appear in a separate communication

Assay of reducing sugar levels (PAHBAH method) — The method of Lever[13,14] was slightly modified Stock solutions were (a) 0 5M *p*-hydroxybenzoic acid hydrazide in 0 5M HCl and (b) 0 5M NaOH plus 0 01M $CaCl_2$ in 0 05M trisodium citrate Before use, solution "a" (10 ml) and solution "b" (90 ml) were mixed to give the PAHBAH reagent solution Phosphate buffer, which was found to interfere with color development, was avoided

*Assay of β-*D-*mannanase* — (*a*) *By use of a soluble-mannan solution* The enzyme preparation (0 1 ml) was incubated with soluble mannan (0 5 ml 0 1%) in 0 1M acetate buffer (pH 5) for 2–10 min at 40° The reaction was stopped by adding PAHBAH reagent solution (5 ml), and the color was developed by incubating the tubes for 6 min at 100°

(*b*) *By use of dyed substrates* The enzyme preparation (0 5 ml) was incubated with dyed galactomannan (1 ml, 0 75%) or mannan (1 ml, 1 5%) for 5–20 min at 40° The reaction was stopped by the addition of ethanol (3 ml), and the mixture was stirred and centrifuged at 2 000 *g* for 5 min The enzyme reaction was monitored by increased absorbance (590 nm) of the supernatant solution The reducing sugar equivalent of the supernatant solution (calc as D-mannose) was determined by evaporating the solution to dryness in a boiling water-bath, dissolving the residue in water (4 ml), and removing aliquots (0 1 ml) for determination by the PAHBAH method With RBB–carob galactomannan (1 15) and RBB-mannan, all incubations were performed in an oscillating incubation-bath

Determination of the effect of prehydrolyzing, with α-D-galactosidase, RBB–carob galactomannans on the action of β-D-mannanase on these substrates — RBB–carob galactomannan (1 50) (10 ml, 0 75%) was incubated with various amounts of purified lucerne α-galactosidase A (0 1–10 ml, 1 5 U/ml on lucerne galactomannan) for 20 h The reaction was stopped and the polysaccharide precipitated by the addition of ethanol (2 vol) Absorbance (at 590 nm) of the supernatant solution was determined after centrifugation The ethanolic supernatant solution was concentrated to dryness and the residue redissolved in water The galactose content was determined on an aliquot with the galactose dehydrogenase method and the reducing sugar produced with the PAHBAH method The precipitated polysaccharide fraction was redissolved in water heated in a boiling-water bath for 2 min to remove ethanol, and the volume adjusted to the original volume (10 ml) with 2M acetate buffer (pH 5) to give a final buffer concentration of 0 3M Aliquots (1 ml) of each of the solutions were used as substrates in the standard β-mannanase assay The level of lucerne-seed β-mannanase B, which was incubated with each of these substrates, was selected to give ~50% release of RBB-dyed fragments from the RBB–carob galactomannan substrate which had not been pretreated with α-galactosidase Aliquots (1 ml) of each of the substrates were hydrolyzed with acid for D-galactose determination by the galactose dehydrogenase method[10]

Preparation and assay of α-D-galactosidase A enzyme of lucerne — This enzyme was assayed at 40° and prepared essentially as previously described[10], with the modification that the initial chromatographic step on DEAE-cellulose was performed at pH 8 0 (Tris), and the second at pH 5 0 (acetate buffer) At pH 8, α-galactosidase A bound to DEAE-cellulose and was eluted at a KCl concentration of 0 15M This gave α-galactosidase A free of β-mannanase A (which did not bind) and essentially devoid of β-mannanase B At pH 5, α-galactosidase A did not bind to DEAE-cellulose, whereas the last traces of β-mannanase B did Chromatography of the enzyme on Sephadex G-100 removed all remaining β-mannosidase activity

RESULTS AND DISCUSSION

Purity and some properties of the β-D-mannanase enzyme employed — The β-mannanase enzyme employed in the present studies was purified β-mannanase B

from lucerne[8] In the present studies, the enzyme was purified 3315-fold from the crude extract with an overall recovery of 27% and a final specific activity of 66 3 U on soluble mannan at 40° and pH 5 0 It gave a single band on polyacrylamide gel disc-electrophoresis, but on isoelectric focusing two bands of protein (pI's 4 4 and 4 5) were detected One of these (pI 4 5) was by far the major component The enzyme preparation was devoid of α-D-galactosidase and β-D-mannosidase activities, and had an optimal pH for activity on carob galactomannan and soluble mannan of 4 4–4 5 At pH 5, with an incubation time of 5 min, the temperature for optimal activity was 55° and the energy of activation on both substrates was 36 4 kJ/mol in the temperature range of 20–35° At temperatures of 40–55°, an energy of activation value of 21 8 kJ/mol was obtained At 40°, the enzyme was relatively unstable in the pH range of 3 5–4 0 At pH 4 5, 30% of the enzyme activity was lost after a 5-h incubation, and at pH 5–6 the enzyme was quite stable during an extended incubation The enzyme had a molecular weight by gel filtration of 28 000 (ref 8) and a value of 41 000 by SDS–polyacrylamide gel disc-electrophoresis (*cf* ref 15)

Development of a simple assay procedure for β-D-mannanase — The method developed employs carob galactomannan dyed with Remazolbrilliant Blue (RBB) The use of insoluble, dyed polysaccharides in assay procedures for a range of *endo*-hydrolase enzymes has previously been described by others[11 16 17] More specifically, McCleary *et al* [18] briefly described the use of Remazolbrilliant Blue (RBB)-dyed mannan in the assay of β-mannanase However, although this technique was simple it lacked sensitivity In the present study, sensitivity has been greatly increased by employing dyed carob galactomannan in place of dyed mannans The assay system measures the hydrolysis of RBB–carob galactomannan in terms of the rate of release of fractions soluble in 66% (v/v) aq ethanol

The rate of release of dyed fragments on hydrolysis of RBB–carob galactomannan was found to be a function of both the dye to anhydrohexose ratio and the concentration of buffer salt in the assay mixture For maximal sensitivity in the assay system, the optimal dye to anhydrohexose ratios in RBB–carob galactomannan were in the range of 1 15 to 1 50

Solution properties of RBB–carob galactomannan (1 15) are greatly affected by buffer salt concentration At salt concentrations above 10mM, the polymer coagulated and rapidly precipitated from solution RBB–carob galactomannan (1 50) also precipitated in the presence of high buffer salt concentrations, but even at 300mM salt concentration, settling took several hours, obviating the necessity to use an oscillating incubator-bath For this reason, the latter substrate was chosen for further studies

β-Mannanase had highest activity on RBB–carob galactomannan (1 50) at salt concentrations of 10mM Activity decreased markedly (about 45%) between 10–200mM but was relatively unchanged from 200–300mM salt concentration Consequently, buffer salt concentrations of 0 3M were used in substrate solutions giving a concentration of at least 0 2M in the final assay mixture The rate of hydrolysis of soluble mannan was unaffected by salt concentration

Standard curves for the conversion of absorbance values (at 590 nm) to enzyme units, on soluble mannan at 40°, are presented in Fig 1 Standard assay conditions were employed with an incubation time of 10 min Release of dyed fragments corresponded directly to increased reducing power The rate of release of dyed fragments from RBB–carob galactomannan was much greater than that from RBB–mannan, or RBB–guar, or lucerne galactomannans The low relative rate of hydrolysis of RBB–mannan is considered to be due to the highly insoluble nature of this substrate RBB–carob galactomannan (1 50), in contrast, is a gelatinuous suspension The low activity on RBB–guar and lucerne galactomannans is due to the high degrees of D-galactose substitution of these galactomannans (33–38 and 45–47%, respectively)[18] in comparison to carob galactomannans[18] (23% Gal)

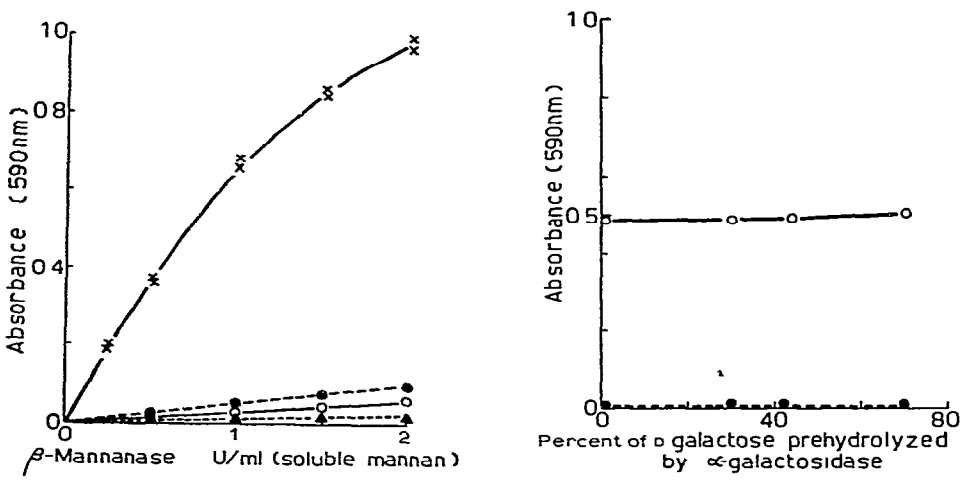

Fig 1 Standard curves relating β-D-mannanase enzyme units on soluble D-mannan to absorbance increase (at 590 nm) on hydrolysis of RBB–carob galactomannan (1 50) (×) RBB–guar galactomannan (1 50) (●), RBB–lucerne galactomannan (1 50) (▲), and RBB–mannan (1 35) (○)

Fig 2 Release of dyed fragments on hydrolysis of RBB–carob galactomannan by α-D-galactosidase (●), and on hydrolysis of α-D-galactosidase-prehydrolyzed substrate by β-D-mannanase (○)

The usefulness of RBB–carob galactomannan as a substrate for the assay of β-mannanase activity in crude enzyme mixtures depends on its specificity One enzyme which might interfere with this assay is α-D-galactosidase Two possible mechanisms by which α-galactosidase might increase the rate of release of dyed fragments from RBB–carob galactomannan were considered the enzyme either might release D-galactosyl units dyed with RBB or, by releasing galactosyl residues, might make the substrate more susceptible to β-mannanase hydrolysis Figure 2 shows the effect that the release of D-galactose (by purified lucerne α-galactosidase A) from RBB–carob galactomannan (1 50) had on absorbance increase at 590 nm, and the effect it had on the susceptibility of the substrate to β-mannanase hydrolysis Since 70% of the D-galactosyl residues were removed with no release of ethanol-soluble,

dyed fragments, it can be concluded that α-galactosidase is unable to remove D-galactosyl residues substituted with Remazolbrilliant Blue dye Furthermore, removal of up to 70% of the D-galactosyl residues gave only a very slight (≈4%) increase in the susceptibility of the substrate to β-mannanase hydrolysis In agreement with this, Marshall[19] has shown that although the exohydrolase, amyloglucosidase, can remove D-glucose from Cibachron Blue-dyed amylose, it cannot concurrently release Cibachron Blue-dyed fragments

β-Mannosidase, like amyloglucosidase[19], is an exo-hydrolase, which sequentially removes D-mannosyl residues from the nonreducing end of manno-oligosaccharides and manno-polysaccharides, and is blocked by D-galactose branch-points[18] Since amyloglucosidase is unable to release dyed fragments from Cibacron Blue-dyed amylose, it might be reasonably concluded that β-mannosidase would be unable to release dyed fragments from RBB–carob galactomannan In the current investigations, however, the author was unable to purify sufficient levels of β-mannosidase activity, devoid of β-mannanase and α-galactosidase activity to obtain direct experimental verification of this assumption

The just described assay procedure has been found to be very useful in the measurement of β-mannanase activity in crude enzyme preparations of both microbial (*e g*, commercial Driselase preparation) and plant origin such as an extract from germinating legume seeds It is also of sufficient sensitivity for use in the routine analysis of fractions being eluted from chromatography columns Figure 3 shows the

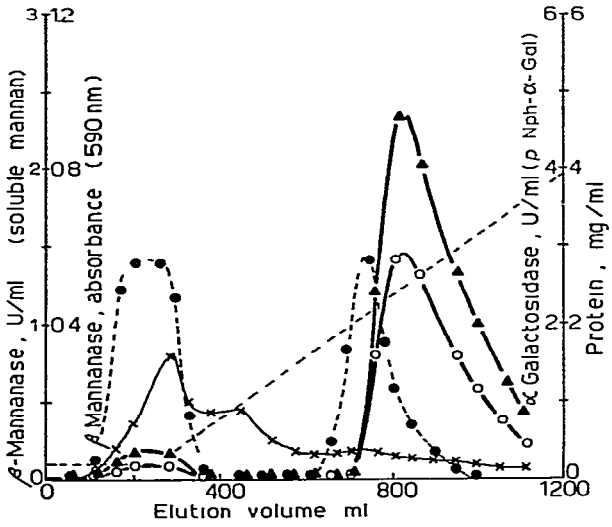

Fig 3 Chromatography on DEAE-cellulose of an enzyme extract from 500 g of lucerne at three days after germination Parameters monitored were β-mannanase activity on soluble mannan (○) and on RBB–carob galactomannan (▲), α-galactosidase activity on *p*-nitrophenyl α-D-galactopyranoside (●), and protein (×) Column 3 5 × 15 cm gradient 20mM sodium acetate (pH 5) to 20mM sodium acetate (pH 5)–400mM KCl Activity of β-mannanase on RBB–carob galactomannan was assayed under standard conditions on 0 5-ml aliquots of column eluate for an incubation time of 10 min

chromatographic separation on DEAE-cellulose of two of the four β-mannanase fractions present in lucerne-seed extracts[8] β-Mannanase activity was quantitatively detected with both soluble-mannan substrate and RBB–carob galactomannan Recently, Villarroya and Petek[15] reported that they could detect only one fraction containing β-mannanase activity in chromatography-column eluates of an extract from germinated lucerne Possible reasons for the different results are that these authors (a) extracted at a different time after germination, (b) employed an assay technique relatively insensitive (*i e*, ~1/10 the sensitivity of the microferricyanide method, and (c) had an enzyme recovery at each chromatography step that was low, and thus the overall enzyme recovery was low

Attempts to determine the K_m of lucerne β-mannanase B for RBB–carob galactomannan (1 50), by measuring the rate of release of dyed fragments, were unsuccessful due to the fact that in dilute substrate solutions (*i e*, ≈0 05%) the bulk of the polymer has to be hydrolyzed to release measurable levels of 66% ethanol-soluble dye Thus, the substrate concentration becomes limiting In previous experiments, a K_m value for lucerne β-mannanase B on carob galactomannan of 4 4mM anhydro-D-mannose (0 095%) was reported[8] In the present experiments, a similar value was obtained for RBB–carob galactomannan by determining the reducing sugar release with the PAHBAH reducing sugar method

Thus, in conclusion, a simple, reliable, relatively sensitive, and highly specific assay procedure for β-mannanase has been developed by use of carob galactomannan dyed with Remazolbrilliant Blue as substrate

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