NOTE

Purification of a Specific Endo-Beta-Glucanase from *Bacillus subtilis* for Beta-Glucan Quantitation

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Several enzymic methods for estimating mixed-linkage (1,3:1,4) β-glucan in cereal grains have been reported (Anderson et al 1978, Fleming et al 1974, Forrest and Wainwright 1977, Henry 1984, Prentice et al 1980). The accuracy of enzymically estimated β-glucan is highly dependent on the specificity and purity of the enzymes used. Because glucose is the end product being specifically measured in most of these methods, the enzymes used must be free of contaminating activities that produce glucose or glucose oligosaccharides from sources other than the mixed-linkage β-glucan. A further consideration is that certain β-glucanases, which degrade the mixed-linkage β-glucan, also have activity on 1,3- or 1,4-linked homo-β-glucans (Barras et al 1969). Thus, it is important that the enzyme selected either be solely specific for the 1,3:1,4 β-glucan or that other potential substrates be absent or not significantly degraded under the conditions used to degrade the mixed-linkage β-glucan. These requirements mean that most commercial sources of β-glucan degrading enzymes must be purified prior to use in β-glucan assays.

Two different types of enzyme have been used in the direct enzymic β-glucan assays: a) cellulase mixture from *Trichoderma viride*, and b) a lichenase from *Bacillus subtilis*. The former enzyme preparation likely contained an endo-1,4-β-glucanase, a β-glucosidase, and possibly a cellobiohydrolase, since it was able to convert polymeric β-glucan to free glucose (Martin and Bamforth 1981). The *B. subtilis* lichenase (EC 3.2.1.73) is a β-glucanase that has been shown to have a high specificity for 1,3:1,4 mixed-linkage β-glucans and no activity on either 1,3- or 1,4-homo-β-glucans (Huber and Nevins 1977).

The objective of the work reported here was to develop a fast, effective way of preparing a functionally pure enzyme for use in β-glucan assays. The *B. subtilis* enzyme was chosen because of its greater specificity, which thereby allows the enzyme to be used directly on raw cereal grist without prior extraction of the β-glucan. Although several purification procedures for this enzyme have been reported (Huber and Nevins 1977, Rickes et al 1962), these were found either to be too long or not entirely effective in removing amylase activity.

MATERIALS AND METHODS

Materials

Cereflo 200L is a commercial bacterial (*Bacillus subtilis*) β-glucanase preparation produced and provided by Novo Industri A/S. Lichenin (*Usnea barbata*) was obtained from Sigma Chemical Co., and laminarin, soluble starch, and soluble carboxymethyl cellulose were obtained from ICN Pharmaceuticals, Fisher Chemical Co., and Nutritional Biochemicals Ltd., respectively. Arabinoxylan was prepared from an aqueous extract of rye flour by ammonium sulfate fractionation. The 40–60%-saturation fraction was treated with a mixture of salivary α-amylase and a purified endo-1,4 β-glucanase (Ballance and Manners 1978) to remove minor glucan contaminants. The enzyme-treated arabinoxylan was precipitated with ethanol and found to contain only arabinose and xylose.

Analytical Methods

Protein was monitored in column effluents as the absorbance at 280 nm. For calculation of specific activities, protein was determined by the method of Lowry et al (1951) using bovine serum albumin as a standard. Reducing power was measured in enzyme digests by the Nelson-Somogyi method using the procedure and reagent formulation outlined by Robyt and Whelan (1968).

β-Glucanase Assay

For detecton of β-glucanase activity in column fractions, aliquots (10 μl) were incubated with 0.6% (w/v) lichenin (200 μl) in 0.2*M* sodium maleate buffer, pH 6.5, at 37° C for 10 min. Activity was reported as the increase in colorimetric absorbance due to increased reducing power for the assay period used. For specific activity measurements, an appropriately diluted enzyme (10 μl) was added to 0.6% (w/v) lichenin (1.0 ml) in 0.2*M* maleate buffer, pH 6.5, and incubated at 37° C. Aliquots were removed for measurement of reducing power at 5-min intervals. A unit of activity was defined as the amount of enzyme that releases one micromole of reducing sugar (as glucose equivalents) per minute under the assay conditions described.

Amylase Assay

To 0.2% (w/v) soluble starch (200 μl) in 0.2*M* sodium acetate buffer, pH 5.5, and containing 0.001*M* $CaCl_2$ was added an appropriately diluted enzyme solution (50 μl). The digest was incubated at 45° C for times ranging from 15 min to 24 hr, depending upon the level of activity. Activity was measured as the increase in reducing power. A unit of amylase activity was defined in the same manner as a unit of β-glucanase activity.

Purification Procedure

An aliquot (50 ml) of a Cereflo 200L sample was adjusted to pH 3.5 with dilute acetic acid. The acidified solution was allowed to stand at 4° C for 1 hr and then centrifuged (10 min × 12,000 *g*) to remove the precipitate. The supernatant fraction was then dialyzed at 4° C for 27 hr against distilled water (3 × 4L) which had also been adjusted to pH 3.5 with acetic acid. The dialyzate was recentrifuged as before to remove further precipitated material. Sodium hydroxide and concentrated sodium acetate buffer were then added to adjust the dialyzate to 0.02*M* sodium acetate buffered at pH 5.5. The acid-treated pH 5.5 dialyzate was divided and chromatographed on two CM-cellulose columns (3.3 × 25 cm) which had been equilibrated at pH 5.5 with 0.02*M* sodium acetate buffer. After loading, each column was washed with the equilibration buffer (200 ml) before a linear NaCl gradient (0–1.5*M*) in this same buffer (1,000 ml total volume) was applied. The effluent was collected in 12-ml fractions. Alternate fractions were analyzed for amylase, β-glucanase, and protein, as indicated above. The fractions containing β-glucanase activity were pooled and concentrated by ultrafiltration over a Diaflo YM-10 membrane (Amicon Corp., Lexington, MA).

β-Glucanase Characterization

The presence of possible contaminating enzyme activities in the final preparation was tested by incubating the enzyme concentrate

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(50 μl) with 0.2% (w/v) solutions (200 μl) of various potential substrates in 0.2*M* maleate buffer at pH 6.5. Activities against soluble starch, laminarin, soluble CM-cellulose, and arabinoxylan were measured after 24 hr incubation at 37° C.

The pH optimum for enzyme activity was determined using the same procedure as described for specific β-glucanase activity, except that the substrate concentration was 0.2%. Digests were buffered at pH 5.0 and 5.5 with 0.2*M* sodium acetate and at pH 6.0, 6.5, 7.0, and 7.5 with 0.2*M* sodium phosphate buffer.

The limit degradation products released from lichenin by the enzyme were separated by paper chromatography in a solution of ethyl acetate, pyridine, and water (10/4/3). Samples (2 mg) of the major trisaccharide product, which had been eluted from the chromatogram, were reduced with sodium borohydride (5 mg) in molar NH_4OH (1.0 ml, 16 hr, 22° C). Reduction was terminated by dropwise addition of acetic acid until effervescence ceased. The samples were then evaporated to dryness and the borate removed by repeated evaporation with methanol (5 × 1 ml). Both reduced and nonreduced samples of the trisaccharide were then methylated and analyzed according to the procedure of Harris et al (1984).

The thermal stability of the enzyme was tested by incubating samples of the purified enzyme in 0.2*M* maleate buffer, pH 6.5, containing 0.02*M* sodium azide at 40, 50, 55, and 60° C. At intervals, aliquots were removed and cooled to 24° C for at least 20 min before being assayed against lichenin using a procedure similar to that used for measuring the specific activity. Activity was expressed as a percentage of activity in unheated samples.

RESULTS AND DISCUSSION

Purification

The technical information provided with the commercial β-glucanase preparation indicated that α-amylase was a contaminant activity. Preliminary attempts to separate these two activities by several column chromatographic techniques were unsuccessful due to varying degrees of co-elution. A treatment involving dialysis at acidic pH was tested as a means of destabilizing or inactivating the α-amylase contaminant because of the general instability of such metallo-enzymes upon removal of the bound calcium (Hsiu et al 1964). At pH 3.5 there was a rapid loss of amylase activity (≈95%) in the first 2 hr followed by a period of slower decline in activity. No apparent loss in β-glucanase activity was observed in the initial 2 hr, but with more prolonged acid treatment there was a significant loss, as indicated by the recovery after the 27-hr treatment (Table I). Some starch degrading enzyme activity still remained after this treatment but was subsequently removed by CM-cellulose column chromatography (Fig. 1). Although a small amount of β-glucanase activity eluted in the frontal peak, most of the activity was tightly bound and required strong salt conditions for elution from the column. Attempts to reduce the acid treatment dialysis time by one half resulted in the appearance of additional starch-degrading activity peaks in the CM-cellulose column profile. Several of these peaks co-eluted with the broad β-glucanase peak. The recovery and specific activity data are summarized in Table I. This purification has been carried out a number of times with two different batches of the commercial enzyme preparation. The results are highly reproducible and recoveries of 35–40% are normal.

Possible contaminant activities in the final preparation were tested at 220 times the concentration of that used to effect complete solubilization of β-glucan from barley grists under comparable incubation conditions. No change in reducing power was observed

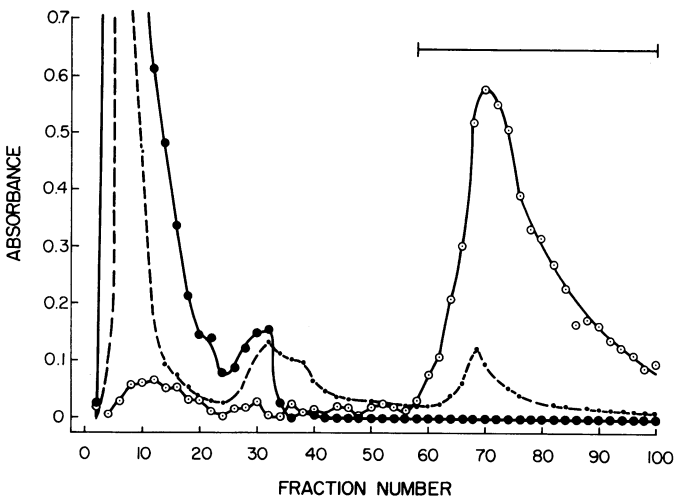

Fig. 1. CM-cellulose chromatograph of acid-treated β-glucanase preparation. Activity against lichenin (⊙——⊙, 10 min incubation) and soluble starch (●——●, 16 hr incubation) are reported as the increase in absorbance at 600 nm. Protein (---) was measured at 280 nm. The elution gradient was started at fraction 20. Fractions under the bar were pooled and concentrated.

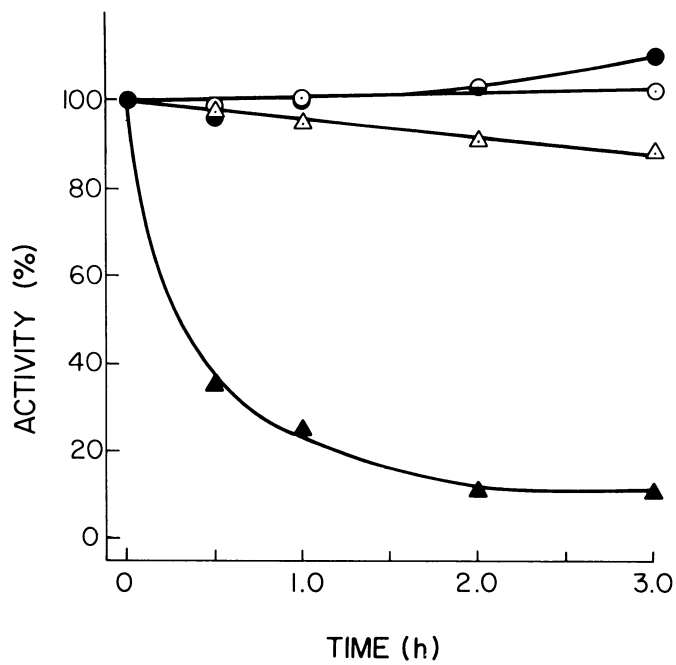

Fig. 2. Thermal stability of purified mixed-linkage β-glucanase. Enzyme was incubated at 40° C, ⊙; 50° C, ●; 55° C, △; and 60° C, ▲; in the absence of substrate.

TABLE I
Purification Data for *B. subtilis* β-Glucanase

Stage	Volume (ml)	Total Protein (mg)	Total Amylase Activity (units)[a]	Total Glucanase Activity (units)[a]	Specific Glucanase Activity (units/mg)	Recovery (%)
Original	50	890	36,300	5,280	5.9	100
Acid-treated dialysate, pH 5.5	90	81.5	58	2,430	29.8	46
Pooled column fractions[b]	1,090	13.8	0	1,910	138	36
Final concentrate	31.8	12.3	0	1,910	155	36

[a] Units are as defined in text.
[b] From both columns.

when the enzyme was incubated with starch, laminarin, CM-cellulose, or arabinoxylan, indicating that potential contaminating activities are either absent or inactive under the conditions used. The pH optimum of the β-glucanase enzyme was found to be between 6.3 and 6.5, which agrees with previous reports (Huber and Nevins 1977, Moscatelli et al 1961).

The enzyme released a tri- and a tetrasaccharide as its major limit degradation products from lichenin. Methylation analysis of the unreduced trisaccharide yielded equimolar amounts of 2,3,4,6-tetra-O-methyl-, 2,3,6-tri-O-methyl-, and 2,4,6-tri-O-methyl-D-glucose derivatives, whereas for the reduced trisaccharide equal amounts of 2,3,4,6-tetra-O-methyl- and 2,3,6-tri-O-methyl-, and a slightly lesser amount of penta-O-methyl-D-glucose derivatives were found. These results indicated that the trisaccharide was 3-O-β-cellobiosyl-D-glucose and thereby confirmed that the enzyme is the same type of specific β-glucanase as purified by Huber and Nevins (1977) and as used by Anderson et al (1978).

The thermal stability of the enzyme is important, because in β-glucan assays extended incubations are used and require that the enzyme remain active. Figure 2 shows the thermal stability of the purified β-glucanase enzyme in the absence of substrate. The enzyme was quite stable at temperatures up to 55° C but lost activity rapidly at 60° C.

CONCLUSIONS

The purification procedure described for the *B. subtilis* endo-β-glucanase is fast, simple, and gives a high yield of a functionally pure enzyme that is free of potentially important contaminating activities. The enzyme is very stable at 40° C under conditions similar to those used in β-glucan assays (Anderson et al 1978).

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LITERATURE CITED

ANDERSON, M. A., COOK, J. A., and STONE, B. A. 1978. Enzymatic determination of 1,3:1,4-β-glucans in barley grains and other cereals. J. Inst. Brew. 84:233.

BALLANCE, G. M., and MANNERS, D. J. 1978. Structural analysis and enzymatic solubilization of barley endosperm cell walls. Carbohyd. Res. 61:107.

BARRAS, D. R., MOORE, A. E., and STONE, B. A. 1969. Enzyme-substrate relationships among β-glucan hydrolases. Adv. Chem. Ser. 95:105.

FLEMING, M., MANNERS, D. J., JACKSON, R. M., and COOKE, S. C. 1974. The estimation of β-glucan in barley. J. Inst. Brew. 80:399.

FORREST, I. S., and WAINWRIGHT, T. 1977. Differentiation between desirable and troublesome β-glucans. Eur. Brew. Conv. Proc. Congr. 16:401.

HARRIS, P. J., HENRY, R. J., BLAKENEY, A. F., and STONE, B. A. 1984. An improved procedure for the methylation analysis of oligosaccharides and polysaccharides. Carbohyd. Res. 127:59.

HENRY, R. J. 1984. A simplified enzymic method for determination of (1-3) (1-4)-β-glucans in barley. J. Inst. Brew. 90:178.

HSIU, J., FISCHER, E. H., and STEIN, B. A. 1964. Alpha-amylases as calcium metalloenzymes. II. Calcium and the catalytic activity. Biochemistry 3:61.

HUBER, D. J., and NEVINS, D. J. 1977. Preparation and properties of a β-D-glucanase for specific hydrolysis of β-D-glucans. Plant Physiol. 60:300.

LOWRY, O. H., ROSEBOROUGH, N. J., FARR, A. L., and RANDALL, R. J. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193:265.

MARTIN, H. L., and BAMFORTH, C. W. 1981. An enzymatic method for the measurement of total and water-soluble β-glucan in barley. J. Inst. Brew. 87:88.

MOSCATELLI, E. A., HAM, E. A., and RICKES, E. L. 1961. Enzymatic properties of a β-glucanase from *Bacillus subtilis*. J. Biol. Chem. 236:2,858.

PRENTICE, N., BABLER, S., and FABER, S. 1980. Enzymic analysis of beta-D-glucans in cereal grains. Cereal Chem. 57:198.

RICKES, E. L., HAM, E. A., MOSCATELLI, E. A., and OTT, W. H. 1962. The isolation and biological properties of a β-glucanase from *B. subtilis*. Arch. Biochem. Biophys. 96:371.

ROBYT, J. F., and WHELAN, W. J. 1968. Amylases and their action on starch. Page 423 in: Starch and Its Derivatives, 4th ed. J. R. Radley, ed. Chapman and Hall: London.

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