Detection and Assay of (1→4)-β-D-Glucanase, (1→3)-β-D-Glucanase, (1→3)(1→4)-β-D-Glucanase, and Xylanase Based on Complex Formation of Substrate with Congo Red[1]

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ABSTRACT

The dye binding of Congo red by cereal (1→3)(1→4)-β-D-glucans, substituted celluloses, O-(carboxymethyl)pachyman, and larchwood xylan may be used to detect and estimate (1→3)(1→4)-β-D-glucanase, (1→4)-β-D-glucanase, (1→3)-β-D-glucanase, and xylanase activities, respectively. Oligosaccharide reaction products, resulting from enzyme activity on these polysaccharides, do not bind Congo red. This can be monitored in solution and was exploited in a gel-diffusion assay system in which radial diffusion of enzyme into a substrate-bearing gel slab was detected by Congo red staining. The diameter of the reaction zone was proportional to the logarithm of the enzyme concentration. Gel diffusion measurements of (1→3)-β-D-glucanase activity in germinating barley corresponded well to viscometrically determined values. The method was applied to survey commercial amylolytic enzymes for β-D-glucanase and xylanase contamination.

Cereal β-D-glucans and substituted celluloses interacted strongly with dyes such as Congo red and Calcofluor in solution (Wood 1980a,b). Cello-oligosaccharides interacted slightly, if at all, suggesting that loss of dye binding might be exploited to monitor hydrolysis and assay enzyme activity. Subsequent studies (Wood 1982) showed that the weak interaction of O-(carboxymethyl)-pachyman (CMP) and O-(carboxymethyl)cellulose (CMC) in aqueous solution or dilute buffer was considerably enhanced by the addition of salt, suggesting the possible use of these also as dye-reactive enzyme substrates. Where a simple visible distinction between polymer and degraded product is possible, a useful assay system may be developed using gel diffusion (Dingle et al 1953), and a preliminary report from this laboratory indicated that staining of β-D-glucans could be used to monitor (1→3)(1→4)-β-D-glucanase, (1→4)-β-D-glucanase, and (1→3)-β-D-glucanase activities using radial diffusion of enzyme into substrate-bearing gel slabs (Wood 1981). We now report further on the basis and development of these techniques. Applications to characterize cellulolytic organisms from bovine rumen (Teather and Wood 1982) and to measure ((1→3)(1→4)-β-D-glucanase in malt (Martin and Bamforth 1983) have been described.

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MATERIALS AND METHODS

General

Oat gum, prepared from the cultivar Hinoat or obtained from R. Hyldon (Quaker Oat Co., Barrington, IL), was extracted by alkali from oats essentially as described by Wood et al (1977) and contained about 75% (1→3)(1→4)-β-D-glucan, the remainder being mainly pentosan and protein. Pure (1→3)(1→4)-β-D-glucan (referred to as oat β-D-glucan) was obtained from a solution of the gum by two successive precipitations with ammonium sulfate. Barley (1→3)(1→4)-β-D-glucan was obtained from V. Bendelow (Agriculture Canada, Winnipeg Research Station). CMP was prepared from pachyman (provided by J. J. Marshall) as described by Clarke and Stone (1962). Tamarind amyloid, a xyloglucan (Srivastava and Singh 1967), was obtained from I. R. Siddiqui of this institute. O-(hyroxyethyl)cellulose (HEC 180GR and HEC 250M), and CMC (7H3 SXF) were obtained from Hercules Incorp., Wilmington, DE. Larchwood xylan was from P. L. Biochemicals Inc., Milwaukee, WI. Congo red (C.I. 22120) was from Canadian Laboratory Supplies Ltd. Malted barley samples were kilned, five-day malts provided by D. E. LaBerge (Agriculture Canada, Grain Research Laboratory, Winnipeg). Endo-(1→3)(1→4)-β-D-glucanase ((1→3)(1→4)-β-D-glucan-4-glucanohydrolase, EC 3.2.1.73; enzyme 1) from *Bacillus subtilis* (Anderson et al 1978) was a gift from B. A. Stone, and endo-(1→3)-β-D-glucanase ((1→3)-β-D-glucan glucanohydrolase, EC 3.2.1.6; from *Rhizopus arrhizus* (QM 1032) enzyme 2) was a gift from E. T. Reese. Commercial enzyme preparations were as follows: 3) α-amylase type IA: pancreatic, from Sigma, lot 99C-8040. 4) α-Amylase type IIA: bacterial, from Sigma, lot 34C-1840. 5) α-

Amylase type IIIA: *Bacillus subtilis*, from Sigma, lot 37C 0186. 6) α-Amylase type VIIIA: malt, from Sigma, lot 71F-0385. 7) α-Amylase type XA: *Aspergillus oryzae*, from Sigma, lot 59C-0022. 8) α-Amylase: *B. subtilis*, from Boehringer-Mannheim, lot 12792502. 9) α-Amylase: fungal, from Calbiochem, lot 703044. 10) α-Amylase: bacterial, from Calbiochem, lot 600093. 11) α-Amylase: *A. oryzae*, from Calbiochem, no lot number. 12) α-Amylase: *B. subtilis*, from Calbiochem, lot 103424. 13) α-Amylase: *A. oryzae*, from Miles lot 7. 14) Amyloglucosidase (Agidex): *Aspergillus*, from BDH, lot 1611020. 15) Amyloglucosidase: *A. oryzae*, from Sigma, lot 29C-0484. 16) Amyloglucosidase: *Rhizopus*, from Sigma, lot 72C-0590. 17) Glucoamylase: *R. niveus*, from Miles, lot 8513A. 18) Amylo-α-1,4-α-1-6-glucosidase: *A. niger*, from Boehringer-Mannheim, lot 7384405. 19) Amylo-α-1,4-α-1,6-glucosidase: *A. niger*, from Boehringer-Mannheim, lot 1139303. 20) Novo 240: bacterial, from Novo Ind. Ltd., lot C1-0741. 21) New Sumizyme 3000: *Rhizopus*, from Shin Nihon Co., no lot number. 22) Pullulanase: *Aerobacter aerogenes*, from Calbiochem, lot 101487. 23) Cellulase: *Trichoderma viride*, from Boehringer-Mannheim, lot 1038402. 24) Onazuka cellulase: from All Japan Biochem., lot 223003. 25) Macerozyme cellulase: from All Japan Biochem., no lot number. 26) Cellulase Type I: *A. niger*, from Sigma, lot 68C-9510. 27) Cellulase Type II: *A. niger*, from Sigma, lot 101F-0666. 28) Cellulase Type IV: *T. viride*, from Sigma, lot 61F-0563. 29) Cellulase: *T. viride*, from BDH, lot 5929922C. 30) Hemicellulase: from Mann Res. Labs, lot 4048. 31) Hemicellulase: *Rhizopus*, from Sigma, lot 30C-0950. 32) Hemicellulase: *A. niger*, from Sigma, lot 41F-0221. 33) β-Glucosidase: Almonds, from Sigma, lot 1C-2720. 34) Glucose oxidase: Source unidentified, from Boehringer-Mannheim, lot 7305382. 35) Glucose oxidase: *A. niger*, from Boehringer-Mannheim, lot 1022301. 36) Glucose oxidase: *A. niger*, from Boehringer-Mannheim, lot 1292301. 37) Glucose oxidase: *A. niger*, from Sigma, lot 62C-2300. 38) Peroxidase: Horseradish, from Boehringer-Mannheim, lot 7185174. 39) Peroxidase: Horseradish, from Boehringer-Mannheim, lot 1380430. 40) Peroxidase: Horseradish, from Boehringer-Mannheim, lot 1400510. 41) Peroxidase: Horseradish, from Sigma, lot 123C-9530.

Monitoring Enzyme Action in Solution

Oat or barley β-D-glucan (25 mg) was stirred and heated (~60° C) in water (4 ml) until dissolved, and 0.25*M* sodium maleate buffer, pH 6.5, containing 25 m*M* azide was added (1 ml), followed by (1→3) (1→4)-β-D-glucanase (enzyme 1) (0.1 ml) to give a final enzyme concentration of 6×10^{-3} units/ml (Anderson et al 1978). The mixture was incubated at 37° C, and two aliquots (0.1 ml) were removed at intervals and heated in a boiling water bath for 5 min. One aliquot, after dilution 1,000-fold with potassium dihydrogen phosphate-disodium hydrogen phosphate buffer (pH 7.0, ionic strength 0.2), was mixed with Congo red (10 μg/ml) and absorbance at 542 nm was measured. The remaining aliquot was directly assayed for reducing sugar (Marais et al 1966). The solution remaining after incubation was immersed in a boiling water bath (5 min), and the soluble portion was concentrated and fractionated on Bio-Gel P-2, with water as eluent (Anderson et al 1978). Fractions were monitored by the cysteine-sulfuric acid assay (Wood et al 1977). Viscosity was monitored in Cannon-Manning semi-micro capillary viscometers (viscosity constants 0.0976 and 0.0141) using oat β-glucan (0.5% w/v) and an enzyme concentration of 7×10^{-3} units/ml.

In similar studies of (1→3)-β-D-glucanase activity, CMP (50 mg) was dissolved in 10 ml of 0.05*M* MES buffer (sodium 2-(N-morpholino) ethane sulfonate), pH 5.5, the solution centrifuged, and the clarified supernatant (5 ml) incubated with the *R. arrhizus* endo-(1→3)-β-D-glucanase (enzyme 2; 0.1 ml, 0.026, and 0.26 mg/ml) at 37° C. Aliquots (0.1 ml) were removed for monitoring reducing sugar as for (1→3)(1→4)-β-D-glucanase. For measurement of absorbance changes in the spectra of Congo red, a second aliquot was diluted 50-fold with molar NaCl before adding Congo red. Absorbance was measured at 525 nm.

To monitor cellulase activity, enzyme solution (enzyme 23; 10–100 μl of 1 and 10 mg/ml) was added to 5 ml CMC (0.5% in 0.05*M* MES buffer, pH 6.5), and samples were incubated at 37° C. Aliquots (50 μl) were removed at intervals, added to 4.5 ml of 1*M* NaCl, and heated in a boiling water bath for 10 min. Aliquots (1.8 ml) of solution were then mixed with 0.2 ml of Congo red (100 μg/ml) and absorbance (540 nm) was determined.

Gel Plate Assay

Substrates (oat gum, tamarind amyloid, CMP, CMC, HEC, and xylan), clarified by centrifugation as necessary, were incorporated (500 μg/ml, but 2 mg/ml for xylan) into gel plates of 0.5% (w/v) agarose 4–5-mm thick. Congo red (40 μg/ml) was incorporated into the gel, or an aqueous solution (1 mg/ml) was layered onto the plate after incubation for ~20 min. A buffer appropriate to the enzyme system of interest was used to prepare the gel, most commonly, in these studies, 0.05*M* MES buffer, pH 5.3–6.5. Agarose (125 mg) was added with stirring to substrate dissolved in buffer (25 ml), and the mixture was heated to boiling. The hot solution was then poured into 9-cm diameter or square plastic petri plates, and wells (routinely 4-mm diameter) were cut in the cooled gel with a cork borer. Enzyme solutions (10 μl) were pipetted into the wells and after appropriate incubation, usually 18 hr at room temperature, the diffusion spot diameter was measured using vernier calipers on two diameters at right angles. For visualisation with CMP, CMC, and xylan, 1*M* NaCl was applied to the stained gel for ~20 min after first rinsing off the layered dye. To stop the reaction 1*M* HCl was sometimes layered onto the plate for ~20 min and then rinsed. The plates, which turned blue, could then be stored for at least two weeks, if kept moist. Temperature and duration of incubation, type of buffer, pH, and substrate concentration, were varied to determine suitable assay conditions. Control incubations were run using gels without added substrate, and with heat deactivated solutions of enzymes. Deactivation was carried out by heating solutions of enzyme in the appropriate buffer, in test tubes, immersed in a boiling water bath (~100° C), taking care that no drops of enzyme solution adhered to the tube walls above the boiling water level. After cooling, the deactivated solutions were centrifuged and the supernatant was used for testing. Blanks of buffer alone were also tested.

For linearity studies, 6–8 levels of enzyme were applied to six plates, each level applied once to a randomly assigned position on each plate.

Barley (1→3)-β-D-Glucanase

Germination of barley. Seeds (cultivar Conquest) were surface sterilized by treatment with 1% (w/v) sodium hypochlorite under reduced pressure for 15 min, then washed three times with sterile water under reduced pressure. Seeds were then germinated under sterile conditions on 1% (w/v) agar gel at room temperature (23° C). Germinated seeds were transferred to a test tube after removal of roots and stem and freeze-dried. The dried seeds were ground using a coffee mill and mortar and pestle prior to enzyme extraction. As a control, some seeds were autoclaved before "germination" treatment. Crude enzyme extracts were prepared by stirring flour from intact, germinated or malted barley with 0.05*M* MES buffer, pH 5.3 for (1→3) (1→4)-β-D-glucanase and pH 5.5 for (1→3)-β-D-glucanase, at room temperature for 1 hr (usually five or 10:1 volume-to-weight ratio). Slurries were centrifuged and the crude supernatant extract used directly for enzyme assay or dialyzed and freeze-dried before use.

Viscometric assay of barley (1→3)-β-D-glucanase. CMP was dissolved by heating (~60° C) and stirring in 0.05*M* MES buffer, pH 5.5, to give a 1% (w/v) solution, which was clarified by centrifugation and filtered through sintered glass. Aliquots (1 ml) were mixed with the MES buffer (1.4 ml) and equilibrated to 37° C. Enzyme solution (0.1 ml) was then added and mixed, this being timed as the start of incubation. An aliquot was removed to charge a Cannon-Manning semi-micro capillary viscometer in which the solvent efflux time (t_s) was 7.4 sec. Efflux times (t_p) of the CMP solution were observed over a period of 1 hr, and plots of $1/\eta_{sp}$ against time prepared, where $\eta_{sp} = (t_p - t_s)/t_s$. Dilutions were used such that plots were linear in the initial periods of incubation, and enzyme activity was determined from $d/dt\ (1/\eta_{sp})$.

(1→3)-β-D-glucanase in germinating barley measured by viscometry and by gel diffusion. Extracts from 1–6 day germinated barley (0.1 g) were assayed by gel diffusion and viscometrically using CMP as substrate. For viscometry, the supernatant was filtered through a 22-μm Millipore filter before assay. Dilutions of the original extract were made using the MES buffer. For the viscometric assays, the extracts from the flour for each day of germination were used to construct a plot of $d/dt\ (1/\eta_{sp})$ against enzyme concentration (and hence viscometric assessment of enzyme activity in the flour at each stage of germination). From these plots, values of $d/dt\ (1/\eta_{sp})$ were obtained for each enzyme extract (and dilutions of these) assayed by gel diffusion. The most active enzyme extracts, which could be directly assayed by gel diffusion, did not give linear plots of $1/\eta_{sp}$ (fluidity) against time unless first diluted. Viscometric values corresponding to each gel diffusion value were obtained, therefore, from the linear plot of $d/dt\ (1/\eta_{sp})$ against enzyme concentration using at least three dilutions for each extract. Diffusion diameters for 0.1 g extracted in 1 ml, also obtained graphically from at least three dilutions, were plotted against the logarithm of $d/dt\ (1/\eta_{sp})$.

RESULTS

Solution Studies

(1→3) (1→4)-β-D-glucan. Assessment of interaction with Congo red was made by absorbance measurements at 540 nm (i.e., at λ_{max} of the difference spectra, Wood 1980b). The absorbance of Congo red (10 μg/ml) is insensitive to changes in concentration of (1→3)(1→4)-β-D-glucan above ~20 μg/ml (Wood 1980b, 1982), and therefore considerable dilution (1,000-fold) of the concentration required for viscometry was necessary to ensure immediate detection of enzyme action by dye. Absorbance values obtained with aliquots from oat and barley β-glucan, calculated to contain ~5 μg/ml of original glucan, were a little erratic but showed a rapid decline before leveling towards values of dye alone (Fig. 1). Enzyme activity could thus be monitored by absorbance changes. Viscosity changes (Fig. 1) using oat β-glucan, like changes in absorbance of Congo red, were characterized by an initial rapid decline followed by leveling off well before significant increase in reducing sugar occurred. The 2-hr incubation point on Figure 1 represents <5% conversion of β-glucan to glucose equivalents. The reaction products of extended incubations with oat and barley β-glucan were mainly tri- and tetrasaccharide, with minor amounts of higher oligosaccharides and some (<5%) insoluble residue.

(1→3)-β-D-glucan. Polyanionic glucans such as CMC and CMP had little effect on the spectra of Congo red in water, but large red shifts (up to 39 nm) were observed in 1 *M* NaCl (Wood 1982). In this medium, λ_{max} of the difference spectra of Congo red in the presence of CMP occurred at ~525 nm, which was the wavelength of choice for examining the effect of CMP concentration on Congo red absorbance. Saturation binding with CMP occurred at higher concentrations of glucan (~200 μg/ml) than with oat β-D-glucan, with an effectively linear relationship between absorbance and concentration of CMP in the range 0–70 μg/ml ($r = 0.995$). Thus, only 50-fold dilution of the enzyme digest (initially ~0.5% CMP) was required when monitoring absorbance change. A comparison of reducing sugar change with absorbance change is shown in Table I for two concentrations of enzyme. A major decrease in absorbance of Congo red was observed with little change in reducing sugar value at the lower enzyme concentration, but reduction to values close to that of dye alone was not observed without significant increase in reducing sugar. The reducing sugar value at this point was equivalent to ~10% conversion of CMP to glucose.

(1→4)-β-D-glucan. CMC behaved similarly to CMP in 1 *M* NaCl. Absorbance changes (540 nm) observed with a dilution of the CMC present in the original digest to 55 μg/ml, shown in Table II, indicate that this system can be used to monitor enzymic hydrolysis of CMC similarly to CMP and (1→3) (1→4)-β-D-glucan.

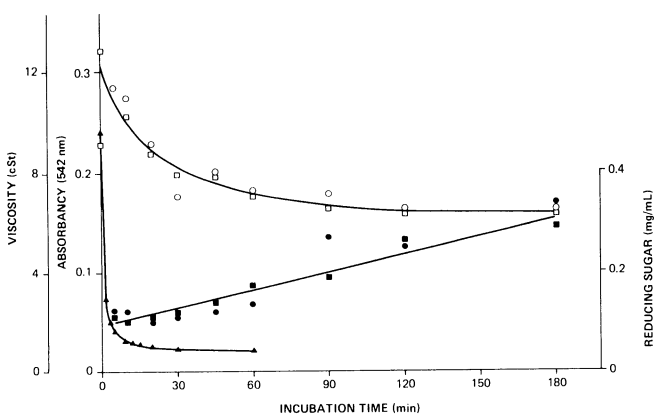

Fig. 1. Effect of *Bacillus subtilis* (1→3)(1→4)-β-D-glucanase (enzyme 1) on solutions of oat and barley β-D-glucan as monitored by changes in viscosity, reducing sugar content, and changes in absorption spectra of Congo red, conditions as described in the text: viscosity of oat β-D-glucan (▲); reducing sugar values with oat (■) and barley (●) β-D-glucan; absorbance of Congo red at 542 nm in the presence of enzyme-treated oat (□) and barley (○) β-D-glucan.

Gel Plate Studies with (1→3) (1→4)-β-D-Glucanase (Enzyme 1)

A typical gel plate incorporating oat β-D-glucan is shown in Figure 2A. Easily distinguished cleared zones shown by α-amylase type XA (enzyme 7; wells 3 and 4), three-day germinated barley (well 8), cellulase (enzyme 23; well 9), and (1→3)-β-D-glucanase (enzyme 2; well 10) were all similar to that shown by the highly purified (1→3)(1→4)-β-D-glucanase (enzyme 1; wells 1 and 2). With a mixture of two enzymes (1 and 7; wells 5 and 6), additive effects were not observed, and the diffusion zone showed the same diameter as the larger zone of enzyme 1 alone.

When dye was incorporated into the gel, the cleared zone was surrounded by a dark ring indicating increased dye concentration. A similar, but fainter, concentric ring was observed with dye layered on the gel after incubation. Measurements of diameter were made to the edge of the cleared zone, which normally could be distinguished easily. The effect of staining method, and of acid

TABLE I
Effect of Incubation of *R. arrhizus* (1→3)-β-D-glucanase on O-(Carboxymethyl) pachyman (5 mg/ml) Measured by Changes in Absorbance of Congo Red at 525 nm and Reducing Sugar Values (glucose equivalents)

Incubation Time (min)	Absorbance (525 nm)		Reducing Sugar Value (μg/ml)	
	0.5[a]	5.0[a]	0.5[a]	5.0[a]
0	0.358	0.355	15	0
10	0.340	0.175	15	200
20	0.331	0.170	15	295
30	0.328	0.168	15	335
60	0.300	0.165	15	445
180	0.318	0.163	15	557
360	0.214	0.160[b]	45	579

[a] Concentration of enzyme, μg/ml.
[b] Essentially equal to absorbance of Congo red alone (0.159).

TABLE II
Effect of Cellulase Action on O-(Carboxymethyl) cellulose as Determined by Absorbance (540 nm) of Congo Red Complex

Incubation Time (min)	Absorbance (540 nm)				
	10[a]	20	40	100	200
0	0.228	0.225	nd[b]	nd	nd
5	0.222	0.215	0.206	0.172	0.131
15	0.211	0.201	0.172	0.126	0.121
30	0.204	0.196	0.135	0.119	0.119
60	0.201	0.178	0.119	0.119	0.120
214	0.129	0.123	nd	nd	nd

[a] Concentration of enzyme 23 in incubation medium (μg/ml).
[b] Not done.

treatment used to stop the reaction, is summarized in Table III. Without acid treatment the staining method had no significant effect on diffusion diameters < 1 cm, but small diffusion areas tend to be obscured by the layering technique. Plots of the logarithm of the enzyme concentration (arbitrary units) (log[E]) against diameter (cm) of diffusion (d) were linear over approximately a 1,000-fold range of concentration ($d = 0.62 \log[E] + 0.91$; $r = 0.994$; $P < 0.005$). No significant between-plate differences were observed ($P > 0.25$).

Control incubations were carried out without substrate (agarose plus dye) and with heat-treated enzyme. No clearing zones were observed in the gel without substrate, showing that there were no interfering dye-enzyme or dye-agarose interactions. Enzyme 1 that had been heated to 100° C for 5 min continued to give a cleared zone that had reduced to ~3% of initial activity after 20 min. After 30 min heating, a halo remained around the well, but distinct clearing was not observed (Fig. 2A, 11).

Decreasing the time of incubation to 7 hr decreased the areas of diffusion. It was routinely convenient to use 18 hr (overnight) incubation at room temperature.

Decreasing oat gum concentration from 1 mg/ml increased the areas of diffusion slightly, but boundaries were less easily disinguished at lower substrate concentration, leading to decreased reproducibility. A substrate concentration of 500 μg/ml was selected as suitable.

The reported optimum pH of 6.5 for the *B. subtilis* (1→3)(1→4)-β-D-glucanase (enzyme 1) (Anderson et al 1978) was used mostly, although tests with 0.05 *M* MES buffer suggested slightly larger diffusion zones might be obtained at pH 5.3. For convenience, however, for example in surveying enzymes of unknown activity, a pH of 6.5 was used.

Gel Plate Studies with (1→3)-β-D-Glucanase (Enzyme 2)

CMP was the substrate of choice for (1→3)-β-D-glucanase measurement (Fig. 2B). Curdlan and pachyman were insoluble, and laminaran did not complex sufficiently strongly with Congo red for diffusion zones to be distinguishable. Gel appearance was improved by centrifuging the CMP solutions before use. A dye layering or dye incorporation technique could be used, and application of molar NaCl was necessary to visualize the zones.

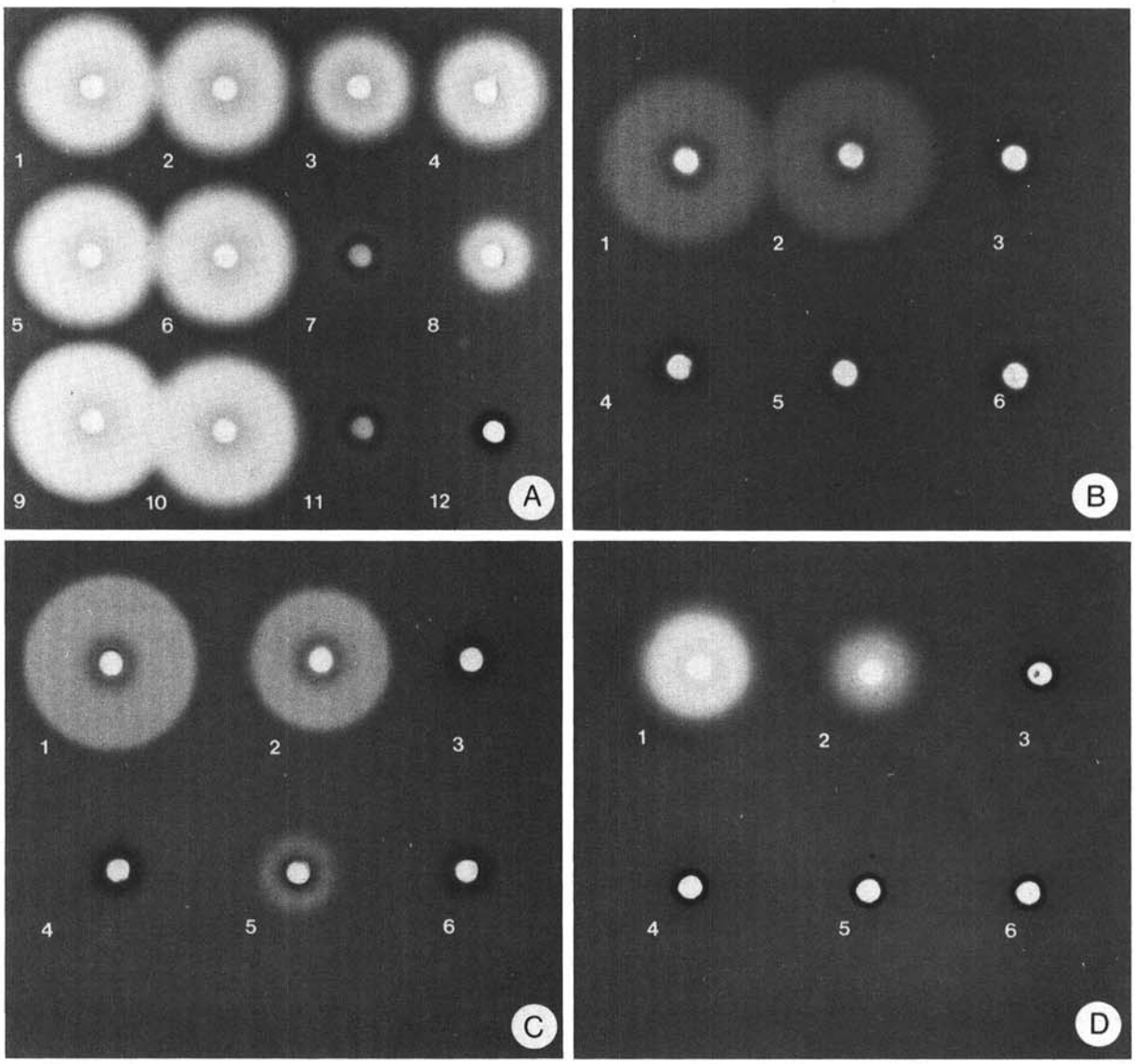

Fig. 2. Gel diffusion plates **A–D:** in each plate, the dye-layering technique was used for staining with Congo red. **A,** Oat gum substrate in 0.05 *M* sodium 2-(N-morpholino) ethane sulfonate (MES) buffer, pH 5.5: **1** and **2,** enzyme 1 (~2×10^{-3} units); **3** and **4,** enzyme 7 (50 μg); **5** and **6,** mixture of enzyme 1 (~2×10^{-3} units) and enzyme 7 (50 μg); **7,** enzyme extract from ungerminated barley; **8,** enzyme extract from three-day germinated barley; **9,** enzyme 23 (100 μg); **10,** enzyme 2 (20 μg); **11,** heat-deactivated enzyme 1 (~2×10^{-3} units); **12,** buffer alone. **B,** O-(carboxymethyl)pachyman substrate in 0.05 *M* MES buffer, pH 6.5: **1** and **2,** enzyme 2 (20 μg); **3** and **4,** enzyme 1 (~5×10^{-3} units); **5,** heat-treated enzyme 2; **6,** buffer alone. **C,** O-(carboxymethyl)cellulose substrate in 0.05 *M* MES buffer, pH 6.5: **1,** enzyme 23 (100 μg); **2,** enzyme 23 (10 μg); **3** and **4,** enzyme 1 (~5×10^{-3} units); **5,** heated enzyme 23 (100 μg); **6,** buffer alone. **D,** Tamarind amyloid substrate in 0.05 *M* MES buffer, pH 6.5: **1,** enzyme 23 (100 μg); **2,** enzyme 23 (10 μg); **3** and **4,** enzyme 1 (~5×10^{-3} units); **5,** heat-treated enzyme 23 (100 μg); **6,** buffer alone.

Using enzyme 2, a linear relationship was observed between log [E] (mg/ml) and diffusion diameter (cm) ($d = 0.56 \log[E] + 2.25$; $r = 0.995$; $P < 0.005$). There were no significant between-plate differences ($P > 0.25$).

Enzyme 1 showed no clearing zone with CMP as substrate (Fig. 2B, wells 3 and 4), again confirming the specificity of this enzyme (Anderson et al 1978), but enzyme 2 showed cleared zones with plates containing oat β-D-glucan (Fig. 2A, well 10), a known activity of this enzyme (Clarke et al 1978).

Enzyme 2 did not produce cleared zones prior to addition of salt, or on plates without substrate, showing that clearing was not caused by nonenzymic artifacts. Heating an aqueous solution of enzyme 2 at 100° C for 5 min reduced activity to <1% of original activity. After heating for 1 hr at 100° C no distinct cleared zone was evident (Fig. 2B, well 5) but a pink residual stain, not evident in the photograph, could be detected. With oat β-D-glucan, after 1 hr at 100° C no reaction zone could be detected (plate not shown).

Gel Plate Studies with (1→4)-β-D-Glucanase (Enzyme 23)

HEC, CMC, and tamarind amyloid were used as (1→4)-β-D-glucan substrates. HEC was a satisfactory substrate, although when dye was incorporated into the gel the diffusion zone was greatly decreased. Tamarind amyloid was also useful (Fig. 2D), but in general clearer and somewhat larger diffusion zones were detected with CMC (Fig. 2C), which, however, requires addition of molar NaCl before diffusion zones become visible. No clearing zone was observed with the (1→3) (1→4)-β-D-glucanase (enzyme 1) from *B. subtilis* (Fig. 2C and D, wells 3 and 4) confirming the reported specificity of this enzyme (Anderson et al 1978), and extending this to demonstrate lack of activity against xyloglucan. Using the commercial cellulase (enzyme 23), a linear relationship was observed between diameter (cm) of diffusion and log[E] (mg/ml) in 0.05 *M* MES buffer, pH 6.5 ($d = 0.49 \log[E] + 1.99$; $r = 0.997$; $P < 0.005$). There were no significant between-plate differences ($P > 0.25$). Enzyme 23 also showed activity in gel plates containing (1→3)(1→4)-β-D-glucan as substrate (Fig. 2A, well 9). With CMC, a cleared spot reduced from 2.40 to 1.50 cm diameter and representing ~3% of initial activity was evident after 1 hr at 100° C. With oat β-D-glucan, there was no evidence of activity after 1 hr of heating the cellulase.

No cleared zones were apparent on plates without substrate or with CMC before adding salt, indicating that clearing was not caused by nonenzymic artifacts.

Application of Gel Diffusion Assay to Survey Commercial Enzymes for β-D-Glucanase and Xylanase Activities

Some commercial enzyme preparations were surveyed for β-D-glucanase and xylanase activities by gel diffusion. The results are summarized in Table IV. Heated (1 hr, 100° C) controls were included because zone clearing continued to be observed with some samples. Controls of gel without substrate showed no clearing zones. It should be noted that rather high concentrations of enzyme were tested in order to detect weak activity. The xylan substrate was slow to stain with Congo red and cleared zones and boundaries were less distinct than for β-glucans.

The results for the glucose oxidase and peroxidase enzymes were difficult to interpret. One preparation (enzyme 40) showed no cleared zone with (1→3) (1→4)-β-D-glucan, CMP, and CMC, but after heating a cleared zone was observed. Similar behavior was observed for three glucose oxidase preparations with (1→3) (1→4)-β-D-glucan as substrate, although the clearing zones observed were small. With CMC all but one of the preparations continued to show cleared zones after heating. This was observed less with HEC, where zone diameters were generally smaller.

Application of Gel Diffusion Assay to Malted Barley

General. Extracts of germinating barley, prepared as described, gave diffusion diameters of 1.07 ± 0.02 cm ($n = 3$) for one-day germinated seed to 1.32 ± 0.04 cm ($n = 5$) for three-day germinated seed when tested with oat gum for (1→3)(1→4)-β-D-glucanase activity.

Larger diffusion diameters were observed using CMP as substrate for (1→3)-β-D-glucanase in both germinated barley and

TABLE III
Effect of Dye Incorporation (40 μg/ml) into Gel Substrate, and Acid, on Diameters of (1→3) (1→4)-β-D-Glucanase (enzyme 1) Diffusion Zones[a]

	Before Acid		After Acid	
Substrate	Dye In	Dye Out	Dye In	Dye Out
Plate 1	1.39 (0.02)[b]	1.40 (0.02)	1.40 (0.01)	1.72 (0.03)
Plate 2	1.37 (0.02)	1.45 (0.03)	1.38 (0.03)	1.77 (0.03)

[a] Incubated 18 hr in 0.05 *M* sodium 2-(N-morpholino) ethane sulfonate buffer, pH 6.5.
[b] Standard deviations ($n = 16$) in parentheses.

TABLE IV
Diameter of Diffusion Zones (cm) in Gel Diffusion Assays of Heated (H) or Unheated (U) Commercial Amylolytic and Cellulolytic Enzymes on Different Substrates[a]

Enzyme	Oat Gum[c]		CMP[d]		CMC[e]		HEC[f]		Xylanase	
Preparation[b]	U	H	U	H	U	H	U	H	U	H
Amylolytic enzymes										
3 (1/10)	0	0	0	0	0	0	0	0	0	0
4 (5)	1.68	0	0	0	0	0	0	0	0	0
5 (5)	2.36	1.00	0	0	0	0	0	0	0	0
6	0.58	0	1.60	0	0	0	0	0	0	0
7 (5)	1.20	0	1.39	0	1.54	0	1.08	0	0	0
8	0.56	0	0	0	0	0	0	0	0	0
9	0.83	0	0.99	0	1.05	0	0.72	0	1.16	0
10	2.22	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0.84	0	0	0	0	0
12	0.60	0	0	0	0	0	0	0	0	0
13	1.41	0	1.78	0	1.84	0	1.43	0	1.30	0
14 (5)	0	0	0	0	0	0	0	0	0	0
15 (1/10)	0	0	0	0	0	0	0	0	0	0
16 (5)	2.00	0	1.92	0	2.06	1.21	0	0	1.23	0
17	0.67	0	0.90	0	0.86	0	0	0	0	0
18 (1/10)	0	0	0	0	0	0	0	0	0	0
19	0.80	0.72	0	0	1.95	1.59	1.76	1.32	1.21	0
20 (5)	2.20	0.71	0	0	0	0	0	0	0	0
21	2.30	0	2.24	0	2.21	0	0.91	0	1.83	0
22	0	0	0	0	0	0	0	0	0	0
Cellulolytic enzymes										
23	1.56	0.54	1.50	0	2.46	1.45	2.16	0.94	nd[g]	nd
24	1.60	0.55	1.88	0	2.32	1.48	1.93	1.12	nd	nd
25	1.79	0	1.74	0	2.96	1.11	0.79	0	nd	nd
26	1.87	0.82	0.81	0	2.49	1.83	2.11	1.55	nd	nd
27	1.87	0.79	0.86	0	2.45	1.83	2.28	1.53	nd	nd
28	0.82	0	1.48	0	2.17	0	1.80	0	nd	nd
29	1.44	0	1.47	0	2.33	1.21	2.09	0.93	nd	nd
30	1.28	0	0	0	1.01	0	0.82	0	nd	nd
31	0.89	0	0	0	1.32	1.13	1.17	0.92	nd	nd
32	0	0	0	0	1.90	1.71	1.98	1.64	nd	nd
33	1.32	1.23	1.10	0	0.87	0	0	0	nd	nd
Glucose oxidase										
34	0	0	0	0	1.50	1.16	1.06	0	nd	nd
35	0	0.73	0.84	0	1.97	1.67	1.97	1.58	nd	nd
36	0	0.83	0	0	2.09	1.58	2.22	1.77	nd	nd
37	0	0.86	0	0	1.22	0.98	0.98	0	nd	nd
Peroxidase										
38	0.92	0.71	1.85	0	1.16	0	0.98	0	nd	nd
39	1.44	1.11	2.23	1.15	1.60	1.19	1.18	0	nd	nd
40	0	1.38	0	1.41	0	1.42	0	0	nd	nd
41	0.62	0.79	1.73	0	1.07	0.97	0.88	0	nd	nd

[a] Enzymes were dissolved in 0.05 *M* sodium 2-(N-morpholino) ethane sulfonate (MES) buffer, pH 6.5, and diameters were averaged from three or four determinations. Heat-treated enzymes were kept at 100° C for 1 hr.
[b] Sample numbers refer to enzyme preparations listed in Materials and Methods. All enzymes were at concentrations of 10 mg/ml of MES, except for those followed by numbers in parentheses: (5) = 5 mg/ml; (1/10) = suspension supplied was diluted 10-fold.
[c] Oat gum, (1→3) (1→4)-β-D-glucanase.
[d] CMP, (1→3)-β-D-glucanase on O-(carboxymethyl) pachyman.
[e] CMC (1→4)-β-D-glucanase on O-(carboxymethyl)cellulose.
[f] (1→4)-β-D-glucanase on O-(hydroxyethyl)cellulose.
[g] nd = Not done.

kilned malts. The diffusion zones of the malt samples, and ungerminated and one-day germinated barley, each contained an inner pink zone. This was shown to be from solubilized barley β-glucan diffusing into the gel, since treatment with the specific (1→3) (1→4)-β-D-glucan-4-glucanohydrolase (enzyme 1) removed the stained zone. Autoclaved, three-day germinated seed showed weak (1→3)-β-D-glucanase activity (which could also be detected viscometrically), but heat treatment (100° C, 5 min) of extracted enzyme completely removed activity. Diffusion zones were not detected before addition of salt or in gel plates prepared without substrate.

Gel diffusion assay of barley (1→3)-β-D-glucanase. Freeze-dried extracts from kilned malts from two cultivars of barley were used over the range 0.5–10 mg/ml to determine suitable conditions for the assay.

Incorporation of dye into the gel (40 μg/ml) lowered diffusion diameters with barley (1→3)-β-D-glucanase. Decreasing substrate concentration in the gel from 500 to 250 μg/ml increased diffusion diameters but resulted in less easily located diffusion boundaries. Diameters were pH sensitive, and were maximum between pH 5.5 and 6.0 (Fig. 3).

An extract of three-day germinated barley was used to determine reproducibility of the assay, using four separate gel plates and nine replicates per plate on two separate days. There was no significant difference between days, but there was a significant difference (at the 1% level) between plates caused by one plate showing low values. The range of diameters observed was 1.60 to 1.77 cm with a coefficient of variation of $<2\%$. Although 0.05 *M* MES buffer (pH 5.5) extracts of germinated barley appear to be stable in the cold for at least two weeks, measurements were normally made within a day or two, or freeze-dried extracts were prepared.

Using six-day germinated barley, a linear relationship between log (barley (1→3)-β-D-glucanase) (arbitrary units) and diffusion diameter (cm) was observed over a 400-fold concentration range ($d = 0.62 \log[E] + 0.77$; $r = 0.997$; $P < 0.005$). No between-plate differences were detected ($P > 0.25$).

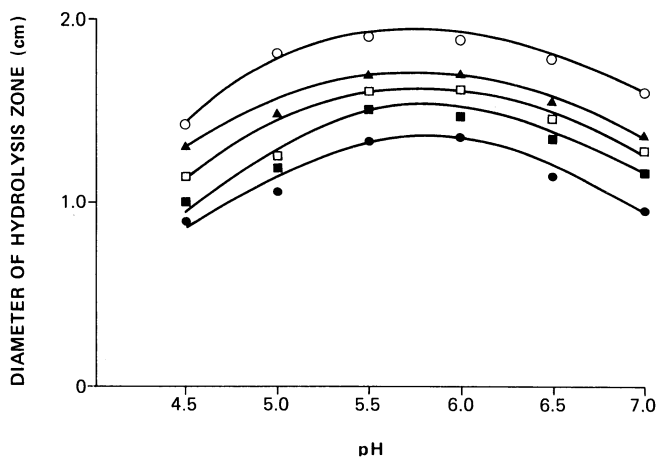

Fig. 3. Effect of pH on diameter of diffusion area of malted barley (cultivar TR905) (1→3)-β-D-glucanase using O-(carboxymethyl)pachyman substrate in the gel. Enzyme concentrations were: ○, 10 mg/ml; ▲, 5 mg/ml; □, 2.5 mg/ml; ■, 1 mg/ml; ●, 0.5 mg/ml. (A similar pattern was obtained with cultivar TR526.)

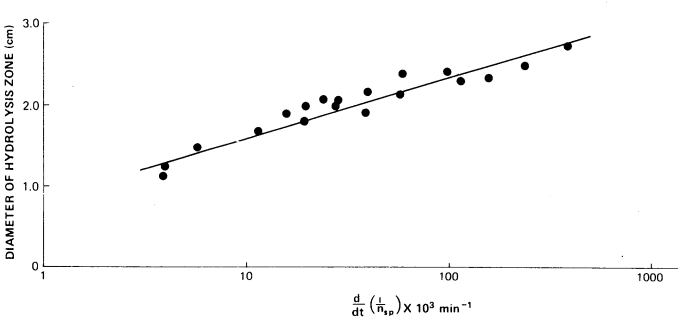

Fig. 4. Relationship between area of diffusion of (1→3)-β-D-glucanase from germinating barley and log (enzyme activity) determined viscometrically.

Relationship of gel assay of barley (1→3)-β-D-glucanase to viscometric assay. Extracts of germinated barley were used to determine the relationship between graphically derived viscometric values of enzyme activity (rather than arbitrarily assigned concentrations) and diffusion diameters. Figure 4 shows a linear relationship between diffusion zone diameter and viscometric units (semilog plot) over a wide range of activities ($d = 0.70 \log[d/dt \text{ fluidity}] + 0.95$; $r = 0.96$; $P < 0.005$). It should be noted that small diameter changes can represent large differences in viscometrically determined activity. For example, the range of diameters observed in the study of reproducibility (1.60–1.77 cm) represents a range of $8–13 \times 10^{-3}$ min^{-1} for d/dt ($1/\eta_{sp}$).

Gel diffusion diameters for one- to six-day germinated barley are compared in Table V with viscometric values reported in terms of the germinated seed weight. Gel diffusion (and viscometric) values are representative of the undiluted extract (0.1 g in 1 ml) and provide an indication of the relative sensitivities of the two methods.

DISCUSSION

Solution studies with (1→3) (1→4)-β-D-glucan in dilute buffers, pH 6–10, have shown λ_{max} of the difference spectra of the complex with Congo red to occur in the region of 540 nm (±5 nm) and saturation binding to occur with <25 μg/ml (Wood 1980b, 1982). With (1→3)-β-D-glucan (CMP) and CMC in sodium chloride, λ_{max} of the difference spectra was ~525 and ~540 nm, respectively, and ~10 times as much glucan was required for saturation binding. With this knowledge, the appropriate dilutions can be determined such that under action of enzyme, the concentration of binding species rapidly falls below levels at which dye is all bound. It is not known at what molecular weight the affinity of CMP, CMC, or oat β-glucan for dye decreases. Comparison of the changes in Congo red absorbance with viscosity losses and reducing sugar production shows that the method monitors endo-activity (Fig. 1 and Tables I and II). Bio-gel P-2 chromatography showed that the final reaction products from the action of *B. subtilis* (1→3) (1→4)-β-D-glucanase (enzyme 1) on oat and barley β-glucan were mainly tri- and tetrasaccharide. Monitoring of endo-enzyme activity by changes in dye-binding appears to be applicable at early to intermediate stages of degradation, similar to viscosity methods, whereas reducing sugar is most useful after extensive degradation. The rate of decline in absorbance is dependent upon enzyme concentration. Thus, for CMC for example, the 5-min value for absorbance (540 nm) of sample minus absorbance of Congo red alone showed an inverse linear relationship with enzyme concentration over a 100-fold concentration range ($r = 0.994$; $P < 0.005$). With increasing incubation time the linear range decreased. Because of the narrow range of absorbance values we

TABLE V
(1→3)-β-D-Glucanase Activity in Germinating Barley (cultivar Conquest) Measured Viscometrically and by Gel Diffusion

Days of Germination	Diameter of Hydrolysis Zone[a] (cm)	Viscometric Activity[b] ($min^{-1}g^{-1}$)
1	1.96	3.9
2	2.18	5.7
3	2.28	11.5
4	2.38	15.9
5	2.50	24.0
6	2.74	39.8
6[c]	0	0.01

[a] Gel diffusion values are for 10 μl of undiluted extract as described in the text.
[b] Viscometric activity is expressed in terms of d/dt ($1/\eta_{sp}$) per gram of germinated barley flour.
[c] Germinated six days, heated to 100° C for 5 min.

have not attempted to develop this approach quantitatively. The wider range possible with fluorescence techniques (Wood 1982, Jørgensen 1985) might provide a useful alternative.

For quantitative measurements, assays based on radial diffusion of enzyme into a substrate-bearing gel slab were developed. This technique has been well described using other dye-substrate interactions (e.g., starch-iodine) and is simple, rapid, and requires little substrate (Dingle et al 1953, Briggs 1962, Schill and Schumacher 1972, Hejgaard and Gibbons 1979). General factors affecting diffusion zone development and accuracy are discussed in these papers.

The basic procedures were established using the well-characterized (1→3) (1→4)-β-D-glucanase (enzyme 1), (1→3)-β-D-glucanase (enzyme 2), and a crude cellulase preparation (enzyme 23). In each case a linear relationship was established between the logarithm of the enzyme concentration and diffusion diameter over 2–3 orders of magnitude in concentration. The method can detect low concentrations of enzyme, but it is insensitive to small differences in activity. Within-plate replication is good, usually ± 0.03 cm for diameters of 1–2 cm. Significant between-plate differences were sometimes noted, but in general between-plate results were comparable.

In general, in studies with (1→4)-β-D-glucan substrate, CMC gave larger zone diameters and was therefore a more useful substrate than HEC. Tamarind amyloid is a natural, relatively lightly substituted cellulose, but in this study it offered no particular advantage over commercial substituted cellulose as a (1→4)-β-D-glucanase substrate. The lack of activity of the (1→3) (1→4)-β-D-glucanase (enzyme 1) from *B. subtilis* against this substrate is a useful result because it allows histochemical distinction between xyloglucan and the (1→3)(1→4)-β-D-glucan (Wood et al 1983). Although residual clearing zones using the heated enzyme preparations were mostly absent or very small with (1→3)-β-D-glucan and (1→3)(1→4)-β-D-glucan as substrate, this was not the case with CMC and HEC as substrate, particularly with the cellulase and hemicellulase preparations. Although such heat stability is not unknown, where heat-treated enzyme continues to produce significant cleared zones, the possibility that nonenzymic artifacts are responsible should be considered.

In view of the current interest in improved analysis of dietary fiber, a survey of β-glucanase activities in commercial α-amylase and amyloglucosidase preparations seemed worthwhile. Because we had observed that, in the presence of 1 *M* NaCl, some xylan preparations could induce significant (~30 nm) red shifts in λ_{max} of the absorption spectra of Congo red, we thought it worthwhile to include a xylan-containing gel plate in this survey. It is not our intention in reporting these results to recommend any particular enzyme preparation but rather to demonstrate the utility of the technique. Nevertheless, the results (Table IV) raise a number of interesting points. It is clear that most commercial amylolytic enzymes contain some β-D-glucanase activity and lack of activity against (1→4)-β-D-glucan or (1→3)-β-D-glucan does not preclude activity against (1→3)(1→4)-β-D-glucan (e.g., enzyme preparations 4, 5, 10, and 20). Surprisingly, one preparation (enzyme 32) apparently has cellulase activity but was inactive against oat gum. Xylanase activity was observed less frequently. The potentially serious consequences of lack of detection of such activity, either by wrong choice of substrate or use of insufficiently sensitive techniques (e.g., release of reducing sugar) in dietary fiber analysis or preparation, or in starch analysis is apparent. The results with glucose oxidase and peroxidase preparations suggests that in some cases cleared zones may be artifactual.

A preliminary communication (Wood 1981) reported that the gel diffusion assay is applicable to measurement of β-glucanase activity in seed extracts. A prime target was development of an assay for (1→3)(1→4)-β-D-glucanase in germinating barley. Martin and Bamforth (1983) have since reported success with such an assay. Their approach produced larger diffusion diameters than we were able to obtain, presumably by increased enzyme volume (100 μl) and lower liquid-to-solids ratio in extraction. A comparison of their 10 m*M* acetate-HCl buffer system with MES-buffer barley extracts showed similar diffusion diameters but more distinct boundaries with the acetate-HCl buffer.

In general we found (1→3)-β-D-glucanase in germinating barley simpler to assay than the (1→3) (1→4)-β-D-glucanase, which is extracted along with its endogenous substrate. This was observed as a pink diffusion zone that, because undegraded oat β-glucan did not diffuse into the agarose gel, presumably was partially degraded (1→3) (1→4)-β-D-glucan.

The results with barley (1→3)-β-D-glucanase showed that the gel diffusion assay was a rapid and simple quantitative method for following this enzyme in germinating and malted barley. This activity has previously been used as an indicator of malt quality (Bendelow 1976). The pattern of development of activity during germination was similar to that previously reported (Ballance and Meredith 1974). As shown in Figure 4, the method compares well with viscometric values over a range of concentrations beyond that possible with viscometry.

Gel diffusion is an indirect technique, and the rate of diffusion of enzyme will not necessarily be controlled by the same factors that influence hydrolytic activity, so the technique may not, for example, determine the true pH and temperature optima for the enzyme. In this report the studies of enzyme action in solution clearly indicated an endo-activity, but the response of a pure exo-enzyme in the gel assay was not tested.

Despite these cautionary notes, the technique is useful for the rapid survey of samples, such as required in breeding programs, and value has been demonstrated for survey of microorganisms and their enzyme products (Teather and Wood 1982). The method may be used quantitatively, but correlation with viscometric results should be established with each system studied and, in view of the relative insensitivity to small changes in activity, consideration given to the accuracy required.

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