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Preliminary communication

The use of dye–polysaccharide interactions in β-D-glucanase assay*

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Certain polysaccharides, in particular cereal β-D-glucans and substituted celluloses, induce bathochromic shifts in the adsorption spectra of such direct dyes as Congo Red[1]. As cello-oligosaccharides showed little or no interaction, it followed that formation of dye–polysaccharide complexes might be useful in studies of β-D-glucanase action. This possibility was investigated by monitoring the digestion of a sample of oat β-D-glucan[2] with purified β-D-glucan-endohydrolase (EC 3.2.1.73) from *Bacillus subtilis*. Solutions of oat β-D-glucan (0.5% w/v) in pH 6.5 buffer [0.05M maleate or 0.05M 2-(*N*-morpholino)ethanesulfonic acid] were treated with enzyme and the viscosity monitored, or aliquots removed at intervals, heated for 5 min to ~100° and tested for dye interaction (absorbance at 542 nm, λ_{max} difference spectra[3]) and reducing sugar[4]. The results (Table I) clearly showed a relationship between loss in viscosity and loss in dye interaction, whereas measurements of reducing sugar were not useful until later stages of degradation. At 24 h, when the absorption spectra were the same as that of dye alone, chromatography on Bio-Gel P-2 showed that the product was mainly trisaccharide and tetrasaccharide, as has been previously reported[5]. The dye-binding technique is extremely sensitive (saturation with ~10 μg/mL of oat β-D-glucan), and hence very low concentrations of substrate are required, providing some advantages over viscosimetry. The somewhat erratic results for absorbance at 542 nm were probably a result of inadequate enzyme deactivation, demonstrated by later gel-plate assays. These assays were developed to overcome problems in quantitation brought about by the small linear range for interaction of β-D-glucan with dye (0–5 μg/mL) and the tendency for precipitates of dye–glucan complex to form.

Radial diffusion of enzyme into a substrate-bearing gel slab is a simple, inexpensive, and sensitive method of assay[6–10]. The area of reaction, which is proportional to log[enzyme], may be visualised in a variety of ways including "clearing" of opaque substrate[6], various staining techniques[6–9] and use of substrate containing covalently attached dye[10]. As oat β-D-glucan, but not its degradation products, interacts with such direct dyes as Congo Red, this dye can be used to visualise the area of β-(1→4),(1→3)-D-glucanohydrolase activity. Similarly, we have found that *O*-(hydroxyethyl)cellulose, *O*-(carboxymethyl)cellulose, and *O*-(carboxymethyl)pachyman interact with Congo Red (the latter two require the presence of salt for useful wavelength shifts to occur) hence

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TABLE I

EFFECT OF β-(1→3),(1,4)-D-GLUCANOHYDROLASE ON OAT β-D-GLUCAN, AS SHOWN BY CHANGES IN REDUCING SUGAR, VISCOSITY, AND DYE INTERACTION

Time (min)	*Viscosity (cSt)* [a]	*Reducing sugar (mg/mL)* [b,c]	*Absorbance (542 nm)* [b]
Before enzyme [d]	9.56	[f]	0.321
0 [e]	[f]	0.13	0.226
1.5	2.95	[f]	[f]
5.0	1.54	0.11	0.219
10	1.20	0.10	0.256
20	1.00	0.11	0.218
30	0.92	0.12	0.196
60	0.84	0.17	0.176
180	[f]	0.29	0.156
24 h	[f]	0.77	0.156

[a] Enzyme 7×10^{-3} units/,L in 0.05 M MES buffer, pH 6.5. [b] Enzyme 6×10^{-3} units/mL in 0.05 M maleate buffer, pH 6.5. [c] Expressed as glucose. [d] Before enzyme added. [e] First measurement immediately after enzyme added. [f] Not done.

allowing detection, and estimation of activity, of three β-D-glucanases of considerable commercial and biological significance.

The technique used was essentially that of Zimmerman[7]. Substrates of oat β-D-glucan, tamarind amyloid[11], *O*-(carboxymethyl)pachyman[12], *O*-(hydroxyethyl)-cellulose 180 GR, or *O*-(carboxymethyl)cellulose 7H3 SXF (the latter two from Hercules Incorporated, Wilmington, Delaware) were incorporated (500 μg/mL) into a gel plate of agarose ~5 mm thick. Congo Red (40 μg/mL) was also incorporated into the gel or, alternatively, was layered onto the plate after incubation. Enzyme solutions were applied into appropriately cut wells and, after suitable incubation (normally 18 h at room temperature), the diameter of the reacted zone measured. For visualization with *O*-(carboxymethyl)cellulose 7H3 SXF and *O*-(carboxymethyl)pachyman, M sodium chloride was applied to the dyed gel. To stop the reaction, M hydrochloric acid was added which changed the dye colour to blue and allowed storage for at least 2 weeks. Fig. 1 (A–D) shows photographs of typical plates demonstrating β-(1→4),(1→3)-D-glucanohydrolase activity, β-(1→3)-D-glucanohydrolase activity, and β-(1→4)-D-glucanohydrolase activity. A linear relationship was observed for log[enzyme] and area of spot for each type of enzyme. Studies with the β-(1→4),(1→3)-D-glucanohydrolase have demonstrated a linear range over three orders of magnitude of concentration, allowing measurement of as little as 3×10^{-4} units[5] of enzyme. The results confirmed the specificity of this enzyme for the mixed-linkage β-D-glucan, as no activity was detected with *O*-(hydroxyethyl)cellulose 180 GR (Fig. 1B) or *O*-(carboxymethyl)pachyman (Fig. 1C). A sample of commercial alpha amylase showed no β-(1→4)-, or β-(1→3)-D-glucanohydrolase activity. It is noteworthy, however, that both of these commercial enzymes showed definite β-(1→4),-(1→3)-D-glucanohydrolase activity. Controls of buffer alone, included in each incubation, showed no action. Similarly, controls of gel without substrate showed there was no spot

formation with enzyme alone. Surprisingly, however, a control of the β-(1→4),(1→3)-D-glucanohydrolase solution, heated for 5 min in a boiling-water bath, showed significant activity against oat β-D-glucan. Even after 20 min, significant activity remained (~3% of original).

For assay of "cellulase" activity, choice of substrate is often a problem. We have used *O*-(hydroxyethyl)cellulose 180 GR, *O*-(carboxymethyl)cellulose 7H3 SXF, and tamarind amyloid[11]. The latter substrate, like cereal β-D-glucan and *O*-(hydroxyethyl)-cellulose 180 GR, interacts strongly with Congo Red, presumably because of the cellulosic nature of its main chain[1]. The development of techniques sensitive only to the

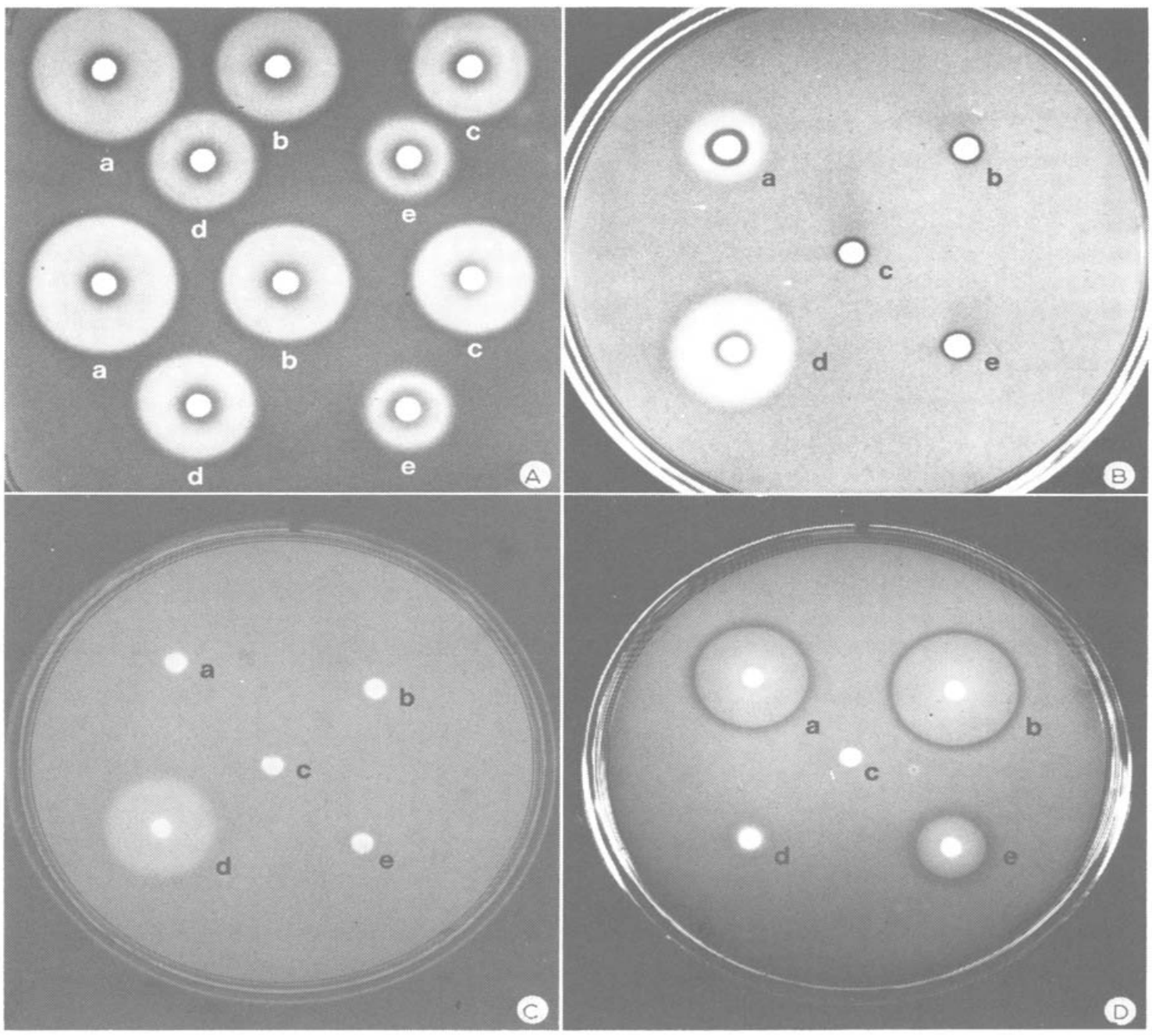

Fig. 1. Photographs of gel plates incorporating oat β-D-glucan (A and D), *O*-(hydroxyethyl)cellulose 180 BR (B), and *O*-(carboxymethyl)pachyman (C). A; duplicate applications (10 μL of *Bacillus subtilis* β-(1→4),(1→3)-D-glucanohydrolase; a, 6×10^{-3} units/mL; b, 3×10^{-3} units/mL; c, 6×10^{-4} units/mL; d, 3×10^{-4} units/mL; and e, 6×10^{-5} units/mL. B and C: a, fungal hemicellulase; b, *Bacillus subtilis* β-(1→4),(1→3)-D-glucanohydrolase; c, buffer control; d, fungal cellulase; and e, bacterial alpha amylase. D: a, Novo 240 bacterial alpha amylase; b, Sigma Type IIIa bacterial alpha amylase; c, buffer control; d, fungal alpha amylase; and e, *Bacillus subtilis* β-(1→4),(1→3)-β-D-glucanohydrolase. In A, the dye was layered on to gel and in D, it was incorporated into the gel.

TABLE II

β-1,4-D-GLUCANOHYDROLASE ACTIVITIES OF SOME ENZYME PREPARATIONS USING *O*-(HYDROXYETHYL)CELLULOSE 180 GR (HEC), *O*-(CARBOXYMETHYL)CELLULOSE 7H3 SXF (CMC, AND TAMARIND AMYLOID

Enzyme [a]	*Source*	*Enzyme activity (Diameter of spot, cm)*		
		HEC	*CMC*	*Amyloid*
Cellulase [b]	*Trichoderma viride*	2.39	3.20	2.15
β-D-Glucosidase [c]	Almonds	1.93	2.13	*d*
Cellulase + β-D-glucosidase [b,c]		2.42	3.13	*d*
Rumen cellulase (crude) [e]	*Ruminococcus albus*	2.04	2.54	1.59
Hemicellulase [f]	*Rhizopus*	1.56	2.17	1.0

[a] Suppliers as noted,. [b] Boehringer Mannheim Canada, Dorval, Quebec. [c] Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A. [d] Not done. [e] J.D. Erfle, Animal Research Institute, Agriculture Canada. [f] Sigma Chemical Company, St. Louis, Missouri, U.S.A.

scission of the main chain might make tamarind amyloid (or xyloglucan) a useful substrate for measurement of β-(1→4)-D-glucanohydrolase activity. The results (Table II) showed some differences in spot diameters, but this is not necessarily an indicator of substrate susceptibility, as substrate may affect diffusion rates differently. Also, it is not known of the limit of the spot is in fact the limit of enzyme diffusion, nor what is the effect of enzyme purity. It should be noted that the β-glucosidase preparation contained β-D-glucanase activity and did not enhance the activity of the cellulose.

The technique outlined here provides a simple, relatively rapid method for surveying β-D-glucanase activity from various sources such as seed extracts. Radial diffusion into gels allows quantitative comparisons, and we have used the technique, in particular, to examine various commercial enzyme preparations used in starch measurement and in removal of starch from cell wall and fibre fractions. The results, which will be reported in detail elsewhere, emphasise that caution should be exercised in the use of these preparations, most of which showed some β-D-glucanase activity (Fig. 1D). Simple demonstration of lack of β-(1→4)- and β-(1→3)-D-glucanohydrolase activity is inadequate, as this does not preclude the presence of β-(1→4),(1→3)-D-glucanohydrolase activity.

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