The Binding of Enzymes to Fungal β -Glucans

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Purified preparations of the extracellular glucan of *Sphacelia sorghi* and the walls of *Claviceps purpurea* were shown to contain enzymically active protein. The glucans and walls were treated in various ways to liberate the protein and determine the nature and strength of its binding, if any, to each matrix. Binding of protein to the glucan appeared to be non-covalent and was specific only in that the native protein seemed to bind more firmly than protein derived from other fungal genera. Binding of protein to walls appeared to be stronger than to the glucan but in each case protein was found to be less stable when freed from its carbohydrate matrix. Protein liberated from glucan showed enhanced activity although its K_m remained the same; thus, this matrix acted in the same manner as a non-competitive inhibitor. Protein liberated from walls was less active than when attached. The differences between the two matrices, with respect to protein binding, are discussed in the light of possible differences in their tertiary structure.

INTRODUCTION

The presence of β -linked glucans in fungi is now well established; they are found both intra- and extracellularly and in the walls (Clarke & Stone, 1963; Perlin & Taber, 1963; Buck *et al.*, 1968; Bartnicki-Garcia, 1970; Dickerson *et al.*, 1970; Wessels *et al.*, 1972; Bartnicki-Garcia, 1973). Wall glucan and extracellular glucans have been proposed as 'elicitors' of host response for fungi that are pathogenic on plants (Keen, 1975; Keen *et al.*, 1975; Anderson-Prouty & Albersheim, 1975; Ayers *et al.*, 1976*a, b, c*; Ebel *et al.*, 1976; Anderson, 1978; Stekol & West, 1978). Glucans of the same type are also known to have antitumour activity (Whistler *et al.*, 1976). The commonest of the glucans is a homopolymer of glucose residues with a main chain of (1 $\rightarrow$ 3)- β -D linkages. Occasionally, other residues are present (Clarke & Stone, 1963) and 'elicitors' have been reported to contain 4% mannosyl residues (Ayers *et al.*, 1976*c*). The main chain of these polysaccharides is frequently branched; the commonest type of branch is a single (1 $\rightarrow$ 6)- β -linked glucose residue, but longer branches are known to exist (Fleet & Manners, 1976, 1977; Sietsma & Wessels, 1977). The molecular weights reported for these polymers range widely, from about 3×10^3 to 5×10^6 (Clarke & Stone, 1963; Anderson-Prouty & Albersheim, 1975; Ayers *et al.*, 1976*c*). Extracellular glucans, in addition to wall glucans, of the above type are a common feature of several fungi including the plant pathogens *Claviceps* and *Sphacelia* (Perlin & Taber, 1963; Buck *et al.*, 1968). During the course of structural studies on purified extracellular glucans from these fungi grown in culture, it was noted that some glucan samples underwent slow hydrolysis (about 1% h⁻¹) when incubated alone in buffers of pH 4.5 to 7.5. Hydrolysis was subsequently found to be due to an associated exo-(1 $\rightarrow$ 3)- β -D-glucanase (Dickerson *et al.*, 1970). Similar phenomena have been reported elsewhere, e.g. from studies on walls of *Schizosaccharomyces* (Fleet & Phaff, 1974) and *Saprolegnia* (Fèvre, 1977). The autohydrolysis of the branched extracellular glucans of *Claviceps purpurea* (Perlin & Taber,

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1963) and *Claviceps fusiformis* (Buck *et al.*, 1968) yielded only glucose; the presence of other enzymes capable of hydrolysing (1 $\rightarrow$ 6)- β linkages was therefore suspected.

We report here studies on the nature of the association between enzymes and fungal glucans with special reference to a β -fructofuranosidase ('invertase') from *Sphacelia sorghi* and an extracellular glucan produced by the same fungus.

METHODS

Organisms. The *Claviceps purpurea* strain (29/4) was the same as that used by Dickerson *et al.* (1976). *Sphacelia sorghi* strain R18B is a derivative of a strain originally isolated from sclerotia on *Sorghum vulgare* in Nigeria (Mantle & Waight, 1968; Mantle, 1968). Basidiomycete QM806 (CMI 155, 771) was supplied by the Commonwealth Mycological Institute, Kew, Surrey.

Medium and growth conditions. These were the same as those described by Dickerson *et al.* (1970).

Analytical methods. Total nitrogen was determined, after digestion of samples in concentrated sulphuric acid and selenium followed by distillation in a micro-Kjeldahl apparatus, using the alkaline phenol reagent (Garza & Weissler, 1967). Total carbohydrate was estimated by the anthrone method (Morris, 1948) and by use of the phenol-sulphuric acid reagent (Dubois *et al.*, 1951, 1956). Protein and nucleic acids in solution were determined from absorbance measurements at 260 and 280 nm; protein was also determined by the method of Lowry *et al.* (1951) and from total nitrogen measurements on the assumption that all the nitrogen was derived from protein. Phosphorus was determined by the method of Fennell *et al.* (1957) using quinoline hydrochloride. Chromatographic procedures for analysis of sugars obtained by acid or enzymic hydrolysis of the glucan were similar to those described by Buck *et al.* (1968). Values are the mean of at least three replicates with a variation of less than 10% overall for both analyses and assay procedures (see below). Unless otherwise indicated, other results given are the mean of values obtained from at least three different preparations (see below) with a variation not exceeding 15% overall.

Production of exo-(1 $\rightarrow$ 3)- β -D-glucanase. This enzyme was isolated and purified from culture filtrates of Basidiomycete QM806 following the methods of Reese & Mandels (1966).

Enzyme assays. The activity of β -glucosidase was determined as described by Dickerson *et al.* (1970) and 'invertase' (β -fructofuranosidase) was assayed by methods similar to those described by Dickerson *et al.* (1970) and Dickerson (1972). Phosphatase activity was estimated by the method of Brandenberger & Henson (1953) using *O*-carboxyphenylphosphate. Incubations were normally carried out with 1 mg protein (or equivalent weight of glucan with the protein) per ml incubate using 1.5% (w/v) substrate. Incubations with exo-(1 $\rightarrow$ 3)- β -D-glucanase were carried out as described by Buck *et al.* (1968). Results obtained as μ mol substrate utilized, or product formed, per ml incubate per mg protein were subjected to first-order plots from which the rate constant (*k*) was determined.

Isolation, purification and analysis of glucan. Glucan was separated from fungal tissue by successive filtrations through filter paper (Whatman no. 54) until no further cell debris could be detected on microscopic examination of the filtrates. Polysaccharide was precipitated by mixing the filtrate with 1 to 2 vol. absolute ethanol or acetone. The precipitated polysaccharide was collected on a glass rod, drained and resuspended in water using the same volume as that of the original filtrate. The polysaccharide was then reprecipitated and collected as before. The procedure was repeated until the supernatant from ethanol precipitation contained no detectable trace of protein; this usually required four further precipitations. The final aqueous suspension was dialysed at 4 °C overnight against 100 vol. distilled water. The final product was freeze-dried from aqueous suspension to give a white, fibrous solid which was stored at 4 °C in vacuum over anhydrous CaCl₂.

Isolation and purification of walls. Fungal wall preparations were obtained using methods essentially similar to those of Fleet & Manners (1976). The cells were crushed using an X-press (Dickerson *et al.*, 1970) and washed in a Mickle disintegrator in the presence of 8 M-urea (Buck *et al.*, 1968). All preparations were checked microscopically to ensure that the walls had no adhering cytoplasm.

Separation of glucan from bound protein. The following methods were used:

(i) A low viscosity solution of glucan (0.4%, w/v) in 8 M-urea, at approximately neutral pH, was stirred for 1 h at room temperature; the polysaccharide was then selectively precipitated by the slow addition of ice-cold absolute ethanol or acetone until a concentration of 45 to 50% (v/v) was reached. With this procedure the protein remained in solution. After removing the polysaccharide with a glass rod, the protein was precipitated by increasing the concentration of solvent to 70% (v/v). The whole process was repeated three more times using the same polysaccharide sample. Protein was recovered from the combined alcoholic mixtures by centrifugation at 15000 *g* for 20 min; it was then washed in 70% (v/v) ethanol and water, dissolved in 5 mM-acetate buffer, pH 5.2, (1 mg ml⁻¹) and dialysed overnight at 4 °C against the same buffer.

The dialysed product was freeze-dried. The whole procedure was repeated replacing the urea by either 6 M-guanidine hydrochloride or 2% (w/v) sodium dodecyl sulphate.

(ii) Glucan solutions, as above, were stirred for 1 h at 25 °C with 0.4% (w/v) DEAE-cellulose (Whatman DE11) in 0.1 M-acetate buffer, pH 4.0, 5.1 or 5.6. The cellulose was recovered by centrifugation at 15000 g for 10 min, washed with the same buffer as used initially, recentrifuged and the residue was brought back to its original w/v by adding 0.2 M-NaCl in 10 mM-acetate buffer at the corresponding pH. The cellulose was removed as before and the supernatant was dialysed overnight at 4 °C against 5 mM-acetate buffer, pH 5.2, containing 0.5 mM-EDTA. The dialysed product was freeze-dried.

(iii) Glucan (4%, w/v) in 50 mM-acetate buffer, pH 5.2, was hydrolysed for 3 h at 30 °C using the exo-(1→3)- β -D-glucanase from Basidiomycete QM806. Protein, initially bound to the glucan, was liberated from the products of hydrolysis (glucose only in unboiled, enzymically active samples; glucose and gentiobiose, in a molar ratio of approximately 2:1, in previously boiled samples) by dialysis as in (ii). The invertase in the dialysis residue was partially purified and freed from exo-(1→3)- β -D-glucanase by passage through a column (46 × 3 cm) of Sephadex G-100 at 4 °C using 10 mM-sodium phosphate buffer, pH 6.0, as eluant. Walls were hydrolysed in a similar manner for 16 h; solid residue following hydrolysis was removed by centrifugation at 15000 g for 10 min before column chromatography.

Gel electrophoresis. Glucan (1.0 mg) was suspended in 100 μ l of 50% (w/v) sorbitol in distilled water. Samples (10 μ l) were applied to 8% (w/v) gels prepared by the method of Weber *et al.* (1972) for undenatured protein and run at 4 °C for 16 h at 7 mA per gel. Gels were stained for protein with Coomassie blue (Chrambach *et al.*, 1967), for carbohydrate with periodic acid-Schiff reagent (Fairbanks *et al.*, 1971) and for invertase specifically with 2,3,5-triphenyltetrazolium chloride after pre-incubation of the gel with 1.5% (w/v) sucrose (Gabriel & Wang, 1969). Stained gels were scanned using a Gilford 240 spectrophotometer with linear transport attachment.

Assay of glucan on gels. Transverse sections of gels were macerated and then incubated overnight with exo-(1→3)- β -D-glucanase in the usual manner (see above). The mixture was then filtered through glass wool to remove gel and the filtrate plus washings were analysed for products.

Assay of β -fructofuranosidase (invertase) on gels. Invertase was specifically identified by the method described above. In addition, transverse sections of gels were macerated and the protein was eluted at 4 °C by immersion overnight in 10 mM-sodium phosphate buffer, pH 6.0, (2 ml per section). Gel was then removed, as above, and filtrates and washings were incubated with 1.5% (w/v) sucrose to assay for invertase activity.

Reconstitution of protein-glucan complex. Protein-free glucan, prepared by the urea treatment, was solubilized in 5 mM-acetate buffer, pH 5.2, and stirred for 1 h at 25 °C with glucan-free protein prepared by the action of exo-(1→3)- β -D-glucanase on glucan with bound protein. The ratio of glucan to protein in the stirred solutions was 10:1 and the glucan concentration was normally 0.4% (w/v). The reconstituted product was purified as above by alcohol precipitation.

Continuous flow system for testing the stability of glucan-bound enzyme. The apparatus comprised a sterilized 100 ml Erlenmeyer flask, containing 60 ml of a sterile solution of sucrose (1.5%, w/v) in 1 mM-sodium phosphate buffer, pH 6.0. The flask, which had a narrow side-arm inserted near the base, was mounted on a magnetic stirrer to permit mixing of the contents by a 10 mm PTFE-coated magnetic flea. A drawn-glass jet was sealed through a rubber stopper used to close the flask. The jet was connected to a 1 l substrate reservoir which was fitted with an air filter. The reservoir contained 600 ml of a sterile solution of sucrose (1.5%, w/v) in buffer, as above. The flow rate was governed by a peristaltic 'Varioperpex' pump (LKB Instruments) connected between the reactor side-arm and a fraction collector. A glucan suspension (1.0 ml; 4%, w/v) was enclosed, using aseptic conditions, in each of three sachets of effective length 4 cm, made of previously boiled, 0.635 cm diameter Visking cellophane tubing. Alternatively, one sachet only, containing 1% (w/v) glucan-free protein, was used. Sachets were introduced aseptically into the reactor. Internal pressure ensured that the reaction volume remained constant. The linear flow rate, even at the lowest rate used (1.4 ml h⁻¹), was sufficient to prevent adventitious contamination by bacteria [the fastest moving bacterium, *Proteus mirabilis*, can achieve velocities of up to 1.0×10^{-5} cm s⁻¹ (Burrows, 1963); the lowest linear flow rate, using 3 mm diameter tubing, was 0.5×10^{-2} cm s⁻¹]. Incubations were carried out at 25 °C and the glucose content of the effluent was normally monitored at intervals of 15 min, and not greater than 1 h.

RESULTS

Samples of purified glucan from *S. sorghi* dried to constant weight at 70 °C had a water content of approximately 10% (w/w). Allowing for the degree of hydration, protein estimations on aqueous solutions of the glucan indicated the presence of between 4 and 14% (w/w) protein for 20 samples tested; the mean was 7.59 ($\sigma_{n-1} = 2.49$). The lower protein

Table 1. *Isolation of protein from fungal β -glucans*

'Purified' glucan (100 mg) was subjected to various treatments to separate protein and carbohydrate as described in Methods.

Treatment	Protein (mg)	Carbohydrate [mg (mg protein) ⁻¹]	Residual glucan [mg (mg protein) ⁻¹]	Invertase activity [10 ⁴ × <i>k</i> (s ⁻¹)]
None	7.2	12.9*	—	1.9
Urea (8 M)	6.3	< 0.1	ND	0
Guanidine hydrochloride (6 M)	6.7	< 0.1	ND	0
Sodium dodecyl sulphate (2%, w/v)	5.9	< 0.1	ND	0
DEAE-cellulose (pH 4.0)	0.3	1.3	1.2	2.6
DEAE-cellulose (pH 5.1)	0.8	1.4	1.2	3.0
DEAE-cellulose (pH 5.6)	1.1	1.0	0.9	3.2
DEAE-cellulose (pH 5.6)†	1.0	0.8	0.7	3.1
Exo-(1→3)- β -D-glucanase	7.0	< 0.1	0	5.8

ND, Not determined.

* Obtained by difference, starting with 100 mg glucan.

† Retreatment of first pH 5.6 treatment.

concentrations were normally found in cultures harvested after 4 to 7 d and the higher concentrations in those harvested after 14 d. The protein concentrations were confirmed by total nitrogen estimations and by analysis of protein and glucose and gentiobiose liberated after treatment of the glucan with the exo-(1→3)- β -D-glucanase. Carbohydrate (i.e. β -glucan) and protein accounted for not less than 98% of the weight of the dehydrated glucan. Absorbance measurements on solutions suggested the presence of nucleic acid to an extent not exceeding 0.5% (w/w); phosphorus estimations showed a concentration of less than 0.1% (w/w), consistent with the presence of a small amount of nucleic acid. Chromatography failed to detect any low molecular weight sugars or amino compounds.

The molar ratio of glucose to gentiobiose liberated by the exo-(1→3)- β -D-glucanase treatment gave the average degree of branching of all the glucan samples tested as approximately one branch on every third residue, as has been found for glucans from *Claviceps* spp. (Buck *et al.*, 1968; Dickerson *et al.*, 1970); this might be expected as *S. sorghi* is a closely related organism (Mower *et al.*, 1973). Associated protein exhibited the following hydrolytic enzyme activities when the glucan was incubated with the appropriate substrate (activities, $\pm 15\%$ for six samples tested, are expressed relative to β -fructofuranosidase activity – see *k* value, Table 1): β -fructofuranosidase (invertase; 1.00), β -glucosidase (0.65), acid phosphatase (2.02), exo-(1→3)- β -D-glucanase (0.04). In the subsequent studies, only invertase activity was routinely monitored. Table 1 shows typical results for the liberation of protein from a purified glucan sample following various treatments. Fractional precipitation with ethanol in the presence of 8 M-urea, 6 M-guanidine hydrochloride or 2% (w/v) sodium dodecyl sulphate removed 80 to 90% of the protein from the glucan. On recovery, the freed protein was enzymically inactive in each case. Addition of DEAE-cellulose to the glucan resulted in some of the protein being transferred to the cellulose. The best results were obtained at pH 5.6 with an equal weight of cellulose, when approximately 15% of the protein was transferred; increasing the ratio (w/w) of cellulose to glucan did not significantly enhance this value. Protein isolated from the cellulose usually contained an approximately equal weight of carbohydrate; as most of this was accounted for by glucose, following hydrolysis of samples using the exo-(1→3)- β -D-glucanase, it was considered to be residual glucan. Approximately 25% of the residual glucan could be removed after further treatment with DEAE-cellulose at pH 5.6 where it was found that all protein recovered from the first treatment re-bound (Table 1). Liberation of protein by hydrolysis of the glucan matrix with exo-(1→3)- β -D-glucanase resulted in preparations that were completely free from contaminating glucan and had qualitatively the same invertase action (Dickerson,

Table 2. *Partial separation of protein and carbohydrate in 'purified' glucan by gel electrophoresis*

'Purified' glucan (100 μ g) in 10 μ l of 50 % (w/v) sorbitol in distilled water was placed on the top of 8 % (w/v) gels. Electrophoresis was carried out at 4 °C for 16 h at 7 mA per gel. Gels were assayed for carbohydrate, protein and β -fructofuranosidase activity as described in Methods.

Distance from top of gel (mm)	Percentage of original glucan carbohydrate	Invertase activity [$10^4 \times k$ (s^{-1})]
0-6	40.0	3.0
6-26	28.8	0
26-46	9.2	0
46-61	< 1.0	0
61-76	0	5.1
76-101	0	0

Table 3. *Reconstitution of protein-glucan complex*

Protein (10 mg) and protein-free glucan (100 mg; 0.4 %, w/v) were stirred for 1 h at 25 °C in 5 mM-acetate buffer, pH 5.2. The polysaccharide was then precipitated with ethanol and taken through the routine purification procedure (see Methods). Associated protein was measured at each stage after resuspension of the precipitated glucan in water (0.4 %, w/v).

No. of ethanol precipitations	Associated <i>S. sorghi</i> protein		Associated yeast invertase	
	[mg (100 mg glucan) $^{-1}$]	$10^3 \times k^*$ (s^{-1})	[mg (100 mg glucan) $^{-1}$]	$10^3 \times k$ (s^{-1})
1	9.0	ND	6.7	ND
2	8.5	ND	4.9	ND
3	8.3	ND	3.7	ND
4	8.2	0.18	3.0	11.4†

ND, Not determined.

* For invertase.

† For the original free enzyme, $k = 18.6 \times 10^{-3} s^{-1}$.

1972) but more than double the specific activity of the associated enzyme. As the K_m of the associated enzyme (4.0×10^{-2} M) did not alter on liberation, the presence of glucan must modify the action of the enzyme in a manner analogous to that of a non-competitive inhibitor.

Partial separation of protein and glucan could be effected by gel electrophoresis (Table 2); over 70 % of the glucan was retained within the top 3 cm of the gel, and small amounts diffused down to about 5 cm. The protein was separated into 15 detectable bands; four of these, comprising 27 % of the total protein, were in the same area of the gel as the glucan. The other 11 bands accounted for the rest of the protein. Invertase activity was found in two bands only: one occupied the top 2 mm of the gel, i.e. the topmost of the protein bands in the area of the gel occupied by the glucan, and comprised about 15 % of the total protein; the other was a single band, comprising about 4 % of the total protein, between 6 and 7.5 cm from the top. The activity of the enzyme in the active areas was similar to that observed for the total protein; the electrophoretic procedure used appears, therefore, to inactivate the enzyme significantly. However, as noted before, the enzyme was less active in the presence of the glucan.

Reconstitution of the protein-glucan complex from protein-free glucan and protein isolated from exo-(1 $\rightarrow$ 3)- β -D-glucanase digests (Table 3; same sample as that referred to in Table 1) gave preparations which had approximately the same enzyme activity as the original material. A commercial preparation of invertase (baker's yeast; Sigma, grade VI) could replace the *S. sorghi* protein but the amount remaining firmly associated with the glucan

Fig. 1

Fig. 2

Fig. 1. Effect of temperature on the enzyme activity of free protein (●) and glucan-associated protein (○). Sucrose (1.5%, w/v) was incubated in 10 mM-sodium phosphate buffer, pH 6.0, with either glucan-associated or free protein (1 mg ml⁻¹) at various temperatures. First-order reaction rates were determined in each case and the results are expressed as μmol substrate utilized (ml incubate)⁻¹ h⁻¹.

Fig. 2. Effect of prolonged incubation on the enzyme activity of free protein (—) and glucan-associated protein (---). Sucrose (1.5%, w/v) was incubated at 25 °C in 1 mM-sodium phosphate buffer, pH 6.0, with either glucan-associated or free protein, each enclosed in dialysis tubing, in the continuous flow apparatus described in Methods. Results are expressed as the concentration of glucose in the effluent from the apparatus.

was much less than that of the 'native' protein. The activity of this enzyme was depressed by the presence of glucan in a similar manner to that of the *S. sorghi* enzyme.

Wall preparations from *C. purpurea* (used instead of *S. sorghi* as little or no glucan is produced in culture), when treated with exo-(1→3)-β-D-glucanase in the same manner as the *S. sorghi* extracellular glucan, showed a dry weight loss following hydrolysis of between 60 and 70%. Glucose liberated during the treatment accounted for 34 to 36% (w/w) of wall material and liberated protein for 4 to 5%. The original wall preparations were enzymically active (mean $k = 5.63 \times 10^{-4} \text{ s}^{-1}$) but residual wall material after hydrolysis showed no activity. As with the glucan, protein liberated by the action of the exo-(1→3)-β-D-glucanase on the walls was also active but, unlike the glucan protein, less so than the associated form (mean $k = 0.72 \times 10^{-4} \text{ s}^{-1}$).

The stability of the enzymes associated with extracellular glucans and walls was investigated as they were not inactivated by the purification procedures for either matrix and resisted inactivation for periods up to 15 min in boiling water. Figure 1 shows the effect of temperature on invertase associated with the extracellular glucan of *S. sorghi* and on the freed enzyme, and demonstrates the increased stability of the former. With walls, the stability of the associated protein at elevated temperatures was not so marked; nevertheless, at 50 °C it retained at least 20% of the enzyme activity that it had at 25 °C. Figure 2 shows that the glucan-associated enzyme, enclosed in dialysis tubing in a continuous flow system, stabilized in activity at 5 d and remained at this level for the duration of each experiment (4 to 5 weeks); its functional half-life at several flow rates was at least 30 times that of freed enzyme under the same conditions.

DISCUSSION

Preparations of the extracellular glucan and walls of *S. sorghi* contained protein that could not be removed by the procedures used to purify the glucan. The amount of protein associated with the extracellular glucan showed some variation; part of this can be accounted for by the tendency of the protein levels associated with the glucan to increase with the age of the culture, but some of the difference might also be attributable to variations in the conformation of both protein and glucan (see below).

The comparatively rigorous methods required for complete separation of the carbohydrate and protein fractions of the mixture, the relative ineffectiveness of DEAE-cellulose in removing protein even with dilute glucan solutions, the ease of reconstitution of the mixture to give a product closely resembling the starting mixture and the thermal stability of the protein associated with the glucan relative to the free protein are all consistent with the protein being non-covalently bound to the glucan. The two polymers, especially if tertiary structure is exhibited and as they are native to the same organism, might be expected to associate by, for example, hydrogen-bonding. The energy derived from this type of association could account for the thermal stability of the bound protein. The native protein, exemplified by invertase, appeared to associate more readily with the glucan than invertase from other fungal sources (e.g. yeast); allowing for the fact that the yeast invertase probably contained inert material in the form of highly denatured protein, this partial specificity might also be explained if the conformation of the native protein molecules corresponded more closely with those of the glucan and thus facilitated binding. Such an affinity might be mediated or enhanced if a specific substrate link between native enzyme and glucan existed. In the continuous flow studies, the initial decline from maximal invertase activity, which was arrested after 2 d, could be attributed to two different invertases associated with the glucan. Alternatively, a single protein only may be represented, the major portion of which is firmly bound and the residue either loosely bound or unbound and hence rapidly inactivated. The electrophoretic studies with glucan-associated protein also demonstrate separable invertase activities; again this may represent two invertases or a single protein with the major portion remaining in the same area of the gel as occupied by the glucan and a minor, more mobile, portion that could represent the loosely bound or unbound enzyme.

The ability of β -glucans, particularly the insoluble variety which normally have a very low degree of branching (Villanueva *et al.*, 1976), to bind proteins has been used to purify, by selective adsorption, hydrolases for which the glucans themselves are the natural substrate (Clarke & Stone, 1965; Villa *et al.*, 1976). Branching increases the solubility of the glucans in aqueous media and from the present work this appears to enhance the degree to which proteins, in addition to those which catalyse glucan hydrolysis and are implicated in wall extension (Villanueva *et al.*, 1976; Kritzman *et al.*, 1978), may bind. From conformational studies a distinct tertiary structure for the unbranched (1 $\rightarrow$ 3)- β -linked glucan has been proposed, consisting of a triple helix stabilized by hydrogen bonds between substituents on C-2 of each residue (Atkins *et al.*, 1969; Rees, 1973). As the turns of the helix result in the substituents for branch formation on C-6 being directed towards the outside of the helix and away from its axis, substitution to give branches should not interfere with the basic tertiary structure. An increased facility for the non-covalent binding and stabilization of proteins may, therefore, be due to the addition of branches to the secondary structure of the polymer or by addition to an existing tertiary structure. The conformational entropy of polysaccharides suggests that a distinct tertiary structure is less likely to occur when the polymer is in solution (Rees, 1973); therefore the triple helix might be the predominant form of the glucan in the fungal wall, but not necessarily in the extracellular glucans elaborated in liquid culture. If this structural difference does exist, it could account for our observation that residual protein remaining in the wall was, after washings in the presence of 8 M-urea to remove cytoplasm, still firmly associated, enzymically active and could only

be removed following partial hydrolysis of the wall with exo-(1→3)- β -D-glucanase. The last procedure resulted in loss of enzyme activity which again may be contrasted with the extracellular glucan where an enhancement was observed. Thus, with respect to β -glucans, both secondary and tertiary structure would seem to be important in any binding and stabilizing process for proteins.

Allowing for other elements in the fungal wall which may play a role in protein binding, the great stability of glucan-associated proteins suggests that the glucan could well be the site in the fungal wall where enzymes involved in assimilation are normally bound *in vivo*; further studies will establish whether other enzymes, concerned with transport and secretion, might also be sited here and whether any binding is more specific than at present suggested. With respect to elicitor activity, wall glucans may owe their role as agents for recognition by plants to their ability to bind to proteins in addition to their ability to bind to other polysaccharides (Moorhouse *et al.*, 1977; Morris *et al.*, 1977). However, if glycoproteins are involved, such binding may merely be another manifestation of carbohydrate interaction.

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REFERENCES

- ANDERSON, A. J. (1978). Isolation from three species of *Colletotrichum* of glucan-containing polysaccharides that elicit browning and phytoalexin production in bean. *Phytopathology* **68**, 189–194.
- ANDERSON-PROUTY, A. J. & ALBERSHEIM, P. (1975). Host-pathogen interactions. VIII. Isolation of a pathogen-synthesized fraction rich in glucan that elicits a defense response in the pathogen's host. *Plant Physiology* **56**, 286–291.
- ATKINS, E. D. T., PARKER, K. D. & PRESTON, R. D. (1969). The helical structure of the β -1,3-linked xylan in some siphonous green algae. *Proceedings of the Royal Society B* **173**, 209–221.
- AYERS, A. R., EBEL, J., FINELLI, F., BERGER, N. & ALBERSHEIM, P. (1976a). Host-pathogen interactions. IX. Quantitative assays of elicitor activity and characterization of the elicitor present in the extracellular medium of cultures of *Phytophthora megasperma* var. *sojae*. *Plant Physiology* **57**, 751–759.
- AYERS, A. R., EBEL, J., VALENT, B. & ALBERSHEIM, P. (1976b). Host-pathogen interactions. X. Fractionation and biological activity of an elicitor isolated from the mycelial walls of *Phytophthora megasperma* var. *sojae*. *Plant Physiology* **57**, 760–765.
- AYERS, A. R., VALENT, B., EBEL, J. & ALBERSHEIM, P. (1976c). Host-pathogen interactions. XI. Composition and structure of wall-released elicitor fractions. *Plant Physiology* **57**, 766–774.
- BARTNICKI-GARCIA, S. (1970). Cell wall composition and other biochemical markers in fungal phylogeny. In *Phytochemical Phylogeny*, pp. 81–103. Edited by J. B. Harborne. London and New York: Academic Press.
- BARTNICKI-GARCIA, S. (1973). Fungal cell wall composition. In *Handbook of Microbiology*, vol. II, pp. 201–214. Edited by A. I. Laskin & H. A. Lechevalier. Cleveland, Ohio: CRC Press.
- BRANDENBERGER, H. & HENSON, R. (1953). Eine spektrophotometrische Bestimmungsmethode für saure und alkalische Phosphatasen. *Helvetica chimica acta* **36**, 900–906.
- BUCK, K. W., CHEN, A. W., DICKERSON, A. G. & CHAIN, E. B. (1968). Formation and structure of extracellular glucans produced by *Claviceps* species. *Journal of General Microbiology* **51**, 337–352.
- BURROWS, W. (1963). In *Textbook of Microbiology*, 18th edn, p. 50. Edited by W. Burrows. Philadelphia: W. B. Saunders.
- CLARKE, A. E. & STONE, B. A. (1963). Chemistry and biochemistry of β -1,3-glucans. *Review of Pure and Applied Chemistry* **13**, 134–156.
- CLARKE, A. E. & STONE, B. A. (1965). β -Glucan hydrolases from *Aspergillus niger*: isolation of a β -(1→4)-glucan hydrolase and some properties of the β -(1→3)-glucan-hydrolase components. *Biochemical Journal* **96**, 793–801.
- CHRAMBACH, A., REISFELD, R. A., WYCKOFF, M. & ZACCARI, J. (1967). A procedure for rapid and sensitive staining of protein fractionated by polyacrylamide gel electrophoresis. *Analytical Biochemistry* **20**, 150–154.
- DICKERSON, A. G. (1972). A β -D-fructofuranosidase from *Claviceps purpurea*. *Biochemical Journal* **129**, 263–272.
- DICKERSON, A. G., MANTLE, P. G. & SZCZYRBAK, C. A. (1970). Autolysis of extracellular glucans produced *in vitro* by a strain of *Claviceps fusiformis*. *Journal of General Microbiology* **60**, 403–415.
- DICKERSON, A. G., MANTLE, P. G. & NISBET, L. J. (1976). Carbon assimilation by *Claviceps purpurea* growing as a parasite. *Journal of General Microbiology* **97**, 267–276.
- DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A. & SMITH, F. (1951). A colorimetric method for the determination of sugars. *Nature, London* **168**, 167.
- DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A. & SMITH, F. (1956). A colorimetric method for the determination of sugars and

- related substances. *Analytical Chemistry* **28**, 350–356.
- EBEL, J., AYERS, A. R. & ALBERSHEIM, P. (1976). Host–pathogen interactions. XII. Response of suspension-cultured soybean cells to the elicitor isolated from *Phytophthora megasperma* var. *sojae*, a fungal pathogen of soybeans. *Plant Physiology* **57**, 775–779.
- FAIRBANKS, G., STECK, T. L. & WALLACH, D. F. H. (1971). Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10**, 2606–2617.
- FENNELL, T. R. W. F., ROBERTS, M. W. & WEBB, J. R. (1957). The semi-micro determination of phosphorus in fluorinated organic compounds. *Analyst* **82**, 639–643.
- FÈVRE, M. (1977). Subcellular localization of glucanase and cellulase in *Saprolegnia monoica* Pringsheim. *Journal of General Microbiology* **103**, 287–295.
- FLEET, G. H. & MANNERS, D. J. (1976). Isolation and composition of an alkali-soluble glucan from the cell walls of *Saccharomyces cerevisiae*. *Journal of General Microbiology* **94**, 180–192.
- FLEET, G. H. & MANNERS, D. J. (1977). The enzymic degradation of an alkali-soluble glucan from the cell walls of *Saccharomyces cerevisiae*. *Journal of General Microbiology* **98**, 315–327.
- FLEET, G. H. & PHAFF, H. J. (1974). Glucanases in Schizosaccharomyces. Isolation and properties of the cell wall-associated β -(1 $\rightarrow$ 3)-glucanases. *Journal of Biological Chemistry* **249**, 1717–1728.
- GABRIEL, O. & WANG, S. F. (1969). Determination of enzymatic activity in polyacrylamide gels. *Analytical Biochemistry* **27**, 545–554.
- GARZA, A. C. & WEISSLER, H. E. (1967). Automatic simultaneous multiple chemical analyses of worts and beers. *Technicon Symposium* **1**, 201–206.
- KEEN, N. T. (1975). Specific elicitors of plant phytoalexin production: determinants of race specificity in pathogens? *Science* **187**, 74–75.
- KEEN, N. T., WANG, M. C., BARTNICKI-GARCIA, S. & ZENTMYER, G. A. (1975). Phytotoxicity of mycolaminarins – β -1,3-glucans from *Phytophthora* spp. *Physiological Plant Pathology* **7**, 91–97.
- KRITZMAN, G., CHET, I. & HENIS, Y. (1978). Localization of β -(1,3)-glucanase in the mycelium of *Sclerotium rolfsii*. *Journal of Bacteriology* **134**, 470–475.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.
- MANTLE, P. G. (1968). Studies of *Sphacelia sorghi* McRae, an ergot of *Sorghum vulgare* Pers. *Annals of Applied Biology* **62**, 443–449.
- MANTLE, P. G. & WRIGHT, E. S. (1968). Dihydroergosine: a new naturally occurring alkaloid from the sclerotia of *Sphacelia sorghi* McRae. *Nature, London* **218**, 581–582.
- MOORHOUSE, R., WINTER, W. T., ARNOTT, S. & BAYER, M. E. (1977). Conformation and molecular organization in fibers of the capsular polysaccharide from *Escherichia coli* M41 mutant. *Journal of Molecular Biology* **109**, 373–391.
- MORRIS, D. L. (1948). Quantitative determination of carbohydrates with Dreywood's anthrone reagent. *Science* **107**, 254–255.
- MORRIS, E. R., REES, D. A., YOUNG, G., WALKINSHAW, M. D. & DARKE, A. (1977). Order–disorder transition for a bacterial polysaccharide in solution. A role for polysaccharide conformation in recognition between *Xanthomonas* pathogen and its plant host. *Journal of Molecular Biology* **110**, 1–16.
- MOWER, R. L., GRAY, G. R. & BALLOU, C. E. (1973). Sugars from *Sphacelia sorghi* honeydew. *Carbohydrate Research* **27**, 119–134.
- PERLIN, A. S. & TABER, W. A. (1963). A glucan produced by *Claviceps purpurea*. *Canadian Journal of Chemistry* **41**, 2278–2282.
- REES, D. A. (1973). Polysaccharide conformation. In *MTP International Review of Science: Carbohydrates. Organic Chemistry, Series 1*, vol. 7, pp. 251–283. Edited by G. O. Aspinall. London: Butterworths.
- REESE, E. T. & MANDELS, M. (1966). β -Glucanases other than cellulase. *Methods in Enzymology* **8**, 607–615.
- SIETSMA, J. H. & WESSELS, J. G. H. (1977). Chemical analysis of the hyphal wall of *Schizophyllum commune*. *Biochimica et biophysica acta* **496**, 225–239.
- STEKOLL, M. & WEST, C. A. (1978). Purification and properties of an elicitor of castor bean phytoalexin from culture filtrates of the fungus *Rhizopus stolonifer*. *Plant Physiology* **61**, 38–45.
- VILLA, T. G., NOTARIO, V. & VILLANUEVA, J. R. (1976). Method of purifying β -(1 $\rightarrow$ 3)-glucanase from *Candida utilis*. *Applied and Environmental Microbiology* **32**, 185–187.
- VILLANUEVA, J. R., NOTARIO, V., SANTOS, T. & VILLA, T. G. (1976). β -Glucanases in nature: biochemistry and function of β -glucanases in yeast. In *Microbiology and Plant Protoplasts*, pp. 323–355. Edited by J. F. Peberdy, A. H. Rose, H. J. Rogers & E. C. Cocking. London and New York: Academic Press.
- WEBER, K., PRINGLE, J. R. & OSBORN, M. (1972). Measurements of molecular weights by electrophoresis on acrylamide gels. *Methods in Enzymology* **26C**, 3–27.
- WESSELS, J. G. H., KREGER, D. R., MARCHANT, R., REGENSBURG, B. A. & DE VRIES, O. H. M. (1972). Chemical and morphological characterization of the hyphal wall surface of the basidiomycete *Schizophyllum commune*. *Biochimica et biophysica acta* **273**, 346–358.
- WHISTLER, R. L., BUSHWAY, A. A., SINGH, P. P., NAKAHARA, W. & TOKUZEN, R. (1976). Noncytotoxic, antitumor polysaccharides. *Advances in Carbohydrate Chemistry and Biochemistry* **32**, 235–275.