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Co-operative Action by Endo- and Exo-β-(1→3)-glucanases from Parasitic Fungi in the Degradation of Cell-Wall Glucans of *Sclerotinia sclerotiorum* (Lib.) de Bary

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1. Two fungi, *Coniothyrium minitans* Campbell and *Trichoderma viride* Pers. ex Fr., were grown on autoclaved crushed sclerotia of the species *Sclerotinia sclerotiorum*, which they parasitize. 2. *In vitro* the crude culture filtrates would lyse walls isolated from hyphal cells or the inner pseudoparenchymatous cells of the sclerotia, in which a branched β-(1→3)-β-(1→6)-glucan, sclerotan, is a major constituent. 3. Chromatographic fractionation of the enzymes in each culture filtrate revealed the presence of several laminarinases, the most active being an exo-β-(1→3)-glucanase, known from previous studies to attack sclerotan. Acting alone this brought about a limited degradation of the glucan, but the addition of fractions containing an endo-β-(1→3)-glucanase led to almost complete breakdown. A similar synergism between the two enzymes was found in their lytic action on cell walls. 4. When acting alone the endo-β-(1→3)-glucanase had a restricted action, the products including a trisaccharide, tentatively identified as 6^2-β-glucosyl-laminaribiose. 5. These results are discussed in relation to the structure of the cell walls and of their glucan constituents.

Jones & Watson (1969), investigating the parasitism of sclerotia of *Sclerotinia sclerotiorum* (Lib.) de Bary by two other fungi, found that the latter produced culture fluids capable of lysing living cells of the host; β-glucanase and chitinase were present in the fluid. Jones (1970) examined the ultrastructure of the cell walls of *S. sclerotiorum* and showed that they contained a β-(1→3)-glucan and chitin. Later Jones *et al.* (1972) identified the glucan by its i.r. spectrum as sclerotan, first found in *Sclerotinia libertiana* (syn. *S. sclerotiorum*) cultures by Kitahara & Takeuchi (1957, 1961) and shown to be a β-(1→3)-glucan with single glucose residues attached at intervals by β-(1→6)-linkages. Studies of a similar glucan from *Sclerotium rolfsii* by Johnson *et al.* (1963) and Batra *et al.* (1969) have emphasized its regular structure. Its complete degradation by an exo-β-(1→3)-glucanase from Basidiomycete QM 806 (cf. Johnson *et al.*, 1963; Buck *et al.*, 1968) yields a mixture of glucose and gentiobiose in a molar ratio usually close to 2:1, although with sclerotan preparations from other species ratios as high as 4.0 and as low as 1.5 have been recorded.

With a view to establishing the enzymic mechanism of cell-wall lysis the enzymes in the culture fluids of the two parasitic fungi were fractionated. Both contained a high activity of exo-β-(1→3)-glucanase, but although this enzyme brought about appreciable degradation of sclerotan and some lysis of cell walls, neither process was completed unless an endo-β-(1→3)-glucanase was also present. The latter enzyme, which is a minor constituent of the culture fluids, had only a limited action on its own. Preliminary accounts have been published (Bacon *et al.*, 1972; Bacon, 1973).

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Experimental

Methods

Culture of micro-organisms. Sclerotia-forming fungi and others parasitic on them were obtained and cultured as described by Jones & Watson (1969), Jones (1970) and Jones *et al.* (1972). *Coniothyrium minitans* was grown on crushed autoclaved sclerotia of *S. sclerotiorum* in a mineral-salts medium (Jones, 1970) at 25°C for about 30 days (Jones *et al.*, 1972); growth of *Trichoderma viride* on the same medium was more rapid and cultures were harvested after 5–10 days.

Preparation of cell walls. Hyphae or sclerotia were broken and the resulting cell walls washed as described by Jones (1970) and Jones *et al.* (1972). They were usually stored in a freeze-dried state.

Chromatography of enzymes. The enzymes in culture filtrates were fractionated at 4°C by using the methods described by Bacon *et al.* (1970). Separations were also made on DEAE-Sephadex and CM-Sephadex (A50 anion-exchanger and C50 cation-exchanger respectively), products of Pharmacia

(G.B.) Ltd., London W5 5SS, U.K. DEAE-Sephadex (2g), swelled in 10mM-Na_2HPO_4 for 2 days, was packed into a glass tube (Pharmacia K15/30) to form a column (15mm×250mm) and washed with 10mM-Na_2HPO_4 until the effluent fluid had the same pH as that applied. CM-Sephadex columns were similarly prepared by substituting 10mM-NaH_2PO_4 for Na_2HPO_4. The columns were eluted with the appropriate gradients described in the text. Flow rates were controlled by a peristaltic pump, usually at 20–30ml/h, and fractions (3ml) were collected.

Concentration of culture filtrates. Cultures were usually harvested when the laminarinase activity of the culture filtrate reached a constant value by filtering the culture medium through Miracloth (Calbiochem Ltd., London W1H 1AS, U.K.) and centrifuging the filtrate at 16000*g* for 30min. The culture filtrate was dialysed against 5 litres of 1mM-sodium phosphate buffer, pH7.0, for 2 days, the buffer being changed twice daily, then freeze-dried in 50ml lots.

Application of samples to columns. One or more lots of freeze-dried culture filtrate were dissolved in 5ml of cold water, dialysed against the gradient starting solution, centrifuged to remove insoluble material and part of the supernatant was carefully applied to a column previously drained to bed level. The sample was washed in with approx. 5ml of starting solution before the appropriate gradient was applied.

Lytic activity. The action of enzyme preparations on living cells was tested as described by Jones & Watson (1969). Procedures for cell walls resembled those of Bacon *et al.* (1970), except that thiol was not added and to economize material the cell-wall concentration and total volume were usually less. Typical incubations comprised enzyme solution (0.25ml), one drop of a suspension of preheated inner pseudoparenchymatous walls of *S. sclerotiorum*, usually 16mg/ml, and 50mM-sodium citrate–phosphate buffer, pH5.0 (0.05ml).

Laminarinase activity. This was measured at pH5.0 as described by Bacon *et al.* (1970). A unit of enzyme activity is defined as that which liberates in 1h reducing power equivalent to 1μmol of glucose.

High-voltage paper electrophoresis. This was carried out on a Gilson Medical Electronics High-Voltage Electrophorator (Anachem Ltd., Luton LU2 7QE, U.K.) with conditions specified in the text.

Other procedures. Physical and chemical examinations of the cell walls were carried out as described by Bacon *et al.* (1969).

Materials

Preparation of glucan from S. rolfsii cultures. It was not considered advisable to handle large quantities of this organism in the living state (cf. Jones *et al.*, 1972). The spent culture medium (Chet *et al.*, 1967) was carefully decanted into ethanol, but very little polysaccharide was precipitated. The mycelial mass was therefore autoclaved, and the firm gelatinous lump produced was disintegrated by using an Ultra-Turrax homogenizer (Janke and Kunkel KG, Staufen, Germany) with additions of water. The resulting gelatinous suspension was centrifuged at 100000*g* for 1–2h at 4°C. The residue was homogenized three more times with water and the resulting supernatants were combined; they were water-clear and colourless, but had a gel-like consistency and usually fell out of the centrifuge tube in one piece.

Ethanol (0.5vol.) was added and the suspension centrifuged as above. The precipitated gel was collected (with some difficulty because air was entrapped), homogenized with water, and centrifuged again to give a firm gel with little or no precipitate. Ethanol (0.2vol.) was added and the suspension centrifuged after degassing. The compact gelatinous pellet was once more homogenized, poured twice through Miracloth, which removed some mycelial debris, and dialysed against water for 3 days at 4°C. One-fifth of the solution was kept at 4°C and the remainder freeze-dried. The first ethanolic supernatant was found to contain some glycogen.

A similar preparation was made from sclerotia of *S. sclerotiorum.*

Partial acid hydrolysis of sclerotan. Glucan solution (10ml; 42mg of dry matter) prepared as above from *S. rolfsii*, was boiled for 1.5h after the addition of 10ml of 4% (w/v) HCl. The solution remained viscous and when cool set to a glassy gel. Addition of ethanol (2vol.) and centrifugation gave a gelatinous pellet which was dispersed in water, dialysed and freeze-dried, yielding 27mg.

Alkali treatment of sclerotan. Freeze-dried glucan (200mg) was heated in 20ml of 3% (w/v) NaOH at 80°C for 1h. The solution became pale yellow and a small precipitate settled out. After neutralization with acetic acid the whole was dialysed for 2 days against water and centrifuged at 50000*g* for 1h (precipitate, 3.9mg). The supernatant, still detectably viscous, contained 6.1mg of dry matter/ml, and an anthrone determination indicated 5.7mg of glucan/ml.

Solution of sclerotan in dimethyl sulphoxide. Freeze-dried glucan (50mg) was shaken in 5ml of dimethyl sulphoxide at 20°C for 6 days, after which most of it had dissolved. A small residue (3mg) was removed by centrifugation and the supernatant fluid dialysed against water. The resulting viscous solution contained a total of 46mg of dry matter.

Results

Assessment of cell-wall lysis

Electron microscopy of sections of the walls of the pseudoparenchymatous cells of sclerotia shows at

least three layers (Jones, 1970). The inner layer contains fibrils, probably of chitin. The outer layer is thin and its composition is unknown. Between them there is a thick layer of electron-transparent material. The dominant constituent of the cell wall, and hence probably of this layer, is a glucan, though whether it is mixed or combined with small quantities of other materials cannot yet be decided. The dry-matter content of sclerotia is around 40%, suggesting a similarly high glucan content in the native cell wall. However, the glucan can form stiff gels at concentrations of 1% or less. Enzymic attack on the cell wall might therefore produce extensive swelling but no loss of continuity. The consequent loss of refraction could lead to its becoming invisible in the light-microscope, use of which would thus not distinguish between this condition and a complete dissolution of the cell wall. Similarly, low-speed centrifugation followed by determination of carbohydrate with anthrone is not satisfactory, because if a thin gel is present additional glucan may be precipitated by increasing the centrifugal force. As recorded below, conditions were found in which suspensions of 2–3mg of cell wall/ml were converted enzymically into unpourable gels.

For these reasons no single procedure could be relied on to assess the extent of lysis and a combination of microscopic observations and chemical examination of the products of lysis was used.

Products of cell-wall lysis

With unfractionated culture fluids the main product detected, both from sclerotial and hyphal cell walls, was glucose; small amounts of oligosaccharides were seen and some *N*-acetylglucosamine. The insoluble residue still had the i.r. spectrum of a β-(1→3)-glucan. Sometimes ethanol would precipitate a little glucan from the medium. No evidence was obtained for liberation of amino acids during lysis.

Fractionation of culture fluid from C. minitans

This was attempted on Sephadex G-100, DEAE-cellulose, and DEAE-Sephadex.

From Sephadex G-100 almost all the laminarinase activity was eluted in one peak, which had lytic action on sclerotial and hyphal cell walls; gentiobiose was among the products. Subsidiary peaks immediately preceding and following it had little lytic action. A large part of the laminarinase activity passed straight through a DEAE-cellulose column at pH8.7, but this fraction showed no lytic activity. A second, much weaker peak emerged later. The suggestion here that more than one enzyme was needed for lysis was confirmed by the use of DEAE-Sephadex.

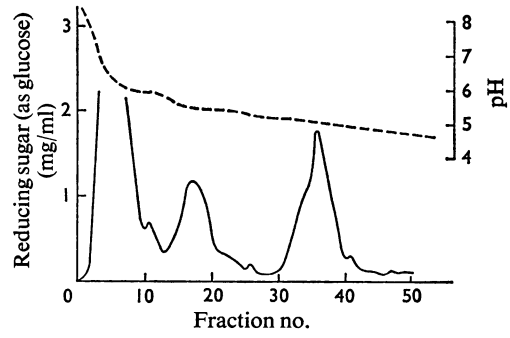

Fig. 1. *Fractionation on DEAE-Sephadex of β-(1→3)-glucanases in a culture fluid of C. minitans*

Material equivalent to 25ml was chromatographed and the fractions were tested as described in the Experimental section. Elution was by a gradient produced by addition of 0.2M-NaH_2PO_4 to 100ml of 0.1M-Na_2HPO_4; the pH of the eluent is indicated (---). Reducing sugar was measured after incubation for 18h at 30°C with laminarin (——).

This separated the laminarinase into three main peaks (Fig. 1), none of which showed much lytic activity when tested separately. However, a mixture of the first and third peaks was moderately active against sclerotial cell walls. The third peak was fractionated on Sephadex G-100. Chitinase activity was eluted early, followed by two peaks of laminarinase activity virtually free from chitinase, each with little or no lytic activity but giving a strong synergistic effect on sclerotial and hyphal cell walls when mixed with the first peak from DEAE-Sephadex, which was also free from chitinase. Therefore it appeared that substantial cell-wall lysis could be obtained in the absence of chitinase.

Further experiments of this nature indicated that the second and third peaks from DEAE-Sephadex, which represented only a small proportion (about 20%) of the total laminarinase activity, contained one or more endo-β-(1→3)-glucanases and a β-glucosidase active against gentiobiose. The presence of the latter explains why gentiobiose was not seen when cell walls were lysed by unfractionated culture fluid. The single laminarinase peak from Sephadex G-100 and the first peak from DEAE-Sephadex were free from the β-glucosidase, and both fractions showed the characteristic products of action of an exo-β-(1→3)-glucanase on sclerotan, glucose and gentiobiose, although in small yield.

It appeared that for lysis two β-(1→3)-glucanases were needed; an exo-β-(1→3)-glucanase, the dominant glucanase in the culture fluid and the first to be eluted from DEAE-Sephadex, and an endo-β-(1→3)-glucanase, which could be contributed by either the

second or third DEAE-Sephadex peaks. Fractionation on Sephadex G-100 removed the β-glucosidase active on gentiobiose, and the chitinase, without affecting lysis noticeably.

Hyphal cell walls were usually given a lower score than sclerotial cell walls in lysis tests with the glucanase mixtures. The explanation probably lies in the persistence of chitin, which constitutes a higher proportion of the hyphal cell walls (Jones *et al.*, 1972).

Action of glucanases on sclerotan

Because of the uncertainties in interpreting lysis referred to above, the enzyme fractions were tested on glucan extracted from *S. sclerotiorum* or *S. rolfsii* (see the Experimental section). Once it had been dried the material was difficult to re-disperse, so part was kept wet throughout its preparation and stored in dialysed solution in the refrigerator.

Effects on viscosity. This 'never-dried' solution, with a glucan concentration of 4mg/ml, was a stiff gel, which had to be diluted by using a glass homogenizer before effects on its viscosity could be examined. Solutions containing 0.5–1.0mg/ml were placed in an Ostwald viscometer and the effect of glucanase fractions was tested. The suspected exo-glucanase produced a fall in viscosity, but no change was detected with the endo-glucanase. There was evidence of co-operative action by the two enzyme fractions.

In preliminary experiments on the extraction of the glucan, alkali had seemed to decrease its viscosity, so its effect was examined in the viscometer. Treatment with 0.1M-NaOH for 1min at 20°C had little permanent effect; 0.5M-NaOH for 30min at 20°C followed by neutralization and dialysis decreased the emptying time of the viscometer from 51s to 25s (water, 17s).

In view of the marked effect of alkali on the physical state of the glucan it seemed advisable to include alkali-treated material in a series of tests with the glucanase fractions. Some glucan was therefore subjected to conditions resembling those often used in the extraction of polysaccharides (see the Experimental section).

Production of reducing sugars. The solution of alkali-treated glucan and the 'never-dried' original preparation were used in a series of incubations in which breakdown was followed by measuring the production of reducing sugar by an alkaline ferricyanide method in the Auto Analyzer (Hoffman, 1937) and of glucose by glucose oxidase (Huggett & Nixon, 1957). Assuming that the only products were gentiobiose and glucose, calculations were made of the percentage breakdown of glucan and the glucose/gentiobiose ratio in the products (cf. Buck *et al.*, 1968).

The first DEAE-Sephadex peak (2.1 units of glucanase/ml of incubation) acting on the alkali-treated glucan (3.2mg/ml) brought about 20% breakdown in 1h, 40% in 4h, 51% in 1 day and 64% in 14 days; a fivefold increase in enzyme concentration after 3h had no additional effect. The unattacked polysaccharide was recovered almost completely by ethanol precipitation. The untreated glucan (2.1mg/ml) was degraded by only 7, 14, 22 and 36% after these times. In both cases the molar ratio of glucose/gentiobiose was 2.3:1. A combination of the first and third DEAE-Sephadex peaks (2.1 units and 0.25 unit respectively) produced almost complete (95%) degradation of both substrates, but the glucose/gentiobiose ratios were much higher (2.6 to 4.3), because of gentiobiose hydrolysis by the β-glucosidase in the third peak. Interpolation between 2.5h when each substrate was about 40% degraded, and 19h when much of the gentiobiose had been hydrolysed, indicated that the primary degradation of the glucan was complete in about 10h: the alkali-treated glucan was not degraded faster under these conditions. From these results it appeared that alkali treatment increased the susceptibility of the glucan to the exo-glucanase, but still did not allow its complete degradation. A combination of the two glucanases acted faster than the exo-enzyme and to completion.

At this stage an endo-glucanase preparation from *Cytophaga johnsonii*, a non-fruiting myxobacterium isolated from the root surface of grasses (Webley *et al.*, 1965), was introduced into the experiments. This preparation, like the exo-enzyme fractions from *C. minitans*, had no action on gentiobiose and so made it possible to study the yields of glucose and gentiobiose at different stages of glucan breakdown. When it was used (0.38 unit/ml) with the exo-glucanase (4.3 units/ml) glucose/gentiobiose ratios of 1.8 and 1.9 were obtained for untreated and alkali-treated glucan respectively, degradation being almost complete with each. Alkali-treated glucan (31mg) was incubated in 20ml with the exo-glucanase (43 units) for 1 day at 30°C; degradation reached 50% with a glucose/gentiobiose ratio in the products of 2.2. Glucan (10.2mg) was recovered by ethanol precipitation and dialysis and 4.1mg incubated (in 2ml) with mixed (9 units of exo-, 0.8 unit of endo-) glucanases for 1 day; it was 80% degraded with a product ratio of 2.0:1.

These results show that the portion of the glucan preparation that resists attack by exo-glucanase must be very similar in basic structure to that which does not. The small differences found here in the glucose/gentiobiose ratios are of doubtful significance, because the method measures gentiobiose by difference, and other substances are probably present in most incubations.

The effect of sequential additions was illustrated in Fig. 1 of Bacon (1973). From this it is evident that

the two glucanases must act simultaneously for complete degradation to be achieved. Addition of various amounts of the *C. johnsonii* enzyme (2.2, 1.1 and 0.22 unit/ml) to a fixed amount of the *C. minitans* exo-glucanase (2.9 units/ml) showed that the rate of degradation of untreated glucan was similar for the two larger amounts, but decreased appreciably for the smallest; after 44.7h the degradation achieved was 79%, 81%, 41% respectively, and 26% for the control without endo-glucanase.

The *C. minitans* exo-glucanase, alone or with the addition of the *C. johnsonii* endo-glucanase, was tested on a sample of sclerotan from *Claviceps purpurea*, kindly provided by Dr K. W. Buck. This material was dispersed with some difficulty (cf. Buck *et al.*, 1968) using a glass homogenizer. Degradation was followed for 4 days, and throughout this period the mixed glucanases gave values more than twice those given by the exo-enzyme alone, reaching 70% and 33% breakdown respectively.

Effects of other treatments on the glucan. Neither partial acid hydrolysis, nor dissolving in dimethyl sulphoxide, gave products essentially different in their resistance to the exo-glucanase. Almost the whole of the glucan dissolved in dimethyl sulphoxide and after removal of the solvent by dialysis it was 38% degraded in 30h, against 35% for the starting material. Acid treatment caused a loss of some 35% as diffusible products, but the residue still formed a viscous solution and was degraded by about 40% by exo-glucanase alone, and completely by the mixed enzymes.

Products of the action of a culture filtrate of C. johnsonii. Unfractionated culture filtrates from *C. johnsonii* grown in the presence of yeast cell walls contain several endo-β-(1→3)-glucanases and endo-β-(1→6)-glucanases (Bacon *et al.*, 1970), but they had no more effect on sclerotan than the partly purified endo-enzyme used above. This was the 'non-lytic' enzyme, a typical endo-β-(1→3)-glucanase giving as end products of its action on laminarin mainly laminaribiose and laminaritriose. The 'lytic' enzyme, which gives no products smaller than laminaripentaose [cf. enzymes from *Myrothecium* and *Rhizopus* examined by Chesters & Bull (1963), and from *Arthrobacter* by Doi *et al.* (1971, 1973*a*,*b*)], had no action on sclerotan, nor did the mixed β-(1→6)-glucanases.

Production of reducing sugar by the unfractionated *C. johnsonii* glucanase mixture was greater with alkali-treated glucan than with untreated glucan. Expressed as glucose production, 23% and 15% breakdown respectively was reached after 2 days and 26% and 15% after 3 days; in another experiment untreated glucan gave 18% breakdown after 11 days. Polysaccharide was still present in the solution and the low-molecular-weight products consisted chiefly of glucose and a suspected trisaccharide. The total conversion of glucan into low-molecular-weight products was therefore greater than the reducing power indicated.

The suspected trisaccharide was separated by chromatography on Whatman 3MM paper. It was homogeneous on high-voltage electrophoresis in borate buffer and distinguishable by this means and by paper chromatography from authentic 3^2-β-glucosylgentiobiose or 3^1-β-glucosylgentiobiose provided as acetates by Dr J. R. Turvey (cf. Peat *et al.*, 1958). m_{Glc} values (glucose = 1.00) after electrophoresis in 0.12M-sodium borate buffer (pH10) for 3h, with 5000V applied to a strip of Whatman no. 1 chromatography paper 1m long, were: gentiobiose, 0.65; laminaribiose, 0.56; 3^2-β-glucosylgentiobiose, 0.35; unknown, 0.40. A minor impurity in the 3^1-β-glucosylgentiobiose sample had m_{Glc} 0.39.

The R_F value of the unknown in ethyl acetate–pyridine–water (10:4:3, by vol.) was 0.35, close to that reported for 6^2-β-glucosyl-laminaribiose (Fleming *et al.*, 1967; M. Fleming, personal communication); 3^2- and 3^1-β-glucosylgentiobiose had R_F values respectively of 0.30 and 0.41. In butan-1-ol–acetic acid–water (4:1:5, by vol.) the unknown had an R_F value of 0.13, against 0.11 for 3^2- and 0.25 for 3^1-β-glucosylgentiobiose.

Only after prolonged incubation was there any action of the *C. minitans* exo-β-(1→3)-glucanase on the unknown compound, the products being gentiobiose and glucose. Once formed it would therefore be likely to survive in the glucanase mixture and hence is probably identical with an oligosaccharide seen in the final products of the sequential experiment referred to by Bacon (1973) and also in sclerotan degradations by the mixed endo- and exo-glucanases. Because of its presence all the values for percentage degradation given in this section are approximate and underestimated.

Fractionation of culture fluids from T. viride

The procedures used with *C. minitans* culture filtrates were applied in the expectation that a similar pattern of glucanase activity would emerge, but although four distinct peaks of laminarinase activity were obtained from DEAE-Sephadex no lytic activity was recovered.

In preliminary separations on CM-Sephadex two peaks of laminarinase activity were obtained, both showing evidence of more than one component. Both brought about lysis of cell walls, the second peak being more effective. Some synergism between the two peaks was found with both *S. rolfsii* glucan and cell walls.

An elution gradient produced by addition of 0.1M-Na_2HPO_4 to 100ml of 0.005M-NaH_2PO_4 gave four distinct peaks (Fig. 2), the products of action on laminarin suggesting mainly endo-glucanase in

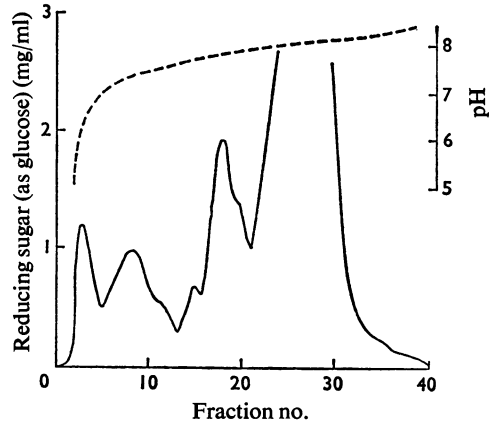

Fig. 2. *Fractionation on CM-Sephadex of β-(1→3)-glucanases in a culture fluid of T. viride*

Material equivalent to 130ml was chromatographed and the fractions tested as described in the Experimental section. Elution was by a gradient produced by addition of 0.1M-Na_2HPO_4 to 100ml of 0.005M-NaH_2PO_4; the pH of the eluent is indicated (---). Reducing sugar was measured after incubation for 23.5h at 30°C with laminarin (——).

the first two. A β-glucosidase was found in the first and fourth peaks, but was absent from the other two. It was tentatively concluded that the results obtained with the first peak were due to a mixture of endo-glucanase and β-glucosidase, and with the third to a mixture of endo- and exo-glucanase, and that the tendency to lose lytic activity was due to the lability of the endo-glucanase. Confirmation was obtained by using the *C. johnsonii* culture filtrate as a source of endo-glucanase free from exo-glucanase.

Acting alone this filtrate abolished the phase-bright appearance of sclerotial cell walls. The middle layer of the cell wall became granulated or was lost completely in places, and the incubation medium became viscous. The same effects were produced by the second *T. viride* peak from CM-Sephadex. Substantial amounts of polysaccharide could be precipitated by addition of ethanol. This material could also be centrifuged down at 230000*g* for 1h, and proved still to have something of the shape of the cell walls from which it was derived. Even after prolonged incubation (up to 11 days) the incubation medium remained viscous.

Addition of *T. viride* exo-glucanase (fourth peak from DEAE-Sephadex) led to a more rapid lysis and a loss of viscosity in the incubation medium. Very little was now precipitated by ethanol and the products included glucose, gentiobiose and the suspected trisaccharide referred to above. Addition of exo-glucanase from *C. minitans* had the same effect. After 1 day with the mixed enzymes the middle layer of the cell wall was no longer visible; some broken outer skins could be seen and some of the inner walls were very thin.

Ultrafiltration of T. viride fractions. In an attempt to avoid inactivation of the *T. viride* endo-glucanase, ultrafiltration was tested as a means of concentrating the culture fluid and the fractions from chromatography. This revealed a further complication arising from the gelatinous nature of sclerotan. Cultures selected when their laminarinase activity was at a maximum still contained glucan. After centrifugation for 0.5h at 16000*g*, the routine procedure, a period at higher speed (1.5h at 190000*g*) brought down from the supernatant fluid a gelatinous pellet with appreciable laminarinase activity (12% of original), and sustained centrifugation at 200000*g* (for 20h) resulted in another 33% of the original activity being precipitated.

When the fractions were tested for glucanase activity, control incubations without laminarin showed considerable increases in reducing power and products consistent with sclerotan breakdown. The supernatant and pellet from centrifugation at 200000*g* were therefore incubated overnight at 20°C and dialysed to remove the degradation products of sclerotan. When the enzymes from the supernatant were concentrated by ultrafiltration through a PM-10 membrane in an Amicon cell (model 52; High Wycombe, Bucks, U.K.) and fractionated on CM-Sephadex only two peaks of laminarinase activity were found. The pellet material was separated to give four peaks resembling those from the original culture fluid.

All the glucanases present in the culture fluid may evidently be adsorbed on sclerotan, and the presence of the glucan probably contributed further to the difficulty of obtaining an endo-glucanase free from exo-glucanase activity.

Lysis of cell walls from S. rolfsii

A concentrated culture fluid from growth of *C. minitans* on sclerotia of *S. sclerotiorum*, which was active *in vitro* against the inner pseudoparenchymatous walls of the sclerotia, was tested on unheated hyphal and sclerotial cell walls from *S. rolfsii*. After 1 day, when the *S. sclerotiorum* cell walls were extensively lysed, the *S. rolfsii* hyphal cell walls were slightly thinned, but the sclerotial cell walls were unaffected. There was no change after a further 1 day's incubation.

Tests for α-(1→3)-glucanase activity

The widespread occurrence of α-(1→3)-glucan has only recently been recognized (cf. Reese *et al.*, 1972). *T. viride* is among the organisms known to produce

an endo-enzyme capable of degrading this glucan (Hasegawa *et al.*, 1969).

We have tested our culture filtrates from *T. viride* and *C. minitans*, and also a commerical enzyme preparation, 'Onozuka cellulase' (All Japan Biochemicals Co. Ltd., Nishinomiya, Japan) said by Reese *et al.* (1972) to contain α-(1→3)-glucanase, on two preparations of α-(1→3)-glucan from the fungus *Polyporus betulinus*, one kindly provided by Dr. E. T. Reese, the other prepared here by fractional extraction with dimethyl sulphoxide.

Release of glucose ceased after breakdown of about 8% of the glucan with the *C. minitans* filtrates and the 'Onozuka cellulase', and less than 2% with the *T. viride* filtrates. That this cessation was not due to inactivation of the enzymes nor to a resistance developed by the insoluble substrate was shown by later additions of enzyme to the test incubations and controls.

It is likely that the glucose was not produced by hydrolysis of the α-(1→3)-glucan, but of β-(1→3)-glucan contaminating the preparations. Small amounts of intermediates could be seen on paper chromatograms, in contrast with the experience of Reese *et al.* (1972) and others who could detect no intermediates during the action of α-(1→3)-glucanases.

Discussion

The main aim was to identify the enzymes by means of which two parasitic fungi lyse the cell walls of *S. sclerotiorum*. This has been achieved in general terms with the discovery that each parasitic fungus produces both chitinase and a β-glucanase mixture capable of attacking sclerotan, the main carbohydrate component of the cell walls. However, the gelatinous nature of sclerotan creates difficulties of interpretation, and as is the case with many studies of this kind one cannot be sure that some minor features of the cell-wall structure are not broken by another type of enzyme altogether.

The i.r. spectrum of the sclerotial cell walls of *S. sclerotiorum* is dominated by sclerotan (Jones *et al.*, 1972). The same spectrum is shown by glucan extracted by autoclaving, as in the present work, or isolated from the culture fluid of *Claviceps purpurea* or *S. rolfsii*. In this state the polysaccharide is degraded to the extent of 35% by exo-β-(1→3)-glucanase from *C. minitans* or *T. viride*. Endo-β-(1→3)-glucanase achieves only about 15% breakdown.

Treatment with alkali removes the distinctive features of the i.r. spectrum, which now resembles that of laminarin and other β-(1→3)-glucans (cf. Bacon *et al.*, 1969; Michell & Scurfield, 1970; Jones *et al.*, 1972). This material is degraded about twice as far as the original glucan, by each enzyme. The action of alkali may not be purely that of a solvent, because glucan dissolved in dimethyl sulphoxide and recovered by dialysis and freeze-drying suffers no change in susceptibility to enzymic attack or in i.r. spectrum.

When the two β-(1→3)-glucanases act together the glucan is completely degraded, whether it has been previously subjected to alkali or not. The main products are glucose and gentiobiose, and these are produced in much the same ratio from the resistant fraction of the glucan as from the susceptible. This confirms the impression received by Johnson *et al.* (1963), who examined the products over the range 10–90% hydrolysis of their *S. rolfsii* glucan by a Basidiomycete glucanase, that in sclerotan one is dealing with a molecule of very regular structure. [This was shown in a different way by the biosynthetic studies of Batra *et al.* (1969).]

From what is known of the mode of action of the two glucanases concerned one would expect that resistance to their attack would arise from quite different aspects of structure. The endo-enzyme, capable of random hydrolysis of internal β-(1→3)-linkages in polysaccharide chains, would be impeded by the glucose side chains, whereas the exo-glucanase, which works along the chain from the non-reducing end and seems to be quite unaffected by the single-residue side groups, could be stopped by an anomalous linkage, perhaps β-(1→6). Co-operation between them could be analogous to that between α- and β-amylases, where the random action of the endo-enzyme α-amylase creates additional non-reducing chain ends for the exo-enzyme β-amylase; if α-amylase is not present, β-amylase is stopped by the α-(1→6)-branch points and a high-molecular-weight dextrin is left.

The unattacked portion of sclerotan after exo-glucanase action could be recovered almost quantitatively by ethanol precipitation, and the endo-glucanase did not lower the viscosity of the glucan appreciably, so in both cases the unattacked fraction was still a high polymer. This would explain the limited effect produced by the enzymes, acting singly, on the appearance of the cell walls.

The synergistic effect seen with our enzyme preparations was unexpected, because in previous studies it had been established independently by Johnson *et al.* (1963) and Buck *et al.* (1968) that the exo-β-(1→3)-glucanase from Basidiomycete QM 806, even when highly purified (Huotari *et al.*, 1968), would degrade sclerotan from several sources to a mixture of glucose and gentiobiose corresponding to 100% of the glucan originally taken. However, an examination of the literature suggests that there may be differences in sclerotan and exo-glucanase preparations from other sources. Thus Ebata & Satomura (1963), whose studies anticipate most of what has been reported subsequently on this topic, used an alkali-treated sclerotan from sclerotia of

S. libertiana and a crystalline exo-glucanase from bran cultures of the same organism. They state that the enzyme, both with laminarin and sclerotan, had an initially rapid rate of reaction (up to 10% hydrolysis with sclerotan), but thereafter acted much more slowly; it achieved 'about 70%' hydrolysis with sclerotan, after 'a long period . . . although the hydrolysis degree attained was dependent to some extent upon the amount of enzyme added'. Perlin & Taber (1963) digested 200mg of sclerotan from *Claviceps purpurea* with 50mg of the Basidiomycete QM 806 exo-glucanase for 18h at 40°C. Glucan (60mg) was recovered by precipitation with 3–4vol. of ethanol, the other products being recovered as glucose (95mg) and gentiobiose (60mg).

There are a few previous reports of the effects of endo-β-(1→3)-glucanases on sclerotan preparations. Satomura *et al.* (1960) found that a *Rhizopus chinensis* endo-glucanase would solubilize the *S. libertiana* sclerotan almost completely, but hydrolysis did not exceed 40%. Perlin & Taber (1963) reported only a small extent of attack on their glucan by a similar preparation. Johnson *et al.* (1963) do not refer to the extent of hydrolysis, but state that the products were glucose, gentiobiose and laminaribiose.

We found very little disaccharide production by endo-glucanase, the chief products being glucose and a trisaccharide which appears to be 6^2-β-glucosyl-laminaribiose, a resistant product of the action of a similar endo-glucanase on soluble laminarin (Fleming *et al.*, 1967).

The studies by Batra *et al.* (1969) on the biosynthesis of sclerotan which established that 'in any given region of the polysaccharide, the interbranch, branch-point and branch residues are inserted at about the same moment in time', taken with analyses by fractionation and partial hydrolysis, emphasise the regularity of structure in sclerotan. One might conclude that the polymer is built up by addition of tetrasaccharide blocks, the main objection to this being some reported divergencies from the glucose/gentiobiose ratio of 2:1 typical of the *S. rolfsii* glucan. The alternative is to assume that insertion of each side group takes place near the growing end of a chain, and is strongly influenced by the position of the previous one.

Assuming that either process could be subject to 'error' it is tempting to speculate that anomalies in the structure could explain (1) the gel-forming capacity of the glucan, (2) its resistance to some exo-glucanases, and (3) its limited susceptibility to endo-glucanase. The variation most likely to fulfil all three conditions is one in which a β-(1→6)-linkage has been inserted into the main β-(1→3)-chain, as in Fig. 3(*a*). This would (1) provide a kink to interrupt helical bonding of the glucan chain with chains from other molecules (Rees, 1969, 1972), (2) stop the exo-glucanase, which requires a terminal β-(1→3)-linked residue, and (3) provide a sequence of unsubstituted residues longer than those in the normal chain, and hence more susceptible to the endo-glucanase. The latter should thus be able to remove the anomalous structure and allow the exo-glucanase to continue its degradative action; in doing so the endo-enzyme might release the trisaccharide 6^2-glucosyl-laminaribiose.

It is also conceivable that this trisaccharide is produced as in Fig. 3(*b*), by attacks on the linkages indicated, because at the branch-point in laminarin these linkages are evidently susceptible to attack. The high frequency of substitution in the sclerotan chain presumably impedes the endo-enzyme greatly, otherwise there seems no reason why the whole molecule should not be degraded in this way. Perhaps some endo-glucanases are less hindered than others.

We were led to the discovery of the co-operative action of the two glucanases by finding a synergistic action of enzyme fractions on cell-wall lysis. These

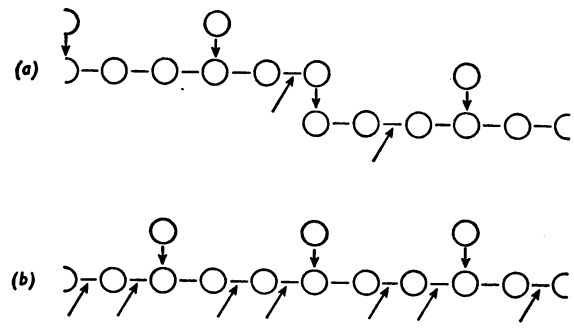

Fig. 3. *Diagrammatic representations of portions of a molecule of sclerotan*

(*a*) Glucan chain with hypothetical 'error'. (*b*) Normal glucan chain. ○, β-glucose residue; ↓, (1→6)-linkage; —, (1→3)-linkage; ↗, point of attack of endo-glucanase.

results illustrate the important part played by glucans in the structure of fungal cell walls and are a further indication of their complexity. It is worth pointing out that sclerotan is not immediately distinguishable from other β-(1→3)-glucans, and may be more widespread in occurrence than one suspects. For instance, it has now been found in *Schizophyllum commune* (Wessels & de Vries, 1973) and so is not restricted to sclerotia-forming fungi.

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