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The Separation of β-Glucanases Produced by *Cytophaga johnsonii* and their Role in the Lysis of Yeast Cell Walls

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1. When *Cytophaga johnsonii* was grown in the presence of suitable inducers the culture fluid was capable of lysing thiol-treated yeast cell walls *in vitro*. 2. Autoclaved or alkali-extracted cells, isolated cell walls and glucan preparations made from them were effective inducers, but living yeast cells or cells killed by minimal heat treatment were not. 3. Chromatographic fractionation of lytic culture fluids showed the presence of two types of endo-β-(1→3)-glucanase and several β-(1→6)-glucanases; the latter may be induced separately by growing the myxobacterium in the presence of lutean. 4. Extensive solubilization of yeast cell walls was obtained only with preparations of one of these glucanases, an endo-β-(1→3)-glucanase producing as end products mainly oligosaccharides having five or more residues. Lysis by the other endo-β-(1→3)-glucanase was incomplete. 5. The β-(1→6)-glucanases produced a uniform thinning of the cell walls, and mannan–peptide was found in the solution. 6. These results, and the actions of the enzyme preparations on a variety of wall-derived preparations made from baker's yeast, are discussed in the light of present conceptions of yeast cell-wall structure.

When the remains of higher plants become incorporated into the soil they are subject to microbial attack, in which fungi play a large part; in fact, dying leaves and stems may have been attacked by fungi before they enter the soil. The subsequent degradation of fungal mycelium has been considered by some authors (see Kononova, 1966) to be an important stage in the formation of the substances typical of soil organic matter.

Until recently little attention has been paid to the composition of fungal cell walls and their susceptibility to enzymic degradation. In beginning an investigation of the abilities of soil organisms to lyse fungal cell walls we therefore thought it advisable to consider the degradation of the cell walls of *Saccharomyces cerevisiae*, which bear some resemblance to those of filamentous fungi, but have been the subject of rather more detailed structural investigations. Our studies have led us to examine some features of the chemistry of the yeast wall (Bacon, Farmer, Jones & Taylor, 1969*a*). In the present paper we refer particularly to the production of wall-lysing enzymes by *Cytophaga johnsonii*, and their action on various preparations from cells of *S. cerevisiae*. Preliminary accounts of part of this work have been published (Bacon, Milne, Taylor & Webley, 1965; Bacon, Gordon & Webley, 1970). The results make it possible to draw a more detailed picture of the structure of the cell wall of baker's yeast than has so far been possible, but it is evident that some features are still elusive.

EXPERIMENTAL

Culture of micro-organisms. Cytophaga johnsonii was isolated from the root surface of grasses (Webley, Duff, Bacon & Farmer, 1965) and maintained on the medium described by Bunt & Rovira (1955). For induction of lytic enzymes the myxobacterium was grown in a basal medium containing mineral salts (Stanier, 1947) and 0.1% Difco peptone, with the addition of the potential inducer at a concentration of 1 mg/ml. Incubation was at 25°C in a shallow, stationary layer of medium, about 60 ml in a 1-litre Pyrex conical culture flask (CX350; A. Gallenkamp and Co. Ltd., London E.C.2, U.K.). The cultures were usually harvested after 3 days by centrifuging at 16000*g* for 30 min. When cell extracts were required cooled suspensions of the cells were treated for 1–2 min in an MSE 60 W ultrasonic generator (Measuring and Scientific Equipment Ltd., London S.W.1, U.K.).

Saccharomyces cerevisiae was obtained as commercial pressed yeast (Distillers Co. Ltd., Glenochil, Clackmannanshire, U.K.). For a few experiments cultures were grown, from cells taken from the interior of a block, on malt extract–yeast extract–glucose–peptone agar or glucose–nutrient agar medium. *Saccharomyces carlsbergensis* and *Saccharomyces fragilis* were obtained from the National Collection of Yeast Cultures, Nutfield, Surrey, U.K., and grown on the same media. The cells were harvested after 5 days at 25°C, washed and suspended in water. If not otherwise stated the term 'yeast' refers to *S. cerevisiae*.

Preparation of cell walls. These were prepared from the *Saccharomyces* species by shaking with glass beads in a Mickle shaker (Crook & Johnston, 1962) or in a Vibrogen Cell-mill (cf. Bacon *et al.* 1969*a*). After being washed with water at 1–5°C they were heated to destroy endogenous glucanases (see below). Their typical appearance is shown in Plate 2(*a*).

Sucrose-density-gradient centrifugation of cell walls. A yeast cell-wall suspension (800mg in 25ml) was pumped at 25ml/min into an MSE type A zonal rotor (Measuring and Scientific Equipment Ltd.; used with Mistral 6L centrifuge) containing a gradient made by mixing 20 and 60% (w/v) sucrose solutions, and displaced from the centre by water. Some material began to move outwards at once, but most of the walls remained as a compact band. The rotor speed was raised from 300 to 1000 rev./min and kept at this speed until most of the material had entered the sucrose gradient. The speed was then lowered to 300 rev./min and the cell walls were recovered as a series of fractions by pumping 60% (w/v) sucrose into the outer zone of the rotor. The whole operation took less than 1h at a rotor temperature of 4°C.

In preliminary experiments several factors, e.g. steepness of gradient, speed of rotation and time of centrifugation, were varied. The separation was watched in the transparent rotor and decisions about the sequence of operations were taken as it progressed. Each fraction was washed with water and examined microscopically.

Gel filtration. Sephadex G-100 [Pharmacia (G.B.) Ltd., London W.5, U.K.] (15g) was suspended in 5mM-Na_2HPO_4 and packed in a glass tube [K25/45; Pharmacia (G.B.) Ltd.] to form a column (25mm × 380mm). The sample was applied in 2–3ml and elution carried out with 5mM-Na_2HPO_4 under gravity, at 24ml/h. The whole operation was conducted at 4°C, and 3ml fractions were collected and stored in the presence of toluene.

Ion-exchange chromatography. DEAE-cellulose (Whatman Microgranular DE32; H. Reeve Angel and Co., London E.C.4, U.K.) (10g) was packed into a glass tube [K15/30; Pharmacia (G.B.) Ltd.] with 10mM-Na_2HPO_4 to form a column (15mm × 250mm) and pumped at 54ml/h until the effluent fluid had the same pH as that applied (pH7.9). The sample, previously dialysed against 10mM-Na_2HPO_4, in about 5ml, was applied and elution begun with a gradient made by adding 200mM-NaH_2PO_4 to 100ml of 10mM-Na_2HPO_4. The whole operation was carried out at 4°C, and the effluent, pumped at 54ml/h, was collected in 3ml fractions.

Measurement of glucanase activity. The substrate, insoluble laminarin or lutean (see Bacon *et al.* 1969*a*), was dissolved by suspending it in water (30mg of polysaccharide/ml), placing the test tube in a beaker of boiling water for exactly 15min and then cooling it in water at 40°C for a few minutes. This procedure was designed to prevent premature precipitation of the substrate. Routine assays were made in AutoAnalyzer cups, with 0.25ml of enzyme solution, 0.25ml of substrate and 0.25ml of buffer (either 50mM-tris–HCl, pH7.5, or 50mM-sodium citrate–phosphate (pH5.0). Thymol was added to prevent bacterial contamination if the incubation was to be prolonged. After a suitable incubation time at 30°C the cups were placed on the AutoAnalyzer sampler module for determination of reducing sugar by the alkaline ferricyanide method of Hoffman (1937), with glucose standards. A unit of enzyme activity is defined as that which liberates in 1h reducing power equivalent to 1μmol of glucose.

Monitoring of effluents from chromatographic columns. An AutoDiluter (Mk II; Hook and Tucker Ltd., Brixton, London S.W.9, U.K.) was used to withdraw 0.3ml from each fraction and expel it with the addition of 0.15ml of buffered substrate (30mg of polysaccharide/ml in one of the buffers mentioned above) into an AutoAnalyzer cup. After incubation overnight at 30°C the cups were placed on the sampler module. The summits of the peaks recorded could then be joined to give the continuous lines reproduced in Figs. 1 and 2.

Chitinase activity. The sample to be tested (0.5ml) was incubated at 30°C overnight with 2mg of colloidal chitin (Hackman & Goldberg, 1964) in a total volume of 1.0ml containing 8mM-sodium citrate–phosphate buffer, pH6.0. The suspension was then centrifuged and *N*-acetylamino sugar determined in the AutoAnalyzer by a slight modification of the method of Swann & Balazs (1965): the sample/wash ratio was decreased to 1:5 and the sampling rate to 20/h; 0.5% *p*-dimethylaminobenzaldehyde was used instead of 1.0%.

Lytic activity. Suspensions of *S. cerevisiae* cell walls were adjusted to a concentration of 16mg of dry matter/ml, determined by freeze-drying 0.25–0.50ml samples. The cell walls (0.2ml in a final volume of 1.0ml) were incubated without agitation at 30°C in small glass-stoppered glass tubes. Buffer was added to maintain the pH at 7.5 (7mM-tris–HCl) or 5.0 (7mM-citrate–phosphate), and 2-mercaptoethanol, when present, was 20mM. Toluene was used as a preservative.

Extensive lysis could be detected through a fall in turbidity, but samples were always examined by phase-contrast microscopy. A very high optical magnification (×2000) was used to confirm the presence of bud-scar residues, which could not always be seen clearly at lower magnifications. Further information was given by electron microscopy of shadowed specimens.

Lysis was also followed by measurements of total carbohydrate by the anthrone method (Fairbairn, 1953) in supernatant fluid and residue after centrifugation at 25000*g* for 30min. The soluble products were examined by paper chromatography. Occasionally macromolecules present were precipitated by addition of ethanol and subsequently hydrolysed with acid.

Paper chromatography. Solvents and spraying reagents were as described by Bacon (1959) and Bacon *et al.* (1969*a*).

Infrared spectra. These were measured on freeze-dried samples in a 12mm KBr disc.

Microscopy. Specimens were washed if necessary to remove salts, dried at 40°C on a Formvar film supported on a copper grid, shadowed with nickel–palladium and examined in a model EM6 electron microscope (A.E.I. Scientific Apparatus Ltd., Harlow, Essex, U.K.).

RESULTS

A culture of *C. johnsonii* grown on the peptone and mineral salts medium has no action on yeast cell walls *in vitro*. If a suspension of living cells of *S. cerevisiae*, *S. carlsbergensis* or *S. fragilis* in a

nutrient medium is inoculated with *C. johnsonii* the bacteria grow well, but no changes are seen in the yeast cells and the culture filtrate again has no lytic activity towards yeast cell walls (Webley, Follett & Taylor, 1967). The same result is found when yeast cells killed by heating at 75°C for 30min in aqueous suspension (hereafter called 'heat-killed cells'), or by treatment with ethyl acetate (cf. Myrbäck, 1957), are used. However, the growing myxobacterium will attack autoclaved cell walls or whole cells, and culture fluids are then capable of lysing unheated cell walls *in vitro* under the conditions described below.

These observations show that some, or perhaps all, of the enzymes needed for lysis of yeast cell walls are inducible, and, incidentally, that the walls have to be degraded to some extent before the inducing agents become accessible to the myxobacterium. These enzymes may therefore be investigated either by physical separation of the proteins in lytic culture fluids, or by attempting to induce them singly by adding purified wall components or related substances to a *Cytophaga* culture. Both approaches have been made and are described below.

It is also possible to incorporate cell walls of other micro-organisms in the cultures, and so, by the enzymes present in the lytic filtrates produced, to detect similarities in their wall composition. Examples of this are given by Webley *et al.* (1967) for *C. johnsonii* and by Jones, Bacon, Farmer & Webley (1968, 1969) for other soil organisms. More information about these organisms and the part they play in the degradation of microbial residues in soil is given by Webley & Jones (1970).

Action of Cytophaga *cultures grown in the presence of autoclaved cell walls.* The action of whole cultures of *C. johnsonii* on unheated yeast cell walls has been described by Bacon *et al.* (1965; Table 1); from this it was apparent that for maximum lysis a thiol, e.g. 2-mercaptoethanol, must be present, or must have been used in a pretreatment of the cell walls. Lysis was more rapid and complete at pH7.5 than at 5.0.

After these results had been published we discovered that the yeast cell walls contained a β-(1→3)-glucanase. In *S. fragilis*, but not *S. carlsbergensis* or *S. cerevisiae*, a glucanase was found to diffuse from whole cells into the suspending medium; this is presumably the exoglucanase described by Brock (1965) and by Abd-el-Al & Phaff (1968). However, wall preparations from *S. cerevisiae* and *S. carlsbergensis* produced oligosaccharides from laminarin and evidently contain an endo-β-(1→3)-glucanase. *S. cerevisiae* cell walls tested on lutean showed no β-(1→6)-glucanase activity. Although there was little evidence of autolytic release of glucose under the conditions of our incubations it seemed advisable to eliminate the β-(1→3)-glucanase in case it should participate in glucan breakdown by exogenous enzymes. In all subsequent experiments every effort was made to shorten the time needed to prepare cell walls (this was usually 25h at 4°C), and the final suspension was heated rapidly to 75°C and held there for 30min before storage at 4°C in the presence of toluene. No evidence was obtained for any differences in the course or degree of lysis as a result of this treatment.

Culture fluids prepared by centrifuging *Cytophaga* cultures at 16000*g* for 30min are as lytic as whole cultures. Ultrasonic disruption of the myxobacterial cells liberates further amounts of the lytic enzymes, but also appreciable amounts of an ineffective glucanase (see below), so for most purposes the fluid was preferred and was used without further manipulation unless purification and separation of the enzymes was intended.

Bacon *et al.* (1965) stated that after incubation at pH7.5 with *Cytophaga* culture and 2-mercaptoethanol the yeast cell walls were no longer present, but their Table 1 shows that the carbohydrate contents of the residues (2.1–2.8mg from 14mg of cell wall) exceeded that of the control *Cytophaga* culture (0.6mg). More careful examination of such residues has now shown that the walls do not completely disappear. The residues are difficult to observe microscopically, possibly because they consist of very thin membranes; occasionally a bud-scar can be seen. The i.r. spectra of such samples indicate that β-(1→3)-glucan is still present, and occasionally there is some absorption attributable to chitin.

The soluble products of lysis include mannan and protein, which may be precipitated by 2 vol. of ethanol. The ethanol-insoluble material from lysis of 128mg of cell wall weighed 65mg; 54mg of this was applied to a column of Sephadex G-200, which was developed with citrate–phosphate buffer, pH6.4, 3ml fractions being collected. The effluent was examined for carbohydrate by the anthrone reagent, and for protein by an automated procedure using the Folin reagent (Bartley & Poulik, 1966). The void volume was 53ml (measured with Blue Dextran) and practically all the protein was found in a sharp peak between fractions 14 (42ml) and 22 (66ml); a large part of the carbohydrate coincided with this (cf. Eddy & Longton, 1969). After ethanol precipitation and dialysis to remove salts, 35mg of dry material was recovered from fractions 14–22. The i.r. spectrum indicated the presence of mannan and peptide, and the products of acid hydrolysis included a little glucose.

In the ethanolic supernatant were present glucose and a series of oligosaccharides, which on hydrolysis yielded only glucose. No free mannose was

ever detected in the incubation mixtures. The oligosaccharides are predominantly of the β-(1→3)-glucan series, laminaritriose being usually the most prominent on paper chromatograms. Smaller amounts of other oligosaccharides include gentiobiose and other compounds with the same colour reaction, but no attempt was made to identify all these substances by rigorous methods. Their production is consistent with the presence of β-(1→3)- and β-(1→6)-glucan structures in the cell walls (see Bacon *et al.* 1969*a*). The chitin content of the walls is so low (about 1%) that the products of its hydrolysis would not easily be seen on paper chromatograms. The culture fluid already contains free amino acids, so it is difficult to detect proteolysis; visual comparison of incubation mixtures with controls on paper chromatograms suggests that little occurs (see also below for lysis by purified glucanase).

No attempt has been made to determine the fate of the wall lipid. Our preparations contain about 2% of easily extractable lipid, the phosphorus content suggesting that a quarter of this could be phospholipid. A further quantity (1–2%) of lipid-like material could be extracted with acid solvents. There is no proof that these substances are derived from the wall; as shown in Plate 2(*c*) a part of the cell contents remains trapped in a few of the cell walls.

Action of Cytophaga *cultures grown in the presence of laminarin.* Since the products of enzymic lysis of cell walls indicated that a β-(1→3)-glucan structure was being degraded, *C. johnsonii* was grown on 'insoluble' laminarin, i.e. the fraction of β-(1→3)-glucan from *Laminaria cloustoni* (*Laminaria hyperborea*; Parke, 1953) that is soluble in water above 80°C but is slowly precipitated at lower temperatures. When autoclaved in the usual nutrient medium (at 1mg/ml) the polysaccharide remained in solution for several days at 28°C, but eventually separated as a deposit on the walls of the culture vessel. Alternatively, 100mg of laminarin was autoclaved dry, suspended in 10ml of ethanol, shaken overnight and the ethanol drained off after centrifugation. The laminarin then dissolved easily in 10ml of water and was shaken gently at room temperature for 2 days, during which time it was reprecipitated. It was then washed five times with 10ml of water. All these operations were carried out aseptically, so that the polysaccharide could then be incorporated into the nutrient medium without heating, and remained insoluble during incubation at 25°C.

The myxobacterium did not always grow well in the presence of laminarin, and the culture filtrates showed great variation in their lytic activities. In more than 20 tests the walls were rarely found to be as completely lysed as by a culture fluid from growth on yeast cell walls; they usually retained about 20% of their original carbohydrate. Microscopic observation showed thinning of the wall, with bud-scars becoming more prominent. The i.r. spectra indicated the presence of β-glucan, with chitin not always easily distinguished.

After the development of methods for its separation, the lytic glucanase component described below was later found to be present in a laminarin-grown culture fluid that had appreciable lytic action.

Action of Cytophaga *cultures grown in the presence of lutean.* Lutean is an extracellular polysaccharide produced by strains of *Penicillium luteum* as its malonyl half-ester, and consists mainly of β-(1→6)-glucan sequences (Anderson, Haworth, Raistrick & Stacey, 1939; Ebert & Zenk, 1967); there is no evidence for or against the presence of small amounts of other linkages. The myxobacterium grew well on a medium in which lutean had been dissolved during autoclaving (it does not dissolve in cold water). These cultures produced a general thinning of the wall and rather more than half the carbohydrate went into solution; comparisons of pH 7.5 with pH 5.0, and of whole culture with culture fluid, gave residues retaining 43, 42, 43 and 43% respectively. An i.r. spectrum showed the wall residue to consist mainly of laminarin-type glucan, with moderate amide absorption and a trace of mannan.

Glucanases in ultrasonic extracts of Cytophaga *cells.* When the organism was grown on laminarin it was noticed that the ultrasonically disintegrated cells contained appreciable β-(1→3)-glucanase activity, but that this was several times greater at pH 7.5 than at pH 5.0, whereas in the culture fluid activities were similar at these values. The ultrasonic extract had little or no effect on yeast cell walls. Cells grown in the basal medium contained negligible β-(1→3)- and β-(1→6)-glucanase activities, but those grown with yeast cell walls yielded a lytic extract with properties similar to those of the culture fluid.

Action of Cytophaga *cultures grown on other carbohydrates.* The organism was grown in the presence of a number of polysaccharides. Some, e.g. cellulose, pectin and agar, which bear no obvious relation to the yeast cell wall, induced no lytic enzymes. Crustacean chitin was not a particularly good substrate for growth and the culture fluid did not attack laminarin or yeast cell walls. A preparation of chitin made by successive acid and alkali treatments of whole yeast cells (cf. Bacon *et al.* 1969*a*) induced the formation of a little β-(1→3)-glucanase, but no β-(1→6)-glucanase activity, and produced a slow lysis of cell walls.

Pachyman, a β-(1→3)-glucan extracted by alkali from *Poria cocos*, was not a good substrate, nor were some preparations of alkali-soluble glucan

Table 1. *Purification of β-glucanases from cultures of* C. johnsonii *grown in the presence of yeast cell walls*

Procedures are described in detail in the text. The values given are drawn from the results of more than 20 purifications. In calculating the percentage recovery of enzyme activity at each stage no account was taken of material removed in testing the fractions at stages (4) and (5) (usually 20%), and other samples removed for various purposes, e.g. of the 3.0ml obtained at stage (3) only 2.0ml was used subsequently (see Fig. 1).

Stage of treatment	Volume (ml)	Glucanase activity at pH5.0 (units/ml)		Recovery of activity (%)	
		On laminarin	On lutean	On laminarin	On lutean
(1) Original culture fluid	267	0.16	0.35	100	100
(2) Concentrated by freeze-drying and redissolving in water	16	2.5	5.1	94	88
(3) Precipitated by $(NH_4)_2SO_4$ to 80% saturation	3	10.3	8.9	73	29
(4) Separation of β-(1→3)-glucanase peak on Sephadex G-100 chromatography	4.9	1.3	1.2	14	6
(5) Separation of non-lytic β-(1→3)-glucanase on DE32 DEAE-cellulose chromatography	4.5	0.9	0.0	9	0

from yeast cell walls, particularly those obtained under conditions where polysaccharide degradation was suspected. However, the organism grew well on glucans extracted with alkali in an atmosphere of N_2, or with dimethyl sulphoxide (see Bacon *et al.* 1969*a*). In these cases both β-(1→3)-glucanase and β-(1→6)-glucanase were produced and the culture fluid lysed cell walls appreciably.

The myxobacterium also utilized laminaribiose and gentiobiose, but the culture fluids did not lyse yeast cell walls, and no glucanases were detected in them.

Separation of glucanases in Cytophaga *culture fluids.* An examination of the action of culture filtrates on laminarin and lutean provided some explanation of the differences noted above. Culture fluids from growth on autoclaved cell walls contained both β-(1→3)-glucanase and β-(1→6)-glucanase activity. Laminarin-grown culture fluids also contained both activities, but usually in smaller amounts. Lutean-grown culture fluids contained a relatively high β-(1→6)-glucanase activity, but hardly any β-(1→3)-glucanase.

No activity towards a mainly β-(1→2)-glucan from *Rhizobium japonicum* (Dedonder & Hassid, 1964) was detectable in a cell-wall-grown culture fluid, and the organism cannot utilize cellulose (Stanier, 1947). It therefore appeared likely that the lytic action was due primarily to β-(1→3)-glucanase or β-(1→6)-glucanase and an attempt was made to separate and purify them.

Both glucanase activities were stable towards concentration by freeze-drying and subsequent ammonium sulphate precipitation (Table 1), although some losses occurred at the latter stage through failure of the precipitate to sediment completely; preferential losses of β-(1→6)-glucanase activity were tolerated when purification of the β-(1→3)-glucanases was the main aim. On a column of Sephadex G-100 the β-(1→3)-glucanase was divided into three fractions (Fig. 1), one emerging with the void volume (region A), another with the ammonium sulphate (region C) and the third between them (region B); β-(1→6)-glucanase was present in regions B and C but not in region A.

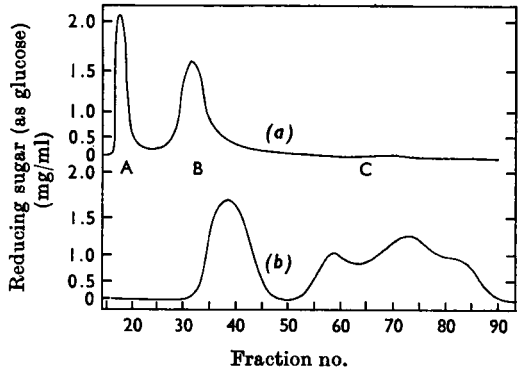


Fig. 1. Fractionation of glucanases on Sephadex G-100. The sample, 2.0ml from stage (3) of the purification scheme (Table 1), was chromatographed and the fractions tested as described in the Experimental section. Reducing sugar was measured after incubation with (*a*) laminarin for 20h [β-(1→3)-glucanase] and (*b*) lutean for 22h [β-(1→6)-glucanase].

The β-(1→3)-glucanase activity in region C was always low, and with some culture fluids there was practically no activity in region A, so attention was directed to region B, where there was some

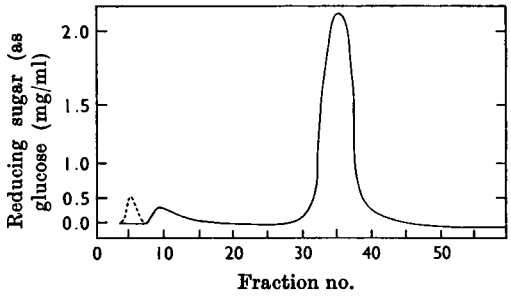


Fig. 2. Fractionation of glucanases on DE32 DEAE-cellulose. The sample, 4.0ml from stage (4) of the purification scheme (Table 1), was chromatographed and the fractions tested as described in the Experimental section. Reducing sugar was measured after incubation for 20h with lutean [β-(1→6)-glucanase] (----) and with laminarin [β-(1→3)-glucanase] (——).

overlap between broad peaks of β-(1→3)-glucanase and β-(1→6)-glucanase activity. The earlier fractions, richer in β-(1→3)-glucanase, were combined and separated on DEAE-cellulose (Fig. 2). The β-(1→6)-glucanase emerged very early and after a considerable interval a large peak of β-(1→3)-glucanase was eluted. Several preparations of the latter were made, free from β-(1→6)-glucanase, but the β-(1→6)-glucanase obtained from these columns always had a little β-(1→3)-glucanase activity. This enzyme was therefore more conveniently prepared from culture fluids obtained by growth on lutean, in which the β-(1→3)-glucanase activity was very low and could be eliminated completely at the DEAE-cellulose stage, giving a similar overall yield of activity of about 10%.

Several preparations of the β-(1→3)-glucanase were tested on yeast cell walls, but were found to have a very limited action (see below); this was not intensified by the addition of the purified β-(1→6)-glucanase. The initial separation on Sephadex was therefore re-examined and it was found that the later-emerging part of region B was lytic. This mixture was resolved on DEAE-cellulose and the fractions immediately after the β-(1→6)-glucose peak were examined (cf. fractions 10–25 in Fig. 2), although they showed little activity towards either laminarin or lutean. When combined and concentrated they proved to have some β-(1→3)-glucanase, but very little β-(1→6)-glucanase, and were actively lytic. Later fractions showed a β-(1→3)-glucanase peak, occupying a similar position to the first β-(1→3)-glucanase to be purified. As expected, this did not produce lysis.

A re-examination of the early part of region B gave a similar result, and fractionation of a culture fluid in the reverse order, i.e. first on DEAE-cellulose and then on Sephadex, indicated that the lytic β-(1→3)-glucanase was distributed in the Sephadex G-100 column effluent in much the same way as the non-lytic enzyme. All β-(1→3)-glucanase fractions from region B were therefore mixtures, which explained why an examination of the products of their action on laminarin had revealed no differences. However, the fractions from DEAE-cellulose were found to differ greatly in this respect, the early fractions, corresponding to the lytic enzyme, producing only higher oligosaccharides, whereas the later fractions produced glucose, laminaribiose and laminaritriose, and relatively little reducing material of smaller R_F value. An examination of the products by paper chromatography thus made it possible to detect the point at which the non-lytic glucanase began to emerge from the column. Those early fractions that contained a little luteanase were excluded and so the lytic β-(1→3)-glucanase was obtained free from both. The chitinase activity of this fraction was tested and found to be negligible in some preparations and very weak in others. The proteolytic activity, on urea-denatured haemoglobin at pH 7.5 (Anson, 1939), solubilized only 0.01μmol/ml of tyrosine equivalents in 20h; under the same conditions an unfractionated culture fluid solubilized 0.26μmol/ml.

Action of the lytic β-(1→3)-glucanase. (*a*) Yeast cell walls. The absence of chitinase activity from the lytic enzyme suggested that the completeness of dissolution of the cell wall would vary according to the chitin content. This was confirmed by the accumulation of debris identifiable as bud-scar residues, and to this extent lysis by the enzyme preparation was less complete than that by unfractionated culture fluids. The solubilization of wall material produced a progressive thinning of the walls; the final residue (Plate 1*a* and 1*b*) contained some of the membranous structures referred to above. The remains of bud-scars differed from the chemically prepared residues illustrated by Bacon, Davidson, Jones & Taylor (1966) and those produced by rupture of spheroplasts (Bacon, Jones & Ottolenghi, 1969*b*) in that the region outside the crater was much more eroded and in some cases the crater rim itself had been lost, leaving a disc studded with granules. The presumption (cf. Houwink & Kreger, 1953) is that these granules are chitin.

In the hope that the lytic process would be better defined if the walls were more uniform, some experiments were carried out by sucrose-density-gradient centrifugation in a type A low-speed zonal rotor (Anderson, 1966). Our first expectation (cf. Lieblová, Beran & Streiblová, 1964) was that the walls could be fractionated according to the number

of bud-scars they carried. Other factors evidently operate, because walls with ten or more bud-scars could be recovered from various points in the gradient, but an increased uniformity of appearance was discernible in some of the fractions. Walls that were more phase-dark and often distorted, or still holding some cell contents, tended to accumulate in the faster-moving fractions, whereas those nearer the centre of the rotor tended to be more rounded and freer of contents. A fraction of the latter kind was lysed almost completely in about 20h, leaving only a very few recognizable cell walls, some of them with many bud-scars; the smaller debris consisted chiefly of isolated bud-scars. Paper chromatography showed that in addition to oligosaccharides barely moving off the base-line there were small amounts of laminaritriose, laminaribiose and glucose.

(*b*) Living yeast. A solution of the lytic enzyme was subjected to ultrafiltration [pore size 0.3μm; Millipore (U.K.) Ltd., Wembley, Middx., U.K.] and added with aseptic precautions to suspensions in 1.0M-sorbitol, with and without 1mM-2-mercaptoethanol, of cells taken from the middle of a block of pressed yeast. Growth of bacterial contaminants was not evident until the second day of incubation at 30°C.

No effect of the enzyme on the yeast wall could be seen, and there was no indication that spheroplasts were being formed. A similar experiment was carried out with actively growing cells from glucose nutrient broth, with the same result.

(*c*) Heat-killed yeast. The lytic enzyme had a slow but noticeable effect on the walls of yeast cells killed by treatment at 75°C for 30min. The action in budding cells was first revealed after 2 days. The bud wall was detached as a cap-shaped structure (Plate 2*c* and 2*d*). The open end, corresponding to the original point of attachment to the wall of the mother cell, was well defined, suggesting that the enzyme had attacked a narrow zone of weakness, perhaps at the point of constriction. The coagulated contents of the bud were thus exposed, and later became detached from the mother cell. The incubation was continued and there was a progressive degradation of the walls, though this was not complete even after 15 days.

(*d*) Autoclaved yeast. The lytic enzyme had a more drastic effect on autoclaved cells, large fragments of wall soon appearing in the suspension. Eventually these were degraded or dissolved and the coagulated protoplast disintegrated, in striking contrast with the heat-killed cells, which retained their rounded appearance even when the incubations were prolonged for many days.

(*e*) Wall 'hydroglucan' preparations. Preparations of (i) yeast cell walls and (ii) alkali-extracted whole yeast (Plate 1*e*) were boiled with 0.5M-hydrochloric acid for 1.5h. The residues (cf. Plate 1*c*), which still retained the shape of the cell and carried bud-scar markings, were incubated with the enzyme, each at a concentration equivalent to that of cell walls in the standard test of lytic activity. The fibrillar wall residues were still evident after incubation for 3 days at 30°C (Plate 1*d* and 1*f*) and determinations by the anthrone method showed that only (i) 44% and (ii) 28% respectively of the carbohydrate had been solubilized. In contrast the yeast cell walls were lysed completely in 1 day, and the wall was removed from the alkali-extracted yeast in 2h.

(*f*) Soluble glucan preparations from cell walls. A water-insoluble glucan prepared by extraction of alkali-extracted yeast with dimethyl sulphoxide (see Bacon *et al.* 1969*a*: p. 560, first column) was quickly solubilized by the lytic enzyme at pH7.5 or 5.0, and paper chromatography showed the production first of higher oligosaccharides, and later of smaller amounts of lower oligosaccharides and glucose.

The 'hydroglucan' prepared from this glucan (illustrated by Bacon *et al.* 1969*a*; Plate 1*a*) was tested similarly; both preparations had been stored in the freeze-dried state. After 10 days a part of the material was still insoluble. The degradation products included a little glucose and lower oligosaccharides.

(*g*) Insoluble laminarin. When the lytic enzyme was tested on a sample of insoluble laminarin obtained from the Seaweed Research Institute in 1955, through the courtesy of Dr E. T. Dewar, reducing power, measured with the alkaline ferricyanide reagent in the AutoAnalyzer, was liberated rapidly, but to a very limited extent. For this reason the enzyme was at first ignored, and even when its lytic properties were discovered it was at first thought of as a minor fraction similar to the main endoglucanase. In fact, their activities cannot be directly compared.

The AutoAnalyzer method, which includes a dialysis step, indicates the liberation of up to 0.31mg of reducing power (as glucose) from 8.5mg of glucan in the laminarin preparation; this would correspond to the breaking of one glycosidic bond in 25. Determinations by the Nelson (1944) and Somogyi (1945) method give higher values, particularly when incubations are prolonged, and paper chromatography shows little material remaining at the origin. In this case the reduction corresponded to one in seven glycosidic bonds broken.

A similar action was found on Sample 1 of insoluble laminarin used by Annan, Hirst & Manners (1965), which from its average degree of polymerization of 24 and chain length of 19 contains about 70% of linear molecules.

If the insoluble laminarin was added in the solid state to a solution of the lytic enzyme in buffer, action was slow and much of the polysaccharide remained insoluble after incubation for 10 days. Paper chromatography showed that some was being degraded to the degree described above.

(*h*) Curdlan. A sample (5mg) obtained from Professor T. Harada of Osaka University was suspended in a dilute buffer solution, pH7.5, and heated in a boiling-water bath. It swelled to form the gel characteristic of this polysaccharide, which Saito, Misaki & Harada (1968) have shown to be a predominantly linear β-(1 → 3)-glucan, with an average degree of polymerization of 453.

The gel was mixed with a solution of the lytic enzyme and incubated at 30°C. After 2 days 53% of the polysaccharide was still insoluble, but paper chromatography showed that some degradation to oligosaccharide had occurred. The insoluble material was freeze-dried and again swollen by heating with buffer solution; a further production of oligosaccharides then occurred on incubation with the lytic enzyme, but some material still remained insoluble.

In a second test curdlan (5mg) was dissolved in 0.5M-sodium hydroxide at room temperature and reprecipitated by acidification to pH4 with 1M-hydrochloric acid. The resulting gel was washed with water and incubated with the lytic enzyme at pH7.5. Very slight action was detected by paper chromatography after 6 days; the enzyme was still active when tested on laminarin. The gel was therefore dehydrated with ethanol and finally dried by the use of acetone and light petroleum. The dry product was swollen with dilute buffer at 100°C and incubated with lytic enzyme. It now behaved like the original material, i.e. part remained insoluble, but oligosaccharides were produced from the portion that dissolved.

The degradation products of curdlan included appreciable amounts of material with a degree of polymerization about 5–6, but also small amounts of lower oligosaccharide and traces of glucose.

Action of the non-lytic β-(1→3)-*glucanase*. This is the β-(1→3)-glucanase referred to by Bacon *et al.* (1969*a*; Table 1).

Yeast cell walls incubated at pH7.5 with non-lytic β-(1→3)-glucanase equivalent to about 10 times the laminarinase activity of a typical culture fluid from growth on yeast cell walls showed signs of uneven attack after several days (Plate 2*b*), but only about half the carbohydrate was solubilized. Three separate experiments gave values of (i) 37 and 43% at 10 and 19 days, (ii) 52 and 51% at 5 and 12 days, and (iii) 47% at 6 days. In experiment (iii) the residue represented 58% in terms of dry matter, and on hydrolysis yielded 20% mannose and 43% glucose.

Similar incubations were made with yeast cell walls that had been autoclaved in water for 15min at 15lb/in^2, washed and autoclaved again in citrate–phosphate buffer, pH7.0. The autoclaving removed 10% of the wall dry matter, but did not increase the susceptibility to enzymic attack.

When autoclaved whole cells were treated similarly, after several days thin skins could be seen floating off some of the coagulated cells, but others appeared to be little affected. After 15 days more enzyme was added, but no further changes were seen.

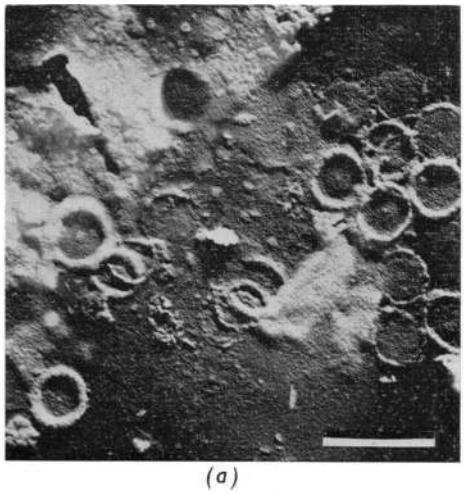
The products of action on cell walls included laminaribiose and glucose, with smaller amounts of higher oligosaccharides. When acting on laminarin the enzyme produced glucose and a series of oligosaccharides in which laminaritriose predominated. Prolonged incubations yielded mainly the mono-, di- and tri-saccharides.

Action of the β-(1→6)-*glucanases*. In early experiments it was not realized that a considerable part of the β-(1→6)-glucanase activity emerged from the Sephadex G-100 column after ammonium sulphate. The preparations made at that time therefore represented the β-(1→6)-glucanase component that accompanies the non-lytic β-(1→3)-glucanase. This fraction was purified from cultures grown in the presence of lutean, which contained negligible amounts of β-(1→3)-glucanase. Some preparations were used straight from the Sephadex G-100 column, others after a further separation on DEAE-cellulose.

Their action on yeast cell walls resembled that of the unfractionated culture fluid. Considerable thinning took place and wall-shaped residues carrying bud-scar markings were left (Plate 2*e*); even after prolonged incubation (e.g. for 19 days at 30°C) these still contained 42% of the anthrone-

EXPLANATION OF PLATE 1

(*a*) and (*b*) Residues of yeast cell walls incubated with lytic β-(1→3)-glucanase at pH7.5 in the presence of thiol for 2 days. The residues included many smooth discs similar to that shown in (*b*), larger than a typical bud-scar. (*c*) Hydroglucan prepared from yeast cell walls, incubated at pH7.5 in the presence of thiol for 3 days. (*d*) As (*c*) but with the addition of the lytic β-(1→3)-glucanase. (*e*) Alkali-extracted yeast cells. (*f*) Hydroglucan prepared from (*e*) and incubated with the lytic β-(1→3)-glucanase at pH7.5 in the presence of thiol for 2 days. All specimens were shadowed with nickel–palladium before examination in the electron microscope. Magnifications are indicated by the bars: (*a*) and (*e*) 2μm; (*b*), (*c*), (*d*) and (*f*) 1μm.



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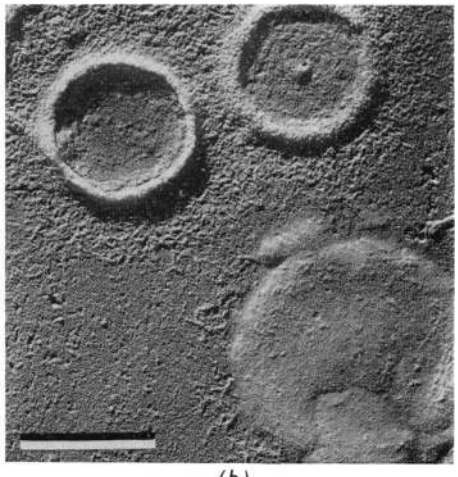

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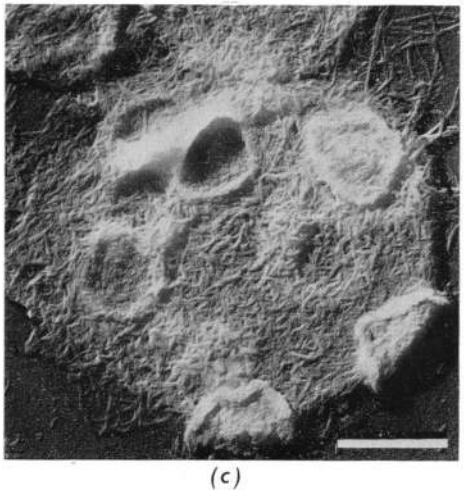

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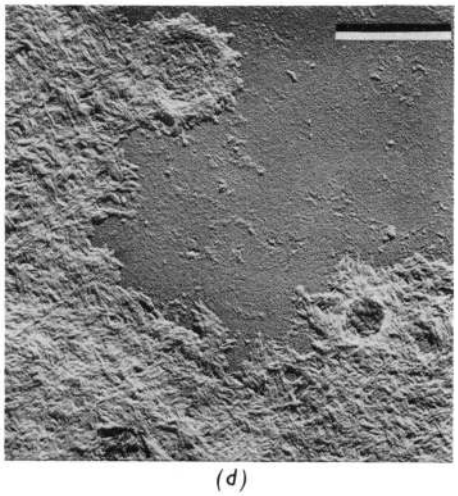

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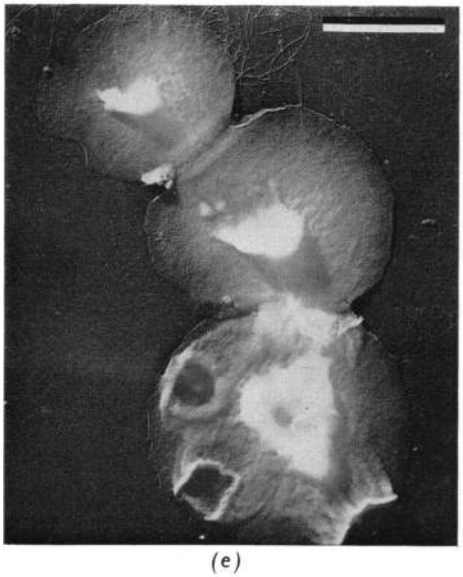
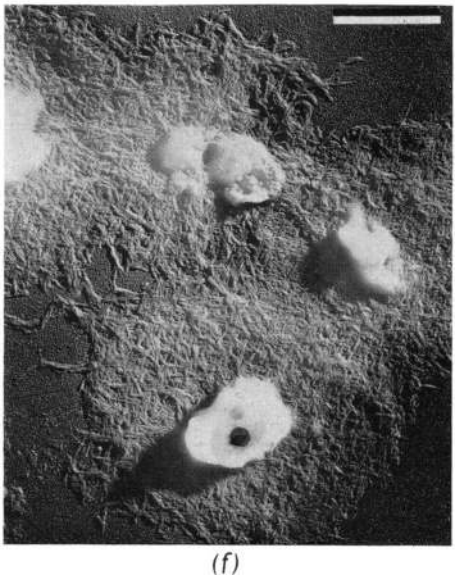
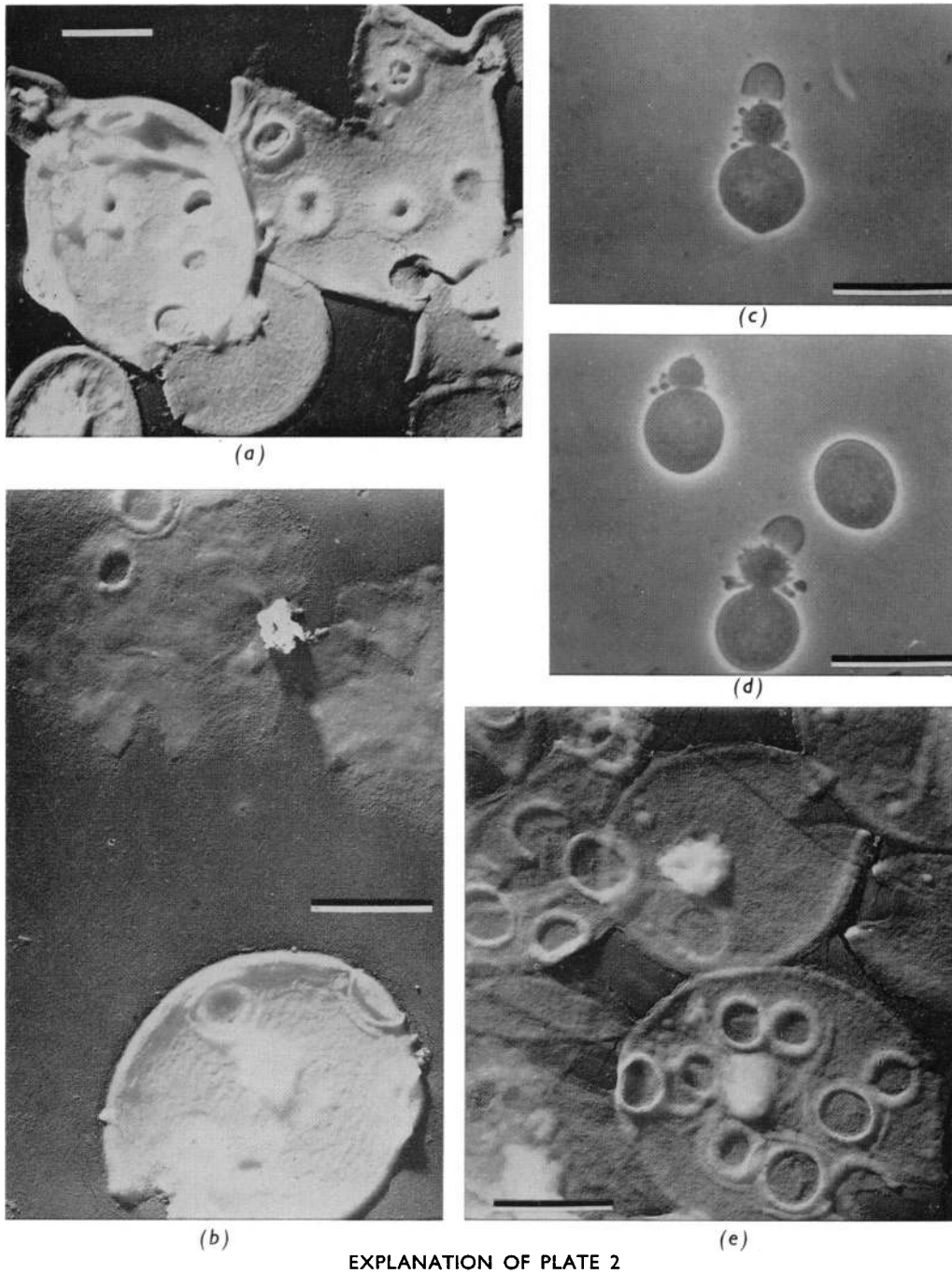


(e)



(f)



EXPLANATION OF PLATE 2

(*a*) Yeast cell walls incubated at pH 7.5 in the presence of thiol (control on sample in Plate 1*a* and 1*b*). (*b*) Yeast cell walls incubated with non-lytic β-(1→3)-glucanase, at pH 7.5 in presence of thiol, for 11 days: the field typifies extremes of the uneven attack, one cell wall being hardly altered, others extensively lysed. (*c*) and (*d*) Heat-killed yeast cells incubated with lytic β-(1→3)-glucanase at pH 5.0 in the presence of thiol for 2 days. (*e*) Yeast cell walls incubated with β-(1→6)-glucanase (purified on Sephadex G-100 only), at pH 7.5 in presence of thiol, for 12 days; the walls are very thin, and some cell contents can be seen in three of them. (*a*), (*b*) and (*e*) are electron micrographs of samples shadowed with nickel–palladium. (*c*) and (*d*) were photographed by phase-contrast microscopy by Mr M. S. Davidson. Magnifications are indicated by the bars: (*a*), (*b*) and (*e*) 2 μm; (*c*) and (*d*) 10 μm.

determined carbohydrate. Hydrolysis yielded only glucose.

The soluble products included glucose, gentiobiose and smaller amounts of laminaribiose and higher oligosaccharides. There was no evidence for proteolysis. Material precipitated by 2 vol. of ethanol had the i.r. spectrum of mannan–peptide.

DISCUSSION

Our earliest experiments showed that growing cultures of *C. johnsonii* would lyse yeast cell walls added to solid or liquid nutrient media, but attempts to obtain lysis of cell-wall suspensions with cells or culture fluids failed. Although puzzling at the time this is now seen to be due to the fact that the cell walls in the nutrient media had been autoclaved. An alternative method of rendering cell-wall preparations susceptible to lysis is by treatment with a thiol, and this was used rather than autoclaving, although a minimal heat treatment was later found to be necessary to inactivate endogenous enzymes. Davies & Elvin (1964) showed that certain effects of thiol treatment on yeast cell walls were demonstrable at pH 7.5, but not at pH 5.0, and so we have used both pH values, in case some significant differences in lytic action should be revealed. Most of the β-glucanases of the myxobacterium are almost equally active at these two values, in contrast with many other microbial glucanases (e.g. fungal laminarinases; Chesters & Bull, 1963*b*) that are much more active at about pH 5.0.

Knowing the chemical complexity of the yeast cell wall, we thought it likely that more than one enzyme in the *Cytophaga* culture filtrate would be needed for lysis, and this impression was deepened when we found that most cultures grown on insoluble laminarin failed to achieve more than a partial degradation of walls. The purification of the main component of the β-(1→3)-glucanase activity afforded further support to this idea, because it also had a limited action on walls.

Attention was therefore directed towards the β-(1→6)-glucanase (cf. Anderson & Millbank, 1966). Fortunately culture fluids grown on lutean were used to provide this, instead of the early fractions from DEAE-cellulose (Fig. 2), which are now known to contain the lytic β-(1→3)-glucanase. The addition of large amounts of β-(1→6)-glucanase activity to the non-lytic β-(1→3)-glucanase did not enhance its lytic capacity. However, the β-(1→6)-glucanase was found to have a well-defined but limited action of its own on cell walls. As H. Tanaka observed with a *Bacillus circulans* enzyme (briefly referred to by Phaff, 1963) the products include oligosaccharides of the β-(1→6)-glucan series, and it is now known (Bacon & Farmer, 1968; Manners & Masson, 1969; Bacon *et al.* 1969*a*) that it is acting on a separate β-(1→6)-glucan component of the cell wall.

Although *C. johnsonii* was described by Stanier (1947) as a chitin decomposer, and our isolate grows well on this substrate, the chitinase activity of the culture fluids from growth on yeast cell walls is low. The addition of a commercial chitinase preparation (Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.) to the non-lytic β-(1→3)-glucanase gave no dramatic increase in lytic action, and where lysis was observed in prolonged incubations with large amounts of addition it was also seen in controls with the chitinase alone. This preparation contained small amounts of β-(1→3)-glucanase (cf. Bacon *et al.* 1969*a*).

A re-examination of the products of chromatography on Sephadex G-100 and DE32 DEAE-cellulose eventually led to the discovery of an enzyme fraction, acting on insoluble laminarin and other β-(1→3)-glucans and hence tentatively called a β-(1→3)-glucanase, that would bring about rapid lysis of thiol-treated cell walls at both pH 7.5 and pH 5.0. The exact specificity of this enzyme remains to be established, but from its action on insoluble laminarin and curdlan it appears to be an endoglucanase acting only on long β-(1→3)-glucan chains, and thus resembles components C and D of laminarinases from *Myrothecium verrucaria* and *Rhizopus nodosus* described by Chesters & Bull (1963*a*), which produced only oligosaccharides with degree of polymerization greater than 5 from laminarin (Bull & Chesters, 1966). This specificity cannot be fully reconciled with the appearance of some lower oligosaccharides and even glucose during its action on yeast cell walls, or on glucan extracted from them, though even in these cases there is a predominance of higher oligosaccharide. This glucanase is much more effective in solubilizing extracted glucan, or the walls of alkali-extracted yeast, than the other endo-β-(1→3)-glucanase. This would provide a basis for the lytic ability of the preparations that contain it, but the possibility still exists that some other essential, but so far unidentified, enzyme is present. We could detect no action on the *Hansenula holstii* phosphomannan (cf. Slodki, 1962; McLellan & Lampen, 1968; McLellan, McDaniel & Lampen, 1970).

The lytic enzyme preparation has been tested on living yeast cells and several preparations derived from them, with the idea of elucidating some structural features of the cell wall.

We have for a long time sought to explain the resistance of the living yeast cell to myxobacterial action. There are certain difficulties in establishing a system in which this resistance can be rigorously defined; for example, it is not possible to grow *Cytophaga* in the presence of mercaptoethanol, nor is it easy to secure simultaneous growth of both

organisms. A similar resistance to lysis is shown by yeast cells killed by a minimal heat treatment ('heat-killed cells'). In both cases the myxobacterium, although it grows, fails to produce any β-glucanases, and it is tempting to conclude that in its natural state the yeast cell wall has no glucan exposed on its outer surface.

If the lytic β-(1→3)-glucanase is added to living yeast cells in the absence or presence of a thiol no action on their walls can be detected, but heat-killed cells in the presence of thiol are attacked to a certain extent, the most susceptible point seeming to be near the junction between mother and daughter cells. From experiments with fluorescent antibody, Chung, Hawirko & Isaac (1965) have suggested that the new cell wall is laid down in an annular band close to the base of the bud. The wall of the bud is detached (Plate 2*c* and 2*d*), apparently along a well-defined line, and floats off. The walls of both cells must then be exposed to attack from within, and remnants of wall carrying bud-scar markings become detached. These must, of course, have been derived from the older multiparous cells.

Yeast cells that have been killed by treatment with ethyl acetate also resist attack in growing *Cytophaga* cultures, and no β-glucanases are produced. The lytic enzyme attacks them in the same manner, but rather less effectively than it does heat-killed cells, and many cells seem to be unattacked. These experiments were also carried out in the presence of thiol, in conditions under which isolated cell walls are fully susceptible. If, therefore, the barrier to lytic action in intact cells is still present in isolated cell walls, it can be circumvented. Perhaps the breaking of the continuity of the wall has permitted the enzyme to act on the wall from the inside where there is no comparable protective layer. The susceptibility of some heat-killed or ethyl acetate-treated cells may arise from a point of weakness where budding is taking place, or occasionally from accidental damage to the wall.

Although the lytic glucanase may not be able to dismantle the walls of living or ethyl acetate-killed cells to the point of lysis, we have some evidence from the escape of invertase (β-fructofuranosidase) (J. S. D. Bacon & A. H. Gordon, unpublished work) that it may be altering its permeability, presumably by solubilizing part of the glucan component.

In a previous paper (Bacon *et al.* 1969*a*) the existence near the outer surface of the wall of a semipermeable membrane composed of a more alkali-resistant glucan fraction and chitin was postulated. Numerous observations of the lysis of walls by the lytic enzyme give limited support to this idea. At an advanced stage of lysis well-formed cell shapes can still be seen with phase-contrast microscopy, but when shadowed with nickel–palladium and examined in the electron microscope they prove to be very thin. The presence of several bud-scars seems to protect the membrane between them, and the only evidence for any persistence of a membrane after prolonged enzyme treatment is given by cell shapes carrying ten or more bud-scar residues. Isolated bud-scar residues can sometimes be seen tumbling freely in the medium, suggesting that they no longer have any wall material attached to them.

One must conclude that protection from glucanase action is given mainly around the bud-scar region (cf. the electron micrographs of wall residues after snail digestive-juice action; Bacon *et al.* 1969*b*), and hence presumably by the presence of chitin. Whether the smooth discs accompanying the typical crater-like bud-scar residues (Plate 1*b*) are also derived from bud-scars or birth-scars cannot be decided on the evidence available. A continuing obstacle to the chemical characterization of all these wall residues is the contamination of the original wall preparations with materials derived from the protoplast. The use of low-speed zonal centrifugation may help in this connexion.

Some of our observations are compatible with the model of wall structure proposed by Lampen (1968). For example, the absence of any induction of glucanase when the myxobacterium grows in the presence of living or heat-killed yeast and the failure of the lytic glucanase to make a general attack on the walls of living or heat-killed cells would both be explained by the presence of an outer layer composed only of mannan. If during wall growth glucan is the first component to be inserted and is only later covered by mannan, this could explain the susceptibility of the bud wall near its point of origin. Other features of the model are not acceptable.

If the mannan layer is held together solely by phosphodiester linkages, how can the wall be solubilized by a purified glucanase? Even in the absence of thiol treatment part of the mannan–peptide material is released by glucanase action, suggesting that it is either trapped by, or covalently linked to, glucan. Invertase is released by the action of thiols at pH 7.5 without any general release of mannan (cf. Davies, 1967). We therefore continue to visualize the mannan component of the wall as being held in place by several types of cross-linkages, which could include phosphodiester linkages, disulphide bridges between the peptide moieties and combination with glucan through attachment to the same polypeptide chain.

The mannan and glucan components are unlikely to be segregated as completely as Lampen (1968) proposes. If this were so one would not expect such marked effects of each on the removal of the other. Unless thiol is added to the system the lytic glucanase fails to attack all the glucan and only

part of the mannan is released, suggesting that the mannan–peptide components are so closely associated with the glucan that the enzyme is prevented from reaching it. [This interpretation is made more plausible by the discovery that the lytic enzyme acts only on long β-(1→3)-glucan sequences.] The thiol, by breaking linkages somewhere in the mannan–peptide fraction, relaxes the structure sufficiently to allow the enzyme to enter. It remains an open question whether the subsequent solubilization of the mannan–peptide is due simply to the disintegration of an entrapping meshwork of glucan, or to the removal of glucan chains from glucan–mannan–peptide complexes.

We have already discussed (Bacon *et al.* 1969*a*) some implications of the insolubility of the wall glucans. Here we must add that, like Bowden & Hodgson (1970), we can see little evidence to support the frequent references to glucan fibrils in the yeast cell wall (cf. Matile, Moor & Robinow, 1969). Lampen (1968) refers to a 'lattice' of glucan fibrils. The linking of glucose residues through glycosidic bonds cannot give a network, only a branched structure; so to form a net it would be necessary for some other form of covalent linkage to be present, or for hydrogen-bonding to occur between linear portions of several branched molecules. The latter structure, a meshwork with fibrillar regions, has some attractions; for example, it would easily be demolished by enzymic attack on the non-crystalline regions. The 'hydroglucan' fibrils seen in acid-treated wall are resistant to attack by the myxobacterial glucanase and so cannot be present in the unmodified wall. A study of the molecular structure of hydroglucan, which dissolves readily in aqueous alkali and is precipitated in the form of fibrils on acidification of the solution, should help considerably in our understanding of the structure of the native glucans.

Since the lytic enzyme will apparently deal only with sequences of ten or more residues its action is likely to be inhibited by close packing of glucan chains; this is confirmed by the slowness of its action on undissolved laminarin and on curdlan. One can therefore visualize considerable variations in the resistance of β-(1→3)-glucans to lysis, depending on their chain length and degree of branching. Such variations could account for the presence of resistant regions in the *Saccharomyces* cell wall, and the varying resistance of other fungal glucans to attack by the *Cytophaga* enzymes.

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