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Exo-β-glucanases in Yeast

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1. A number of yeast species were examined for the presence of β-glucanases. Extracts obtained by cell disruption of *Saccharomyces cerevisiae*, *Fabospora fragilis* and *Hansenula anomala* hydrolysed laminarin and pustulan with the production of glucose. Enzymic activities were also detected in the culture fluids of *F. fragilis* and *H. anomala* grown aerobically in buffered mineral medium with glucose as the carbon source. 2. *F. fragilis* and *H. anomala* possessed approximately sevenfold higher β-(1→3)-glucanase activity than *S. cerevisiae*. 3. Intracellular exo-β-glucanase from baker's yeast was purified 344-fold from the dialysed cell extract. 4. Exo-β-glucanase from *F. fragilis* was purified 114-fold from the dialysed culture fluid and 423-fold from the dialysed intracellular extract. The purified extracellular and intracellular enzymes had similar properties and essentially the same specific activity, 79 enzyme units/mg. of protein. 5. Extracellular exo-β-glucanase of *H. anomala* was purified 600-fold. 6. The optimum pH of the enzymes from *F. fragilis*, *S. cerevisiae* and *H. anomala* was 5·5 in each case. Chromatographic evidence indicated that the three enzymes remove glucosyl units sequentially from laminarin as well as pustulan. 7. The ratio of activities towards laminarin and pustulan remained constant during purification of the exo-β-glucanase obtained from the three species, suggesting a single enzyme. Additional evidence for its unienzymic nature are: (i) the two activities were destroyed at exactly the same rate on heating of the purified enzyme from *F. fragilis* at three different temperatures; (ii) the competitive inhibitor glucono-δ-lactone gave the same value of K_i when tested with either substrate; (iii) quantitative application of the 'mixed-substrate' method with the purified enzyme of *S. cerevisiae* gave data that were in excellent agreement with those calculated on the assumption of a single enzyme. 8. The purified exo-β-glucanases of the different species of yeast had different kinetic constants. The ratios of maximal velocities and K_m values with laminarin and pustulan differed markedly. Comparison of $V_{max.}$ and K_m values suggests that the rapid release of spores from asci in *F. fragilis* might be explained in terms of an enzyme with higher maximal velocity and higher affinity to the ascus wall than that present in baker's yeast. 9. The estimated molecular weights for exo-β-glucanases from *F. fragilis*, *S. cerevisiae* and *H. anomala* were 22000, 40000 and 30000 respectively.

Though β-glucanases have been studied extensively in bacteria and fungi (Reese, 1963; Tanaka & Phaff, 1965; Bull & Chesters, 1966), their occurrence and function in yeast have received relatively little attention. The presence in yeast of an enzyme system capable of hydrolysing the β-(1→3)-glucosidic linkage was inferred from the observation that certain yeasts were able to grow on extracts of *Laminaria cloustoni* (Morris, 1955). Laminarin may constitute 30% of the dry weight of *L. cloustoni* fronds.

Brock (1965) reported the partial purification of an intracellular β-glucanase from compressed baker's yeast. No activity was found in the culture fluid, and unbroken cells exhibited only negligible activity on laminarin. Paper-chromatographic analysis of the products of hydrolysis by the intracellular enzyme showed that glucosyl units were

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successively removed from laminarin and from pustulan. During the course of a 100-fold purification of a crude cell extract of baker's yeast the ratios of activities with laminarin, pustulan and *p*-nitrophenyl β-D-glucoside remained constant. Brock (1965) also reported that these three activities remained together during starch-block zone electrophoresis and were equally inhibited by 1mg. of glucono-δ-lactone/ml.

Brock (1961) had shown earlier that conjugation in *Hansenula wingei* requires protein synthesis, because amino acid analogues inhibit this process. He postulated that conjugation involves the induction of a wall-softening enzyme that acts on the mating cells in a localized area of the walls. Brock (1964) subsequently found that β-(1→3)-glucanase activity rises sharply during conjugation; cells during the vegetative growth phase had low β-(1→3)-glucanase activity. He did not specify whether the glucanase from *H. wingei* was an exo- or an endo-splitting enzyme.

The present work was undertaken to repeat the work of Brock (1965) and to obtain further evidence for the existence of a unienzymic system in the hydrolysis of laminarin and pustulan and to expand specificity and kinetic data. The work was further extended by the inclusion of several yeast species whose asci (in contrast with those of baker's yeast) lyse rapidly at maturity, thus liberating the spores. Because of the importance of β-glucans as major structural components of the cell walls of yeasts (Phaff, 1963), it seemed reasonable to assume that one or more glucanases are involved in the lysis of ascus walls. Such walls are the equivalent of what were previously the cell walls.

With these points in mind, several yeast species were studied with respect to the presence and concentrations of β-glucanases. The enzymes were extensively purified and the characteristics of the purified enzymes were determined.

MATERIALS AND METHODS

Organisms and culture conditions

Compressed baker's yeast (Red Star Yeast Co., Milwaukee, Wis., U.S.A.) was purchased in 1lb. blocks at a local market. The following strains of pure cultures were obtained from the yeast culture collection of the Department of Food Science and Technology, University of California (Davis, Calif., U.S.A.) under the numbers indicated: *Saccharomyces cerevisiae* no. C–299; *Fabospora fragilis* no. 61–293 (syn. *Saccharomyces fragilis*); *Hansenula anomala* no. C–317. For the purpose of surveying the enzyme-producing ability of these species they were grown for 24hr. in a liquid medium consisting of glucose (5%, w/v) and powdered yeast autolysate (Albimi, Brooklyn, N.Y., U.S.A.) (0·5%, w/v) in Erlenmeyer flasks on a rotary shaker at 30°. If intracellular enzyme was to be studied the cells were centrifuged, washed twice with water and disrupted by ultrasonic oscillation. The centrifuged cell extracts or the supernatant culture fluids were dialysed against 5mM-sodium succinate buffer, pH5·5, before determination of β-glucanase activity with laminarin or pustulan as substrates.

If large quantities of cells or culture medium were required for enzyme purification it was preferable to use a medium without organic non-diffusible nitrogen. In this case the organisms were grown in an 80l. fermenter (Stainless Steel Products Co., St Paul, Minn., U.S.A.). The medium contained Yeast Nitrogen Base (Difco Laboratories, Detroit, Mich., U.S.A.), or a mixture of the individual components as recommended by Wickerham (1951), glucose (5%) and 50mM-sodium succinate buffer at pH6·0 as the starting pH. A 4l. volume of a 24hr. culture was used to inoculate 70l. of medium. Aeration was kept at approx. 7l./min. The temperature of growth was maintained at 30°. Cells were harvested after 24hr. by centrifugation in a large Sharples Centrifuge. The resulting yeast cake (usually 1200–1500g. per run) was used as a source of intracellular enzyme. The culture fluid was concentrated fourfold by vacuum distillation. Concentrated culture fluid was dialysed against 5mM-sodium succinate buffer, pH5·5.

Preparation of enzyme extracts

Ultrasonic oscillation. A 15g. sample of compressed yeast cells was suspended in 30ml. of 0·1M-sodium succinate buffer, pH5·5. The suspension was subjected to ultrasonic oscillation (20kcyc./sec.) (Biosonic; Bronwill Scientific, Rochester, N.Y., U.S.A.) until 50–95% of the cells were ruptured. Baker's-yeast cells showed a higher rate of breakage than those of *F. fragilis* and *H. anomala*.

Mechanical shaking. To a special 75ml. flask were added 50g. of glass beads (0·45–0·50mm.; Bronwill Scientific) and approx. 20ml. of yeast suspension (10g. of compressed yeast + 10ml. of 0·1M-sodium succinate buffer, pH5·5). The stoppered flask was shaken in a Braun homogenizer (Bronwill Scientific) for 1–2min. at 4000oscillations/min. with sufficient liquid CO_2 delivered to the chamber to prevent heating. One min. was usually sufficient for 99% breakage of *S. cerevisiae* cells whereas *F. fragilis* required about 2min. for the same degree of breakage. Chains of *H. anomala* cells were difficult to break, even after subjecting the cells twice to a 2min. treatment. In this case, only 50% breakage was obtained. The glass beads were separated from the broken cells by filtering through a fine nylon netting.

Use of a colloid mill. For rupturing large quantities of yeast cells, the Eppenbach model QV–6 laboratory colloid mill (Gifford–Wood Co., Hudson, N.Y., U.S.A.) was chosen. The method employed was that described by Garver & Epstein (1959). Milling was continued for 60 and 90min. to attain 95–99% breakage of cells of *S. cerevisiae* and *F. fragilis* respectively. Glass beads (0·12mm.) were separated by allowing them to settle for a few minutes and decanting the mixture of cell walls and cytoplasmic contents.

After rupture of yeast cells by any of the above methods, clear intracellular enzyme preparations were obtained by centrifugation at 13000*g* in a Sorvall centrifuge for 1hr.

Substrates

Laminarin. Cold-water-insoluble lamarin was purchased from K & K Laboratories (Plainview, N.Y., U.S.A.).

Modified laminarin preparations. Laminarin modified at both ends was obtained by periodate oxidation according to the procedure of Goldstein, Hay, Lewis & Smith (1965). Laminarin in which only the reducing end was modified was prepared by hypoiodite oxidation according to the procedure of Jansen & MacDonnell (1945).

Laminaribiose. Small samples of laminaribiose were obtained through the courtesy of Dr S. Kirkwood, University of Minnesota (Minneapolis, Minn., U.S.A.), and of Dr W. Z. Hassid, University of California (Berkeley, Calif., U.S.A.). In addition, laminaribiose was isolated from a partial acid hydrolysate of laminarin, the oligosaccharides being separated by gel filtration on Sephadex G-15.

Pustulan. Pustulan was initially obtained through the courtesy of Dr B. J. D. Meeuse of the University of Washington (Seattle, Wash., U.S.A.) and of Dr E. T. Reese of the Quartermaster Research and Engineering Center (Natick, Mass., U.S.A.). Later, pustulan was prepared from the lichen *Umbilicaria pustulata* by the procedure of Lindberg & McPherson (1954) and subjected to final purification by the procedure described by Reese, Parrish & Mandels (1962). The yield of the purified product was approx. 7%. The *U. pustulata* powder was kindly furnished by Dr J. R. Villanueva (Madrid, Spain). The extraction and purification procedure described by Lindberg & McPherson (1954) is lengthy and laborious. In spite of the many steps recommended in their procedure the product was still quite dark and it was only after heating with 0·33 N-H_2SO_4 at 100° for 15 min. followed by fractional precipitation with ethanol as recommended by Reese *et al.* (1962) that a white powder was obtained. Application of fractional precipitation at an earlier stage may prove to be a better and hopefully a shorter procedure.

Oat glucan. This was given by Dr P. A. J. Gorin of the Prairie Regional Laboratory (Saskatoon, Sask., Canada).

Crown-gall polysaccharide. This material, produced by *Agrobacterium radiobacter*, was a gift from Dr J. F. T. Spencer of the Prairie Regional Laboratory (Saskatoon, Sask., Canada). This glucan has mainly β-(1→2)-glucosidic bonds.

Cellulose dextrin. This was prepared by the method of Fuller & Norman (1942).

Glucono-δ-lactone. This was purchased from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.). Since it was found to contain trisaccharide impurities it was recrystallized from methylCellosolve (2-methoxyethanol) as described by Isbell, Holt & Frush (1962). The product was chromatographically homogeneous.

Reference proteins for molecular-weight determination. Bluefin-tuna myoglobin was kindly supplied by Dr W. D. Brown, University of California (Berkeley, Calif., U.S.A.). Horse heart cytochrome *c*, ox pancreas α-chymotrypsin and pepsin were obtained from Mann Research Laboratories Inc. (New York, N.Y., U.S.A.). Horseradish peroxidase was obtained from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.).

Other substrates and materials were commercial products of the highest purity available.

Analytical measurements

Reducing sugars. These were determined by the Nelson–Somogyi method (Somogyi, 1952).

Glucose. This was determined with the Glucostat reagent as recommended by the manufacturer (Worthington Biochemical Corp.).

Protein. This was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Crystallized bovine serum albumin was used as the standard.

Paper chromatography

The epiphase of butan-1-ol–acetic acid–water (4:1:5, by vol.), the epiphase of ethyl acetate–acetic acid–water (3:1:3, by vol.), or ethyl acetate–pyridine–water (10:4:3, by vol.) mixture was used for descending development of chromatograms on Whatman no. 1 paper at 30°. Detection of spots was done by spraying with a benzidine–trichloroacetic acid reagent (Bacon & Edelman, 1951) or by an ammoniacal $AgNO_3$ reagent (Trevelyan, Procter & Harrison, 1950).

Determination of enzymic activities

Assay of exo-β-glucanase. Routine assays were done as follows: 1 ml. of 1% β-glucan (or other substrate) in 0·1 M-sodium succinate buffer, pH 5·5, was equilibrated at 30° in a water bath. Then 1 ml. of enzyme solution, separately equilibrated at 30°, was added. Samples (0·5 ml.) were withdrawn periodically and placed in boiling water for 2 min. to stop the enzymic reaction. Glucose determinations were done with the Glucostat reagent. Glucose values were plotted as a function of time and the enzyme activity was calculated from the slope. The initial reaction rate was a linear function of enzyme concentration in the range in which assays were done. One unit of exo-β-glucanase is defined as the amount of enzyme that releases 1 μmole of glucose/min. under the above conditions. Activity units are expressed with reference to the particular substrate used.

Column chromatography

DEAE-cellulose (Eastman no. 7392) was obtained from Distillation Products Industries (Rochester, N.Y., U.S.A.). It contained some ultraviolet-absorbing materials, which were removed by treating the anion-exchanger with 1 N-NaOH, followed by water to neutrality, then ethanol and finally ether. The product was dried at room temperature and equilibrated at pH 5·5 with 10 mM-sodium succinate buffer. Columns were packed according to the procedure described by Peterson & Sober (1962).

Proteins were adsorbed on the DEAE-cellulose column under flow conditions induced by gravity. Proteins were eluted from the column by an increasing convex gradient of NaCl in sodium succinate buffer. The gradient was produced by an apparatus similar to that described by Palmer (1955).

Protein concentrations in the effluent were estimated from the E_{280} values (measured with a Beckman model DB spectrophotometer equipped with a recording unit) or the E_{254} values (measured with a ISCO model UA recording ultraviolet analyser). The effluent was collected in fractions by use of a fraction collector designed to deliver constant volumes to the collection tubes.

Gel filtration

Columns of Sephadex (Pharmacia, Uppsala, Sweden) were prepared by standard procedures as recommended by the manufacturer.

Blue dextran 2000 was used to determine the void volume and for checking column packing.

Samples to be chromatographed were added to the top of the gel bed in a few millilitres of buffer, and washed into the gel with several small portions of buffer before continuous elution was started. Elution volumes were taken from the initial addition of the sample to the peak of the eluted protein.

Molecular weights of enzyme proteins were estimated by the procedure of Whitaker (1963).

RESULTS

Exo-β-glucanase from baker's yeast

Preliminary experiments. The partially purified intracellular exo-β-glucanase preparation used by Brock (1965) showed specific activities of 24 and 17 (mg. of glucose released/mg. of protein/hr.) with 0·1% pustulan and 0·1% laminarin respectively. Because Brock (1965) did not determine the effect of substrate concentration, a comparison was made between two concentrations of each of the two substrates with a crude extract of commercial baker's yeast. The results showed that on increasing the laminarin concentration from 1 mg./ml. to 4 mg./ml. the specific activity increased from 0·0014 to 0·0058 μmole of glucose/min./mg. of protein, and in a similar experiment with pustulan the specific activity rose from 0·0020 to 0·0046 μmole of glucose released/min./mg. of protein. Hence with glucan concentrations of 0·1% (as used by Brock, 1965) the enzyme is far from saturated and with 0·4% substrate concentrations the enzyme exhibits the higher specific activity towards laminarin than towards pustulan.

Exo-β-glucanase activity in budding and non-budding cells. In the manufacture of compressed baker's yeast, the cells are harvested in the stationary phase, with very few, if any, budding cells present. If the formation of buds in a growing culture is related to the synthesis of β-glucanase for the purpose of softening or dissolution of cell wall at the location of new buds, then the glucanase activity should be higher in budding than in non-budding cells.

A 5 g. sample of compressed baker's yeast was inoculated in 1 l. of yeast autolysate (0·5%, w/v) plus glucose (5%, w/v). The culture was aerated for 4 hr. at 30°, after which most of the cells were actively budding. Known dry weights of the budding and of the original resting cells were broken in a Braun homogenizer and after centrifugation the extracts were dialysed against 5 mM-sodium succinate buffer, pH 5·5. The results showed that the specific activity in the non-budding cells decreased from 1·118 μmoles of glucose/min./g. dry wt. of original cells to 0·755 in the budding cells. In the cell extract, the corresponding change was from 0·0052 to 0·0044 μmole of glucose/min./mg. of protein. Thus the actively budding cells contained less soluble and extractable enzyme than the non-budding cells in the compressed cake. The lack of a measurable increase in exo-β-glucanase activity does not mean necessarily that this enzyme is not involved in the budding process, since the cells may have a sufficient activity even in the resting condition. Another possibility is that there is an increase in enzyme activity but that in budding cells the enzyme is more tightly bound to the newly synthesized glucan. In contrast, in the conjugation process of *H. wingei*, Brock (1965) showed an increase in the exo-β-glucanase activity.

Purification of intracellular exo-β-glucanase from baker's yeast. A colloid mill was used to disrupt compressed baker's-yeast cells. After settling of the glass beads the homogenate was centrifuged to separate cell debris and supernatant fluid. Next the clear extract was dialysed against 5 mM-sodium succinate buffer, pH 5·5.

Preliminary experiments showed that the dialysed solution could be heated at 50° for 10 min. without significant loss in exo-β-glucanase activity. This treatment resulted in the coagulation of much unwanted protein, which was removed by centrifugation. The supernatant fluid was then applied to a DEAE-cellulose column (15 cm. × 1·5 cm.) equilibrated at pH 5·5. The use of an upper limit of 0·6 M-sodium chloride–0·1 M-sodium succinate buffer, pH 5·0, and 600 ml. of 5 mM-sodium succinate buffer, pH 5·5, at the lower limit produced a gradient that successfully separated exo-β-glucanase from most of the proteins adsorbed on the column. The eluate from DEAE-cellulose that contained exo-β-glucanase activity was dialysed again against 5 mM-sodium succinate buffer, pH 5·5, and concentrated by application on a short column of DEAE-cellulose followed by elution with concentrated buffer. The concentrated eluate was applied on a Sephadex G-100 column (2·5 cm. × 100 cm.). The protein was eluted with 0·1 M-sodium chloride–0·1 M-sodium succinate buffer, pH 5·5. The flow rate was 60 ml./hr. and the elution volume for exo-β-glucanase was 325 ml.

Table 1 summarizes the various steps in the purification. The data show a 343-fold overall purification based on the dialysed homogenate of the yeast cake.

Properties of purified exo-β-glucanase from baker's yeast. (*a*) Optimum pH. pH–activity curves were determined with 50 mM-sodium succinate buffer, pH 4–6, with sodium citrate buffer, pH 3–4, and with sodium phosphate buffer, pH 6–8. The optimum pH of the action of the purified enzyme on laminarin, pustulan and *p*-nitrophenyl β-D-glucoside was 5·5.

(*b*) Substrate specificity and dependence of exo-β-glucanase activity on substrate concentration.

Table 1. *Summary of various steps applied in the purification of intracellular exo-β-glucanase from baker's yeast*

Activity units were determined with 0·5% laminarin as the substrate. The total eluate from the Sephadex column was the result of three separate applications.

	Step	Volume (ml.)	Exo-β-glucanase activity (unit/ml.)	Concn. of protein (mg./ml.)	Sp. activity (units/mg. of protein)	Yield (%)
1	Dialysed intracellular preparation	180	0·200	28·7	0·007	100
2	Heating at 50° for 10min.; centrifugation	140	0·210	19·2	0·011	81·5
3	DEAE-cellulose column chromatography	210	0·085	0·056	1·520	49·5
4	Sephadex G-100	200	0·080	0·033	2·4	44·5

Table 2. *Action of purified intracellular exo-β-glucanase from baker's yeast on various substrates*

The enzyme assays were designed to detect 0·001 unit by the Glucostat method. Zero indicates that $V < 0·001$ unit with 0·5% substrate concentration.

Substrate	$V_{max.}$	K_m
Laminarin	9·51	14mg./ml.
Pustulan	3·78	3·61mg./ml.
Gentiobiose	0·17	$1·27 \times 10^{-2}$M
p-Nitrophenyl β-D-glucoside	0·66	$2·15 \times 10^{-3}$M
Oat glucan	0	
Cellodextrin	0	
Cellobiose	0	
Agrobacterium radiobacter glucan	0	
Phenyl β-D-glucoside	0	
Methyl β-D-glucoside	0	
Methyl α-D-glucoside	0	

The substrates shown in Table 2 were tested for susceptibility against the exo-β-glucanase. The reaction was followed by measuring the rate of glucose release and by paper-chromatographic analysis of the products formed. For the substrates that were hydrolysed the initial reaction rates were then measured as a function of substrate concentration. The maximum velocities, $V_{max.}$, and the Michaelis constants, K_m, were determined graphically in each case from Lineweaver–Burk plots.

The data in Table 2 show that the maximum velocity with laminarin is 2·5 times that with pustulan. Brock (1965) reported the activities of 100-fold-purified exo-β-glucanase from baker's yeast against various substrates at concentrations of 1mg./ml. At this concentration he found that the rate of pustulan hydrolysis was 1·4 times that of laminarin; this observation was confirmed here from the Lineweaver–Burk plots with purified enzyme and also with crude enzyme as shown above. At the concentrations of laminarin (5mg./ml.) and pustulan (2·5mg./ml.) used for assays in this work, laminarin was hydrolysed 1·6 times as rapidly as pustulan. These findings emphasize the undesirability of comparing rates of hydrolysis of two or more substrates in the same concentration by a single enzyme if the K_m values prove to vary greatly with the different substrates.

(c) Pattern of action. Preliminary experiments with crude extracts showed that the enzyme produced only glucose from laminarin and pustulan. The endwise hydrolysis of these substrates, suggested by the results obtained, was then studied in more detail with the purified enzyme. Substrate (5mg.) was dissolved in 0·5ml. of 0·1M-sodium succinate buffer, pH 5·5, and 0·5ml. of appropriately diluted exo-β-glucanase solution in 5mM-sodium succinate buffer, pH 5·5, was added. Incubation was done at 30° with 0·01% merthiolate added as a preservative. This compound showed no effect on the enzyme activity. Heated samples were spotted on Whatman no. 1 paper together with standards of glucose and appropriate oligosaccharides; development was done with the epiphase of butan-1-ol–acetic acid–water (4:1:5, by vol.). Sugars were located on the paper by the ammoniacal silver nitrate reagent.

Two reaction mixtures were prepared of laminarin containing 0·5 and 0·05 unit of the purified enzyme/ml. respectively. With the higher enzyme concentration glucose appeared as the sole reaction product. With the lower concentration of enzyme glucose appeared initially and was followed by a transient very faint spot corresponding to laminaribiose.

Chromatographic analysis of a reaction mixture with pustulan as the substrate and containing 0·5 pustulan unit/ml. also showed glucose initially as the sole product, with a faint spot of gentiobiose appearing towards the end of the hydrolysis. With tenfold-higher enzyme concentration a faint spot of gentiobiose appeared transiently during the reaction. These results point to an endwise attack on laminarin and pustulan and confirm the results obtained by Brock (1965).

Evidence for a single enzyme. Brock (1965) reported that during a 100-fold purification of exo-β-glucanase from baker's yeast the activities of the enzyme against laminarin, pustulan and

Table 3. *Relative activities of intracellular exo-β-glucanase from baker's yeast on different substrates during the various steps of purification*

The rates with laminarin were taken as 100%.

Substrate	Relative reaction rates			
	Dialysed homogenate	Heated supernatant	DEAE-cellulose eluate	Sephadex G-100 eluate
Laminarin (0·5%)	100	100	100	100
Pustulan (0·25%)	61	58	64	62
Gentiobiose (0·25%)	2·3	2·3	2·9	2·5
p-Nitrophenyl β-D-glucoside (0·25%)	7·8	9·9	6·9	7·0

p-nitrophenyl β-D-glucoside remained together and the ratio of their activities was unchanged. To verify these findings the 343-fold-purified enzyme obtained in this work was tested with gentiobiose in addition to the three substrates used by Brock (1965). The same was done at key points during the purification. As shown in Table 3, the ratios of hydrolysis rates with these substrates at all stages of purity of the enzyme remained constant.

Brock (1965) also reported that the activities towards laminarin, pustulan and *p*-nitrophenyl β-D-glucoside remained together during starch-block zone electrophoresis and were equally inhibited by 1 mg. of glucono-δ-lactone/ml.

This last evidence does not appear valid in view of the very different K_m values for laminarin and pustulan of the more highly purified enzyme obtained in the present work (cf. Table 2). Because of the different affinities of exo-β-glucanase towards the two glucans, one would not expect a given concentration of the competitive inhibitor glucono-δ-lactone to inhibit both reactions to the same extent.

It was therefore felt desirable to obtain at least one additional piece of supporting evidence for the presence in baker's yeast of a single enzyme that can degrade both laminarin and pustulan in an endwise fashion. Quantitative application of the mixed-substrate method with purified enzyme could provide evidence whether one or more enzymes are involved. In the presence of two substrates that are being hydrolysed by the same enzyme, each substrate will act as a competitive inhibitor for the enzymic reaction with the other substrate, since presumably the same active centres act on both. Webb & Morrow (1959) have derived the following formula:

$$v_t = \frac{v_a(1+\alpha)+v_b(1+\beta)}{1+\alpha+\beta}$$

where v_t is the total velocity (mixed-substrate velocity), v_a is the velocity with substrate A, v_b is the velocity with substrate B, α is the relative (effective) substrate concentration for substrate A, defined as:

$$\phi_A = \frac{s_A}{K_m \text{ for substrate A}}$$

and β is the relative (effective) substrate concentration for substrate B, defined as:

$$\phi_B = \frac{s_B}{K_m \text{ for substrate B}}$$

This formula was used to calculate the expected v_t if one enzyme is acting on two substrates. The results are presented in Table 4. The measured values of v_t are in excellent agreement with those calculated from the equation. This is consistent with the hypothesis that a single enzyme is responsible for the hydrolysis of laminarin and pustulan.

β-Glucanase in other yeasts

It was decided to compare several species of yeast for the activity and properties of β-glucanases. Three species were compared. One was a pure culture of *S. cerevisiae*, a yeast that forms asci of which the ascus wall is not lysed when the spores are mature. The other two species were *F. fragilis* and *H. anomala*, both examples of yeasts in which the ascus wall lyses rapidly on maturity of the spores. It was thought that the lytic phenomenon of the ascus wall might be associated with a higher enzyme activity than was found in baker's yeast (*S. cerevisiae*). The yeasts were grown in glucose–yeast autolysate.

This survey showed that cell extracts obtained by ultrasonic treatment of all three species hydrolysed laminarin and pustulan with the production of glucose as the sole product, as determined by chromatographic analysis. In addition there was a significant exo-β-glucanase activity in the dialysed culture fluids of *F. fragilis* and *H. anomala*, whereas that of *S. cerevisiae* showed only negligible activity.

Table 4. *Mixed-substrate method application on the purified exo-β-glucanase of baker's yeast*

The various symbols used in this Table are explained in the Results section. v_a+v_b represents the sum of the two separate velocities that would have been obtained experimentally if two enzymes were acting on the two substrates.

Concn. of substrate (s) (mg./ml.)		Relative concn. of substrate ($\phi=s/K_m$)		Velocity (μg. of glucose/ml./min.)			Total velocity (v_t)	
Laminarin (A)	Pustulan (B)	α (laminarin)	β (pustulan)	v_a	v_b	v_a+v_b	Measured	Computed from equation
3·9	1·0	0·278	0·278	12·50	4·92	17·42	14·6	14·35
8·75	4·38	0·625	1·210	18·96	11·60	30·56	20·26	19·90

Table 5. *Comparison of the β-(1→3)-glucanase activities in* F. fragilis *and* S. cerevisiae

All activity units were determined with 0·5% laminarin as the substrate.

	Culture fluid		Intracellular preparation		
				Sp. activity	
Organism	Activity (unit/ml.)	Sp. activity (unit/mg. of protein)	Activity (unit/ml.)	(unit/mg. of protein in cell extract)	(units/g. dry wt. of whole cells)
F. fragilis 61–293	0·047	0·750	0·193	0·063	6·8
S. cerevisiae C–299	0·004	0·100	0·037	0·009	1·1

A comparison of *S. cerevisiae* and *F. fragilis* (Table 5) shows that the latter's specific activity was about seven times that of the former with laminarin as the substrate. The enzyme activity in *H. anomala* was comparable with that of *F. fragilis*.

A detailed study of the enzyme of *F. fragilis* and *H. anomala* was undertaken next.

Exo-β-glucanase from F. fragilis

Purification of extracellular exo-β-glucanase. Since appreciable exo-β-glucanase activity was found in the culture medium (cf. Table 5) it was decided to study the extracellular enzyme first. The organism was grown in Yeast Nitrogen Base plus glucose in an 80l. fermenter as described in the Materials and Methods section. The concentrated dialysed culture fluid (21l.) was first stirred with 20g. of DEAE-cellulose (equilibrated at pH 5·5) for 30min. followed by filtration through a coarse sintered-glass filter. The enzyme was recovered by elution with 1·0M - sodium chloride – 0·1M - sodium succinate buffer, pH 5·0. In addition to the decrease in volume (21l. to 0·28l.) this step provided a 44-fold increase in the specific activity. The eluate was dialysed against 5mM-sodium succinate buffer, pH 5·5.

Chromatography on DEAE-cellulose was applied next and the elution pattern is shown in Fig. 1. This step led to an approximately twofold increase in specific activity. Fractions containing enzymic activity were combined and concentrated on a short

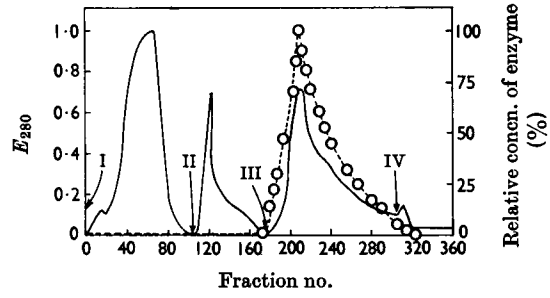


Fig. 1. DEAE-cellulose chromatography of extracellular exo-β-glucanase from *F. fragilis*. Eluents: I, 5mM-sodium succinate buffer, pH 5·5; II, gradient to 0·1M-NaCl–20mM-sodium succinate buffer, pH 5·5; III, gradient to 0·5M-NaCl–50mM-sodium succinate buffer, pH 5·0; IV, gradient to 1M-NaCl–0·1M-sodium succinate buffer, pH 5·0. Fraction volumes were 5ml. The column (1·5cm. × 14cm.) was equilibrated against succinate buffer at pH 5·5. ——, Protein concentration; ----, relative enzyme activity with 0·5% laminarin as the substrate. The volume of starting buffer in mixing flask was 300ml.

DEAE-cellulose column as described for the purification of exo-β-glucanase of baker's yeast.

The concentrated eluate in 1·0M-sodium chloride–0·1M-sodium succinate from DEAE-cellulose was finally chromatographed on Sephadex G-100 and the elution pattern is shown in Fig. 2. Representative fractions were examined for enzymic activity towards laminarin, pustulan and *p*-nitrophenyl

β-D-glucoside. Fractions tested had nearly the same ratio of activities towards the three substrates. Table 6 summarizes the results and shows a 114-fold overall purification.

Purification of intracellular exo-β-glucanase of F. fragilis. Approximately equal quantities by weight of *F. fragilis* press cake and 50mM-sodium phosphate buffer, pH 6·0, were ground at 25° with Ballotini beads in a colloid mill for 90min. followed by centrifugation at 13000*g* for 1hr. The supernatant fluid was dialysed against 50mM-sodium succinate buffer, pH 5·5. The dialysed enzyme preparation was subjected to the following treatments.

(*a*) Heating. Preliminary experiments showed that the solution could be heated at 55° for 10min.

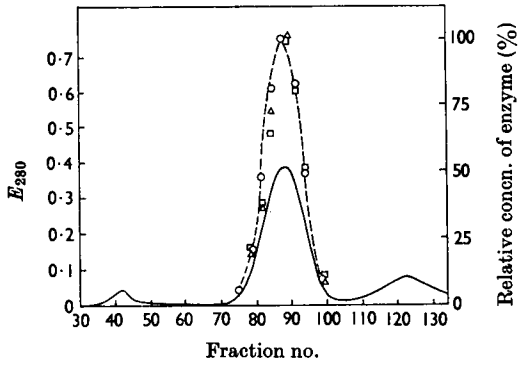


Fig. 2. Elution pattern of exo-β-glucanase from *F. fragilis* during gel filtration over a column (2·5cm. × 120cm.) of Sephadex G-100. The protein was applied in a volume of 5ml. and eluted with 0·1M-NaCl–0·1M-sodium succinate buffer, pH 5·5. The fraction volume was 4·2ml., the temperature 25° and the flow rate 60ml./hr. The void volume was 182ml. The elution volume of the enzyme was 377ml. Enzyme activity was measured with 0·5% laminarin (○), 0·25% pustulan (□) and 0·25% *p*-nitrophenyl β-D-glucoside (△). Activity in the most active fraction was taken as 100%. ——, Protein concentration.

without loss in exo-β-glucanase activity. Application of this step led to coagulation of much unwanted protein, which was removed by centrifugation. The specific activity in the supernatant fluid increased two- to four-fold.

(*b*) Protamine sulphate precipitation. When protamine sulphate was used to precipitate nucleic acids, it was found that exo-β-glucanase was co-precipitated. The enzyme could be recovered subsequently by eluting the precipitate with 0·2M-sodium succinate buffer, pH 5·5. The best results were obtained by the addition of 3ml. of 5% (w/v) protamine sulphate solution to 100ml. of the supernatant liquid obtained from the heating step. Approx. 20–30% of the exo-β-glucanase was not precipitated. However, the seven- to ten-fold increase in specific activity and decrease in volume justified this step.

(*c*) DEAE-cellulose column chromatography. On dialysis of the protamine sulphate eluate against 0·02M-sodium succinate buffer, pH 5·5, the enzyme was unexpectedly co-precipitated with other material. However, nearly all of the activity could be recovered by eluting the centrifuged precipitate with 0·02M-sodium succinate buffer. This eluate could be applied directly on a DEAE-cellulose column (18cm. × 2cm.). Presumably, the enzyme is co-precipitated with substances soluble in 0·2M but not in 20mM buffer.

Elution gradients similar to those used in the purification of extracellular exo-β-glucanase were applied. Fractions containing enzyme activity were collected and concentrated on a short DEAE-cellulose column.

(*d*) Gel filtration on Sephadex G-75 (superfine grade). The molecular weight of the enzyme was estimated to be about 20000 from the behaviour of the extracellular enzyme on Sephadex G-100 (see below). This suggested that a better purification could be expected on Sephadex G-75 than on Sephadex G-100 because the former has a lower exclusion limit (the approximate exclusion limits of Sephadex G-100 and G-75 are 100000 and 50000

Table 6. *Purification of extracellular exo-β-glucanase from* F. fragilis

All activity units were determined with 0·5% laminarin as the substrate. The total eluate from the Sephadex column was the result of three separate applications.

Step	Volume (l.)	β-Glucanase activity (units/ml.)	Concn. of protein (mg./ml.)	Sp. activity (units/mg. of protein)	Yield (%)
Culture fluid	82	0·061	0·089	0·683	100
Dialysed concentrated culture fluid	21	0·222	0·328	0·680	93·5
Batch adsorption on DEAE-cellulose followed by elution	0·28	8·66	0·343	29·7	48·5
DEAE-cellulose column chromatography	0·82	2·28	0·039	58·8	37·4
Sephadex G-100	0·28	5·25	0·067	78·3	29·4

Table 7. *Purification of intracellular exo-β-glucanase from* F. fragilis

All activity units were determined with 0·5% laminarin as the substrate. The total eluate from the Sephadex column was the result of two separate applications.

Step	Volume (ml.)	β-Glucanase activity (units/ml.)	Concn. of protein (mg./ml.)	Sp. activity (units/mg. of protein)	Yield (%)
Intracellular preparation	800	5·58	29·3	0·19	100
Heating at 55° for 20min.; centrifugation	750	5·50	11	0·50	92·5
Protamine sulphate precipitation; elution	265	8·78	2·8	3·40	52
Dialysis→precipitate→eluate	420	4·61	1·2	3·84	44
DEAE-cellulose column chromatography	540	3·54	0·117	30·2	43
Sephadex G-75 (superfine grade) chromatography	100	12·6	0·156	80·5	28·4

respectively). A 5ml. sample of concentrated exo-β-glucanase was applied to a column (195cm. × 1·9cm.) of Sephadex G-75 (superfine grade) and eluted with 0·1M-sodium chloride–0·1M-sodium succinate buffer, pH5·5, at a rate of 8ml./hr. Elution volume for exo-β-glucanase was 270ml. This step led to a 2·7-fold increase in specific activity.

Table 7 summarizes the various steps and shows an overall 423-fold purification.

Properties of purified extracellular exo-β-glucanase from F. fragilis. (*a*) Optimum pH. Though the approximate optimum pH was initially determined with crude enzyme, a more accurate pH–activity curve was determined with the purified enzyme with 50mM-sodium succinate, sodium phosphate and sodium citrate buffers as described for baker's yeast. Maximal activity was obtained at pH5·5 with laminarin. Pustulan and *p*-nitrophenyl β-D-glucoside had identical pH–activity curves.

(*b*) Substrate specificity. A number of substrates (Table 8) were treated with purified exo-β-glucanase and the course of the reaction was followed by paper chromatography and by determination of the rate of glucose released by the glucose oxidase reagent. For most of the substrates that were attacked, the initial reaction rates were measured also as a function of substrate concentration in the presence of 50mM-sodium succinate buffer, pH5·5. Michaelis constants, K_m, and maximum velocities, $V_{max.}$, were determined by plotting the reciprocal of the velocity against the reciprocal of the substrate concentration. It should be realized that the absolute value of the K_m may vary with different preparations of the polymers. Factors that influence K_m are the degree of polymerization and extent of branching.

Table 8 summarizes the action of purified extracellular enzyme on various substrates. Maximum velocity of the purified enzyme with laminarin was ten times that with pustulan. Homopolymers with β-linkages other than β-(1→3)- or β-(1→6)-linkages were not attacked. The rate of hydrolysis with 0·2% laminaribiose indicated that the enzyme has significant activity on this disaccharide. In contrast, gentiobiose was hydrolysed only slowly in comparison with pustulan and with laminaribiose.

Pattern of action of purified exo-β-glucanase from F. fragilis. Paper-chromatographic analysis showed the enzyme to attack laminarin and pustulan in an endwise manner, releasing glucose units sequentially from the two substrates.

The possibility that the immediate production of glucose might be due to the presence of an active oligosaccharide-splitting enzyme acting on intermediates produced by a much weaker endo-β-glucanase was investigated by using glucono-δ-lactone as a selective inhibitor. This compound has relatively little effect on β-glucanases, but has been reported as a very effective inhibitor of β-glucosidases (Conchie & Levvy, 1957). Thus, in a reaction mixture containing laminarin, an endo-β-glucanase, a β-glucosidase and glucono-δ-lactone, one might expect the accumulation of oligosaccharides if a randomly splitting enzyme is present.

When glucono-δ-lactone was added so that the rate of glucose formation from laminarin was approx. 70% inhibited, chromatographic analysis of the reaction products again showed glucose as the sole product.

When the enzymic activity was assayed by measuring the rate of glucose release (glucose oxidase) and the rate of increase in reducing power (Nelson–Somogyi procedure), identical values in terms of glucose equivalents were obtained. On addition of glucono-δ-lactone to the reaction mixtures, both of the measured rates were lowered to the same extent. Similar results were obtained with an unpurified extract of *F. fragilis*. The combined evidence indicates that the hydrolysis of laminarin is catalysed solely by an exo-β-glucanase, which sequentially releases glucose units from this polymer.

To establish the mode of attack of the exo-β-glucanase on laminarin, the rates of attack of the

Table 8. *Action of purified exo-β-glucanase from* F. fragilis *and* H. anomala *on various substrates*

The enzyme assays were designed to detect 0·001 unit by the Glucostat method. Zero indicates that $V < 0{\cdot}001$ unit with 0·5% substrate concentration.

Substrate	Main linkage	$V_{max.}$		K_m	
		F. fragilis	*H. anomala*	*F. fragilis*	*H. anomala*
Laminarin	β-(1→3)	82·60	54·00	1·24 mg./ml.	5·00 mg./ml.
Laminaribiose	β-(1→3)	34·50*	—	—	—
Pustulan	β-(1→6)	7·40	7·15	1·85 mg./ml.	5·90 mg./ml.
Gentiobiose	β-(1→6)	0·58	0·17	$2{\cdot}56 \times 10^{-2}$ M	$2{\cdot}9 \times 10^{-2}$ M
Cellodextrin	β-(1→4)	0	0		
Cellobiose	β-(1→4)	0			
Agrobacterium radiobacter glucan	β-(1→2)	0	0		
Oat glucan	β-(1→4) and β-(1→3) (mixed)	0	0		
p-Nitrophenyl β-D-glucoside		2·20	1·82	$5{\cdot}5 \times 10^{-4}$ M	$5{\cdot}35 \times 10^{-3}$ M
Phenyl β-D-glucoside		0			
Methyl β-D-glucoside		0			
Methyl α-D-glucoside		0			
Amygdalin		0			
Salicin		0			
Arbutin		0			

* $V_{max.}$ was not determined with laminaribiose. The value is that obtained with 0·2% laminaribiose.

Table 9. *Comparison of the rates of attack of crude extracellular exo-β-glucanase of* F. fragilis *on laminarin with that of terminally modified derivatives of the polysaccharide*

Substrate (5 mg./ml.)	Initial rate of hydrolysis	
	Glucose release (μg. of glucose/ml./min.)	Increase in aldehyde groups (μg. of glucose equiv./ml./min.)
Laminarin	34·0	36·0
Hypoiodite-oxidized laminarin	20·2	18·0
Periodate-oxidized laminarin	0·6	0·2

enzyme with derivatives of the polysaccharide were compared with that on native laminarin. Periodate-oxidized laminarin (both ends modified), hypoiodite-oxidized laminarin (aldehydic end oxidized to a carboxyl group) and laminarin were incubated at a concentration of 0·5% with dialysed culture fluid of *F. fragilis* at pH 5·5. Table 9 shows the results. Laminarin modified only at the reducing end of the molecule was hydrolysed at 60% of the rate with laminarin. In contrast, laminarin modified at both ends of the chains was attacked at a negligible rate. Similar results were obtained with purified extracellular enzyme and with crude cell extract of *F. fragilis*. The results confirm that the β-glucanase from *F. fragilis* attacks laminarin in an endwise fashion, successively removing glucosyl units from the non-reducing ends of the chains.

Extent of degradation. The action of purified intra- or extra-cellular exo-β-glucanase on laminarin and on pustulan was followed by measuring glucose release as a function of time. The results are shown in Fig. 3. With pustulan, the reaction proceeded linearly until about 22% of the β-(1→6)-glucan was hydrolysed and then the rate gradually slowed down. After degradation of approx. 45% of the substrate (8 hr.) the rate became extremely low. Laminarin was hydrolysed at a linear rate until the hydrolysis was half complete; then the reaction proceeded at a far lower rate.

A final check after 4 days showed essentially complete hydrolysis of the two glucans.

Evidence for a single enzyme. The following results support the conclusion that, as in baker's yeast, in *F. fragilis* a single enzyme acts on both pustulan and laminarin.

The ratios of activities with 0·5% laminarin and 0·25% pustulan were determined after each step in the purification of extracellular and intracellular enzyme. The ratio of the rates with laminarin and pustulan remained constant (approx. 20:1) during purification. Similarly, fractions comprising a

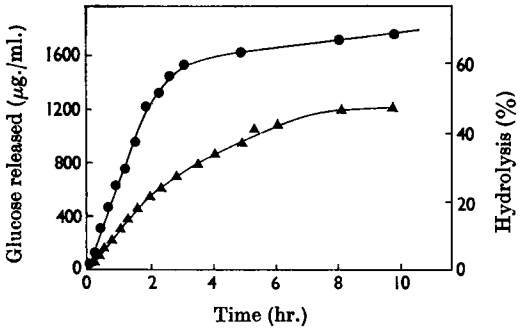
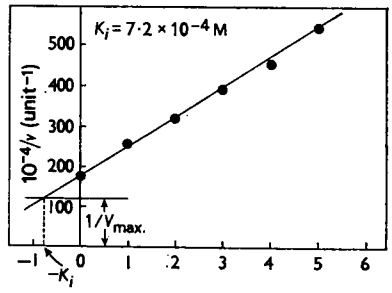


Fig. 3. Extent of degradation of laminarin (●) and pustulan (▲) by purified exo-β-glucanase from *F. fragilis*. The reaction mixture contained 0·25% substrate in 50mM-sodium succinate buffer, pH 5·5. The temperature was 30°.



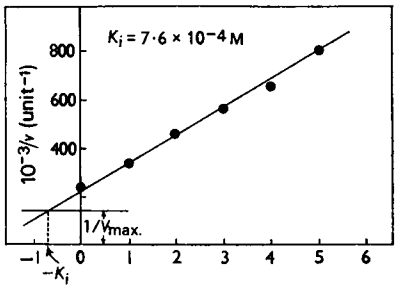
Concn. of glucono-δ-lactone (mM)

Fig. 4. Graphic determination of inhibitor constant, K_i, with laminarin as substrate. The enzyme was purified exo-β-glucanase from *F. fragilis*. The laminarin concentration was 2·5mg./ml. Enzyme activity with 0·25% laminarin in the absence of inhibitor was 0·045 μmole of glucose/min./ml. of reaction mixture.

single peak during gel filtration on Sephadex or during DEAE-cellulose column chromatography always had nearly identical ratios of activities towards the two glucans.

Glucono-δ-lactone was found to inhibit exo-β-glucanase activity competitively with both pustulan and laminarin. By determining the velocity with a series of inhibitor concentrations, with the substrate concentration kept constant, a straight line was obtained on plotting $1/v$ against i, the inhibitor concentration. The intersect of the extrapolated inhibitor graph and a horizontal line representing the value of $1/V_{max.}$ corresponds with $-K_i$ (cf. Dixon & Webb, 1964). Application of this method on results obtained with laminarin and pustulan (Figs. 4 and 5) gave inhibitor constants of $7·2 \times 10^{-4}$M and $7·6 \times 10^{-4}$M respectively.

The third line of evidence for a single enzyme is based on the finding that a constant ratio between the two activities was maintained as the enzyme underwent heat inactivation at three different temperatures. The results are shown in Fig. 6.



Concn. of glucono-δ-lactone (mM)

Fig. 5. Graphic determination of inhibitor constant, K_i, with pustulan as substrate. The enzyme was purified exo-β-glucanase from *F. fragilis*. The pustulan concentration was 2·5mg./ml. Enzyme activity with 0·25% pustulan in the absence of inhibitor was 0·035 μmole of glucose/min./ml. of reaction mixture.

Exo-β-glucanase from H. anomala

Next, the β-glucanase system of *H. anomala*, one of the common species of the genus *Hansenula*, was chosen for study. This species forms large hat-shaped ascospores that are rapidly liberated from the ascus at maturity by a lytic process.

H. anomala C–317 was grown at 30° in Fernbach flasks on a shaker in Yeast Nitrogen Base with 5% glucose and buffered with 50mM-sodium succinate at pH 6·0 as the starting pH. Cultures were harvested after 24hr.

The cells were broken in a Braun homogenizer. On dialysis of the extract against 5mM-sodium succinate buffer, pH 5·5, for 1–2 days at 5°, much of the β-(1→3)-glucanase activity was lost, a

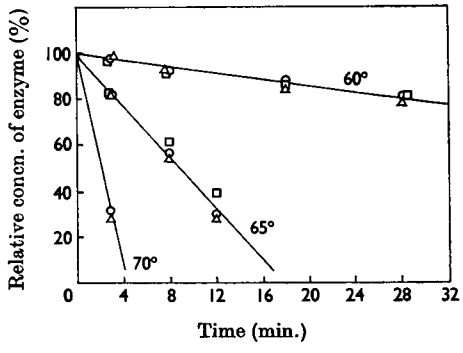


Fig. 6. Rates of inactivation of purified exo-β-glucanase at various temperatures, with laminarin (○), pustulan (△) and *p*-nitrophenyl β-D-glucoside (□). The enzyme was heated in 0·1M-NaCl–0·1M-sodium succinate buffer, pH 5·5.

Table 10. *Purification of extracellular exo-β-glucanase from* H. anomala

All activity determinations were done with 0·5% laminarin as the substrate.

Step	Volume (ml.)	β-Glucanase activity (unit/ml.)	Concn. of protein (mg./ml.)	Sp. activity (units/mg. of protein)	Yield (%)
Dialysed culture fluid	3000	0·028	0·76	0·036	100
Batch adsorption on DEAE-cellulose, followed by elution	100	0·482	1·74	0·276	57·5
DEAE-cellulose column chromatography; eluate	30	0·855	0·124	6·9	30·4
Gel filtration on Sephadex G-75; eluate	63	0·366	0·016	22·9	27·4

phenomenon not encountered with the previous two species. Since the enzyme was found to be much more stable in the culture fluid, the centrifuged supernatant fluid was used as the enzyme source.

Purification of extracellular exo-β-glucanase from H. anomala. On the basis of experiments with the enzymes from *F. fragilis* and baker's yeast, initial purification was tried with DEAE-cellulose. It was found that the enzyme could be adsorbed from the dialysed (5mM-sodium succinate) culture fluid at pH 5·5 and then eluted with 1M-sodium chloride–0·1M-sodium succinate buffer, pH 5·0.

After the preliminary batch purification and concentration, the enzyme was further purified by column chromatography on DEAE-cellulose. The dialysed enzyme preparation from the previous step was applied to a column (15cm. × 1·5cm.) and an elution gradient similar to that described in the purification of baker's yeast enzyme was employed. The DEAE-cellulose was concentrated by application on a short column of DEAE-cellulose.

A 10ml. sample of the concentrated DEAE-cellulose eluate was applied to a column (194cm. × 1·9cm.) of Sephadex G-75 (superfine grade) and eluted with 0·1M-sodium chloride–0·1M-sodium succinate buffer, pH 5·5, at a rate of 8ml./hr. Elution volume for the enzyme was 244ml.

Table 10 summarizes the purification results and shows an overall purification of 600-fold. The ratio of activities against laminarin and pustulan remained constant during the purification.

Pattern of action. Chromatographic analysis showed that initially glucose was the only product formed from laminarin and pustulan as substrates. Faint transient spots of the corresponding disaccharides appeared late during the reaction. The results indicate an endwise attack on the β-(1→3)- and β-(1→6)-glucans, glucose units being released sequentially from the two polymers.

Properties of purified exo-β-glucanase from H. anomala. Exo-β-glucanase activity was measured as a function of pH as was done for the enzyme from baker's yeast. The optimum pH of the action of the purified extracellular enzyme on laminarin and pustulan was 5·5.

Substrate specificity and dependence of exo-β-glucanase activity on substrate concentration were determined next. Table 8 summarizes the results obtained with various substrates. The maximum velocity of exo-β-glucanase with laminarin was about eight times that with pustulan. On prolonged incubation of the enzyme with oat glucan, β-(1→2)-glucan or cellodextrin, no action was detected by paper chromatography or by the glucose oxidase reagent.

Estimation of the molecular weights of the various exo-β-glucanase preparations by gel filtration

The separation of solutes on cross-linked dextrans according to molecular weight is well documented. Barring certain complications, Whitaker (1963) found an excellent linear correlation between the logarithm of molecular weight of a protein and the ratio of its elution volume, V_e, to the void volume, V_0, of a Sephadex column.

To estimate the molecular weights of the exo-β-glucanases from *F. fragilis*, *S. cerevisiae* and *H. anomala*, a column (195cm. × 1·9cm.) of Sephadex G-75 (superfine grade) was calibrated with several proteins of known molecular weights. Proteins were applied to the top of the gel bed in 1ml. of 0·1M - sodium chloride – 0·1M - sodium succinate buffer, pH 5·5, and eluted with the same buffer at a rate of 8ml./hr. at 20°. The void volume of the column was 175ml. as determined with blue dextran 2000. A standard calibration curve was prepared with horse heart cytochrome *c*, bluefin-tuna myoglobin, ox pancreas α-chymotrypsin, pepsin and horseradish peroxidase. The logarithms of their molecular weights formed a straight-line function of V_e/V_0. Table 11 shows the elution volumes for the exo-β-glucanases from the different yeast species together with their molecular weights estimated from the standard curve.

Table 11. *Properties of the exo-β-glucanases of three yeast species*

Purified enzyme from	Elution volume (V_e)	V_e/V_0†	Mol.wt. determined graphically	$V_{max.}$ (μmoles of glucose/min./mg. of protein) Laminarin (I)	$V_{max.}$ (μmoles of glucose/min./mg. of protein) Pustulan (II)	K_m (mg./ml.) Laminarin	K_m (mg./ml.) Pustulan	$V_{max.}$ (I) / $V_{max.}$ (II)
*F. fragilis**	271	1·55	22500	82·6	7·4	1·2	1·8	11·1
S. cerevisiae	215	1·23	40000	9·5	3·8	14·0	3·6	2·5
H. anomala	244	1·40	29500	54·0	7·1	5·0	5·9	7·5

* Purified intracellular and purified extracellular enzyme had the same elution volume.
† The void volume, V_0, of the column was 175ml.

DISCUSSION

This work was initiated to study yeast β-glucanases and to correlate the amount and properties of β-glucanases in various species with the susceptibility of mature asci to lysis.

F. fragilis and *H. anomala* are yeasts whose asci lyse rapidly on maturity, thus releasing the ascospores. The ascus wall in *S. cerevisiae* remains intact until it is ruptured by what is thought of as a swelling process of the spores during germination. These species appear to produce exo-β-glucanase constitutively, but the two groups mentioned above differ greatly in their β-glucanase activity. For example, *F. fragilis* possessed approximately sevenfold greater β-(1→3)-glucanase activity (per g. dry wt. of cells or per mg. of protein in cell extract) than *S. cerevisiae* (Table 5).

The enzymes of *F. fragilis*, *S. cerevisiae* and *H. anomala* have certain properties in common, but there are certain differences. The optimum pH values were 5·5 in all cases. Chromatographic evidence indicated that all three glucanases can be classified as exo- or endwise-splitting enzymes. They sequentially remove glucosyl units from the glucan. The purified exo-β-glucanases all attacked laminarin as well as pustulan. The ratios of activities towards the two glucans remained constant in the various fractions during the purification from the crude extracts to the most highly purified preparations in the three species. This suggested that a single enzyme possessed both β-(1→3)- and β-(1→6)-glucanase activity.

Additional evidence for this viewpoint has been obtained. Activities with laminarin and pustulan were destroyed at exactly the same rate when the enzyme solution was subjected to heating at three different temperatures (Fig. 6). The competitive inhibitor glucono-δ-lactone gave the same K_i value when tested with either substrate (Figs. 4 and 5). Quantitative application of the 'mixed-substrate' method with the purified enzyme of *S. cerevisiae* gave strong evidence that both activities reside in a single enzyme (Table 4). Brock (1965) reported that the two activities of 100-fold-purified exo-β-glucanase of *S. cerevisiae* remained together and their activity ratios did not change during starch-gel electrophoresis.

Thus there is strong evidence that in each of these yeasts a single exo-β-glucanase can hydrolyse β-(1→3)- as well as β-(1→6)-glucans. In this respect the exo-β-glucanase of yeast is different from fungal exo-β-glucanase. The latter enzyme, isolated and purified from basidiomycete QM806 (Nelson, Scaletti, Smith & Kirkwood, 1963), was shown to be specific for the β-(1→3)-linkage only. This fungal exo-β-glucanase also differed from the yeast enzymes in that it had no action on laminaribiose. With purified exo-β-glucanase from *F. fragilis*, the rate of hydrolysis of 0·2% laminaribiose was about one-third of the maximum velocity of the enzyme with laminarin. The β-(1→6)-linked disaccharide gentiobiose, however, was hydrolysed at a rate far lower than pustulan by the exo-β-glucanases of *F. fragilis*, *S. cerevisiae* and *H. anomala*. The exo-β-glucanases from the basidiomycete and yeasts were similar in their pattern of action on laminarin in that the attack took place from the non-reducing ends of the chains. The changes in hydrolysis rates by modifying the reducing end or both ends of the laminarin chain, observed by Nelson *et al.* (1963) for the fungal enzyme, were very close to those observed for the *F. fragilis* enzyme.

The purified exo-β-glucanases have different kinetic constants and molecular weights, as shown in Table 11.

The observation that the ratios of maximum velocities of exo-β-glucanase towards laminarin and pustulan were different in the three yeasts may be of evolutionary significance. The substrate specificity of an exo-β-glucanase of a particular yeast species may be such that its cell-wall glucan is efficiently hydrolysed. Our present knowledge on the structure of yeast cell-wall glucans is not adequate to support or refute this possibility. Further, in most of the studies with yeast glucans, investigators have been

unaware of the presence of β-glucanases. In the absence of heating or alkali extraction, these enzymes could conceivably act on cell-wall glucans during preparation and initial purification of cell walls, especially if the enzyme has high affinity for the walls. This could account for the differences in the degree of substitution of the main chains and in the lengths of the side chains reported for different samples of baker's-yeast glucan (Manners & Patterson, 1966).

Though the velocity constants have not been determined for any of the enzymes studied, the large difference in K_m values between the purified enzymes from *F. fragilis* and *S. cerevisiae* indicate that the exo-β-glucanase from the latter has lower affinity towards laminarin and pustulan than that of *F. fragilis*. If this difference in affinity can be extended to a glucan with mixed linkages (i.e. cell-wall glucan), the rapid release of spores from asci in *F. fragilis* could be explained in terms of an enzyme with higher maximal velocity and higher affinity to the ascus wall than the enzyme found in baker's yeast. The K_m values for exo-β-glucanase from *H. anomala* occupied a position intermediate between those obtained with *F. fragilis* and *S. cerevisiae*.

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