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ACTION PATTERNS OF (1→3)-β-D-GLUCANASES FROM *Oerskovia xanthineolytica* ON LAMINARAN, LICHENAN, AND YEAST GLUCAN*

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ABSTRACT

Four D-glucanases in the crude culture-broth of *Oerskovia xantineolytica* act synergistically to degrade the walls of viable yeast. Enzyme I has a molecular weight of ~18,000, but does not lyse viable yeast cells, even though it displays high activity against laminaran, and will readily depolymerize lichenan. It is an endo-glucanase (E.C. 3.2.1.6). Enzyme II is also an endo-glucanase (E.C. 3.2.1.29) having a molecular weight of ~29,000 and is active against both viable yeast cells and laminaran, but it exhibits restricted activity against lichenan. Enzymes IIIa and IIIb have molecular weights of 27,000 and 29,000, respectively, and exhibit high lytic activity against viable yeast cells, but release few reducing groups from laminaran or yeast glucan. They exhibit higher specific activity against yeast glucan than against laminaran and yield an oligosaccharide of degree of polymerization (d.p.) 5 as the eventual hydrolysis-product. In the initial stages of hydrolysis, they act in an endo-manner, but they effect cleavage of a d.p. 14 substrate in an apparent exo-manner. They have high specificity for straight-chain, β-(1→3)-linked D-glucan. The proposed systematic name for enzymes IIIa and IIIb is (1→3)-β-D-glucan pentaosehydrolase. When yeast glucan was hydrolyzed by enzyme IIIb, ~40% of the glucan was recovered as the products of d.p. 5 and 9; 20% was solubilized, but remained as oligosaccharides having d.p. of between 15 and 35; 24% had a d.p. >35; and 10% was insoluble material that resembled yeast-bud scars microscopically.

INTRODUCTION

Oerskovia xantineolytica[1] produces extracellular (1→3)-β-D-glucanases that rapidly degrade the walls of yeast[2–4] and germinating fungi if they contain (1→3)-β-D-glucan as a structural polymer[5]. Some of these enzymes are unique in that they disrupt the integrity of yeast and fungal walls without releasing large quantities of

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reducing groups. The crude *Oerskovia* enzyme is more cell-lytic than crude exo- and endo-(1→3)-β-D-glucanases from *Rhizopus arrhizus*, *Trichoderma viride*, and[5] basidiomycete QM 806. Other investigators[4,6–15] have found enzymes that exhibit high yeast lytic activity and release only small amounts of reducing sugar into the mixture. The primary products are generally β-(1→3)-linked oligosaccharides having a degree of polymerization[16] (d.p.) of 4 or 5 hence, degradation of the polymer proceeds more rapidly than if the product were glucose.

The present research determined action patterns of four (1→3)-β-D-glucanases from *Oerskovia xanthineolytica* on several glucans. The approach used gel-permeation chromatography to isolate and identify the intermediate products in sequential samples of enzymic hydrolysis. The amounts of each product were measured by reducing-group assay. In addition, quantitative analysis of residual oligosaccharides and polysaccharides resulting from extensive enzymolysis of yeast glucan yielded some evidence of the structure of yeast glucan.

RESULTS

Separation of the enzymes by gel chromatography. — Glucan-desorbed enzyme solution was purified by molecular-sieve chromatography (Fig. 1). A major protein peak eluting near the void volume exhibited α-D-mannanase and proteinase activity, but neither laminaranase nor yeast lytic activity. Three other proteins had laminaranase activity. Yeast lytic activity was associated with two of these laminaranases (fractions 15–25), but not with the other enzyme I (fractions 31–38). Fractions containing yeast lytic activity were pooled, dialyzed against mM potassium phosphate buffer, (pH 6.5) in 10mM 2-mercaptoethanol, and applied to DEAE-Bio-Gel A. Approximately 30% of the lytic activity applied was recovered from the DEAE column (Fig. 2), 83% of which eluted in the void volume. This lytic peak possessed <10% of the recovered laminaranase activity and was termed enzyme III. The remaining 90% of the laminaranase activity (2.87 U; 0.95 U/mg) was eluted in a second peak at 36mM potassium chloride. This peak possessed <15% of the viable yeast lytic activity (68 U; 22 U/mg) and was termed enzyme II. Fig. 2 shows that the peak containing enzyme III had more activity against yeast glucan than against laminaran; the reverse was true for enzyme II. Enzyme III possessed 0.9 U (34%) and enzyme II possessed 1.73 U (66%) of the total glucanase activity. DEAE-Bio-Gel fractions containing the major yeast lytic activity (enzyme III) were pooled and dialyzed for 30 h at 4° against 10mM potassium phosphate buffer, pH 6.0, containing 10mM 2-mercaptoethanol and chromatographed on *O*-carboxymethyl-Bio-Gel A (Fig. 3). Two peaks of yeast lytic activity were separated. The first peak that eluted was termed enzyme IIIa, the second was termed IIIb. Each of these enzymes exhibited laminaranase and yeast glucanase activity.

Synergism of purified enzyme preparations. — Significant losses of yeast lytic activity occurred during separation of the enzyme fractions, and the ratio of yeast lytic to laminaranase activity was not constant in different preparations. These results

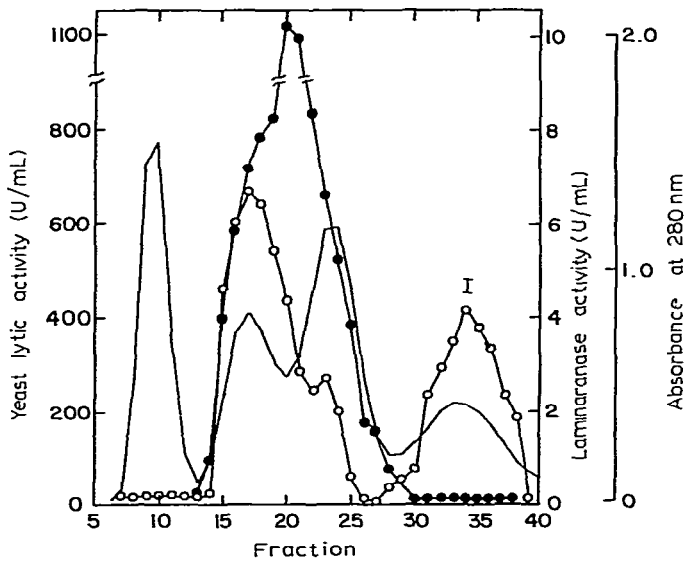

Fig. 1. Chromatography on Bio-Gel P-60. Glucan-desorbed enzyme (11.0 mL, 40,000 lytic units) was applied to the bottom of a column (2.5 × 88 cm) of Bio-Gel P-60 that was eluted with 50mM potassium phosphate buffer, pH 6.5, in 10mM 2-mercaptoethanol. After collecting ~70 mL in a single volume, fractions (4.7 mL) were collected and assayed for protein content (solid line), laminaranase (○), and viable yeast lytic activity (●). The V_0 was ~92 mL and the flow rate was ~18 mL/h with a 60-cm head pressure.

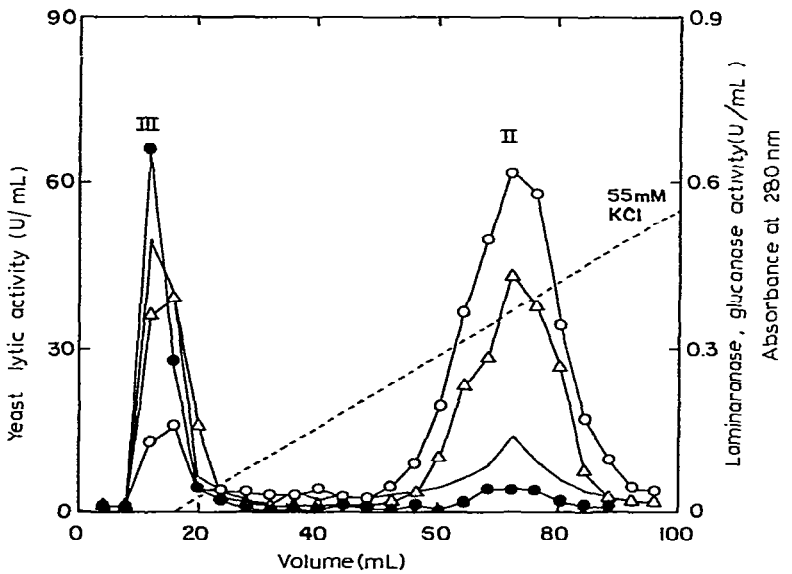

Fig. 2. Chromatography on DEAE-Bio-Gel A. The column (0.9 × 19 cm) was equilibrated with 10mM potassium phosphate buffer, pH 6.5 in 5mM 2-mercaptoethanol. Following application of the enzyme (0.5 mL, 1,630 U) the gel was washed with 12 mL of the equilibrating buffer and then developed with a linear gradient (----) (0–0.2M potassium chloride in 10mM potassium phosphate buffer, pH 6.5) in a total volume of 400 mL. Fractions (4.0 mL) were collected and assayed for protein content (solid line), laminaranase (○), glucanase (△), and viable yeast lytic activity (●).

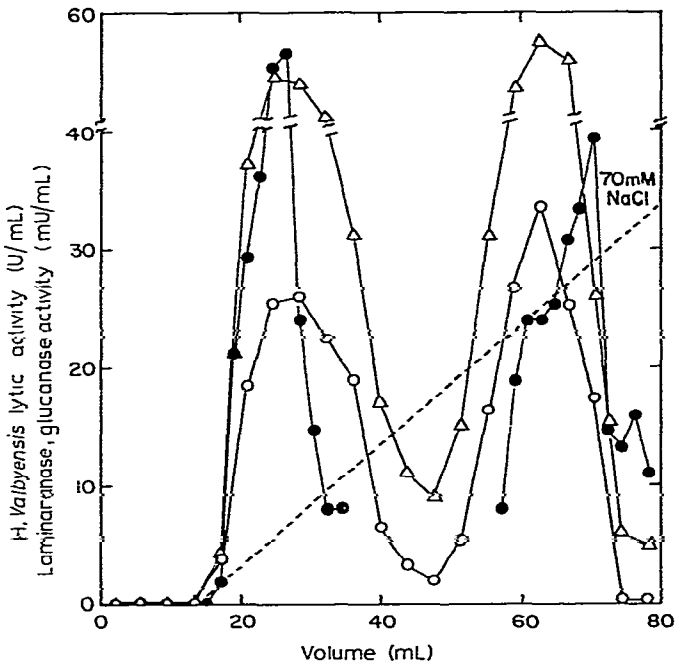

Fig. 3. Chromatography on CM-Bio-Gel A. Enzyme III (13 mL, 850 *H. valbyensis* lytic units 170 *S. cerevisiae* lytic units) from DEAE Bio-Gel A chromatography (Fig. 2) was applied to the top of a column (0.9 × 27 cm) of *O*-carboxymethyl-Bio-Gel A that had been equilibrated with 0.1M potassium phosphate buffer, pH 6.0, in 10mM 2-mercaptoethanol. The column was eluted with 200 mL of a gradient (---) of 0–0.1M sodium chloride in 10mM potassium phosphate buffer, pH 6.0, and 10mM 2-mercaptoethanol. Fractions (2.0 mL) were collected and assayed for laminaranase (○), glucanase (△), and *H. valbyensis* yeast lytic activity (●).

TABLE I

COMPARISON OF OBSERVED AND EXPECTED ACTIVITIES OF VARIOUS COMBINATIONS OF ENZYMES PURIFIED BY GEL AND ION-EXCHANGE CHROMATOGRAPHY

Enzyme	*Volume (μL)*	*Viable* H. valbyensis *lytic activity (total U)*		
		Observed	*Expected (additive)*	*Ratio*
I	10	0.00	0.00	
II	100	0.39	0.39	1.0
III	10	1.07	1.07	1.0
I	10	0.60	0.39	1.5
II	100			
I	10	1.03	1.07	1.0
III	10			
II	100	3.37	1.46	2.3
III	10			
I	10			
II	100	5.2	1.46	3.6
III	10			

could be explained if the four enzymes acted synergistically to hydrolyze the walls of viable yeast. Therefore, enzyme preparations were assayed singly and in combinations for their activities against *H. valbyensis* cells. The results are summarized in Table I. Combination of enzyme preparations II and III resulted in a 2,3-fold increase in activity over what was expected from a strictly additive relationship. This synergism could account for the single yeast lytic peak observed following chromatography on Bio-Gel P-60 (Fig. 1). Assayed altogether, the three enzyme preparations exhibited a 3,6-fold synergism over the separate activities.

Molecular weights of purified enzymes. — Enzyme I had the smallest apparent molecular weight (18,000); enzyme II had the largest (29,000). Enzymes IIIa and IIIb had very similar molecular weights of ~27,000 and 26,000, respectively.

Action patterns on laminaran and lichenan: Enzyme I. — The action pattern of enzyme I on laminaran was determined by separating and measuring the amounts of glucose and laminaran oligosaccharides released from the substrate (Fig. 4). At zero time, the molecular-weight distribution of the laminaran substrate was broad, but most of the material eluted near the void volume. Within 20 min, some of the laminaran had been degraded to glucose, laminarabiose (L_2), tri- (L_3), tetra- (L_4), penta- (L_5), and higher β-(1→3)-linked-oligosaccharides. Within 20–40 min, the concentration of higher oligosaccharides reached a maximum and began to decrease. The concentrations of glucose, L_2 and L_3, increased steadily from 0–80 min.

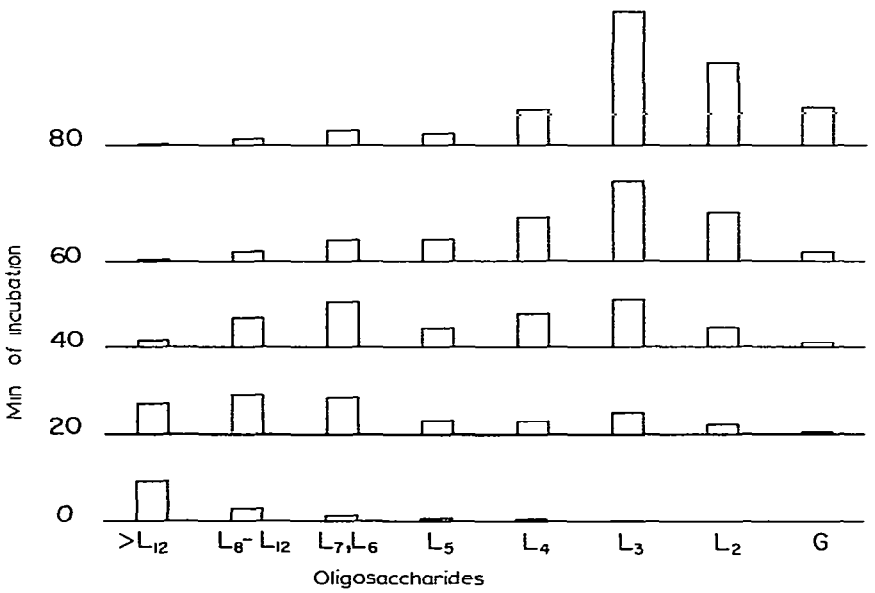

Fig. 4. Action pattern of enzyme I on laminaran. Laminaran (20 mg) was digested with 0.15 laminaranase units of enzyme I in a final volume of 5.0 mL of 50mM potassium phosphate buffer, pH 6.5. Samples were removed periodically and analyzed for reducing sugars by chromatography on Bio-Gel P-2. After 80 min, the reducing equivalents (all as μmol of glucose) were as follows: glucose, 1.4; L_2, 2.9; L_3, 4.7; L_4, 1.26; L_5, 0.4; $L_7 = L_6$, 0.5; L_8–L_{12}, 0.2; and $>L_{12}$, 0.05.

Enzyme I was tested for its ability to hydrolyze the hetero-linked β-(1→3)-β-(1→4)-D-glucan, lichenan. Initially, the enzyme released 10 nmol of glucose equivalents $mL^{-1}.min^{-1}$. The rate decreased gradually to 3 $nmol.mL^{-1}.min^{-1}$ as the reaction continued. The enzyme I preparation possessed 66 laminaranase units and 2.0 lichenase units per mL. The action pattern of enzyme I was also examined with lichenan as a substrate. The products were analyzed by gel chromatography. No reducing groups were detected initially, but within 20 min, several hydrolysis products were apparent. Two major product-peaks eluted at approximately 65 and 78 mL, corresponding to d.p. ~6 and 3, respectively. The product that eluted at 65 mL reached a maximum at 40 min and decreased thereafter. The amount of product eluting at 78 mL increased throughout the entire reaction. These latter fractions were combined and were shown by paper chromatography to contain Glc-(1→4)-Glc-(1→3)-Glc only.

Enzyme II. — The action pattern of enzyme II against laminaran, determined similarly, showed the initial formation of large oligosaccharides which were then degraded into glucose, L_2, L_3, L_4, L_5, L_6, and some higher oligosaccharides in an endo-pattern similar to that exhibited by enzyme I. Under the same conditions, lichenan yielded only a single product, which eluted from the Bio-Gel column near the void volume. The initial activity of enzyme II against laminaran (0.6 U/mL) remained unchanged during such incubations, but that against lichenan (3.6 U/mL) fell to less than half within 20 min.

Yeast glucan (100 mg) was hydrolyzed with laminaranase enzyme II for 36 h. Products were analyzed by molecular-sieve chromatography on Bio-Gel P-6 and P-2. They consisted predominantly of glucans and laminarabiose. Some L_3 and L_4 were also present. Unhydrolyzed residual glucan removed by centrifugation accounted for 28%, and the material eluting near the void volume on P-6 accounted for 12% of the recovered dry weight.

Enzymes IIIa and IIIb. — Enzymes IIIa and IIIb did not have activity against lichenan. The action pattern of enzyme IIIb on purified laminaran suggested endo hydrolysis (Fig. 5) in which major hydrolysis products, which began to accumulate within 40 min, still persisted after 120 min of incubation. The second of these, eluting at 67 mL, was identified as L_5 by paper chromatography. The product(s) eluting at 55 mL consisted of a heterogeneous collection of larger oligosaccharides of $\overline{\text{d.p.}}$ 9. The action pattern of enzyme IIIa on laminaran was found to be essentially the same as that of enzyme IIIb. The smallest oligosaccharide observed was L_5.

The hydrolysis of laminaran and yeast glucan by enzyme IIIb were compared in a larger-scale experiment. With laminaran, there were greater amounts of the d.p. 5 and 9 products and no significant quantities of larger saccharide materials, as observed with glucan (Fig. 6). The hydrolysis products from the digestion of yeast glucan were recovered by lyophilization. Of the 100 mg of original glucan, 97% was accounted for. Of the dry weight, 31% was recovered in the major product (d.p. 5) peak; 9% was present in the d.p. 9 peak; and 24% eluted between fractions 33 and 48. This material was believed to consist of β-(1→3)-β-(1→6)-linked oligosaccharides;

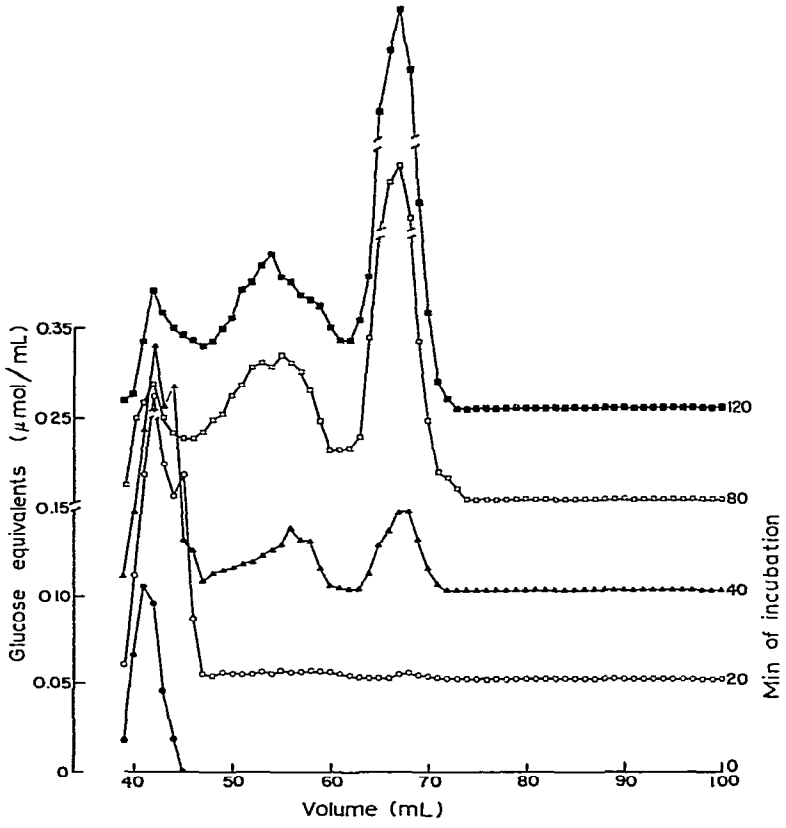

Fig. 5. Action pattern of enzyme IIIb on laminaran. The mixture contained Bio-Gel P-6-purified laminaran (16 mg) and 0.018 laminaranase units of enzyme IIIb in a final volume of 5.0 mL of 60mM potassium phosphate buffer, pH 6.5. Samples were removed periodically and analyzed by chromatography on Bio-Gel P-2. Zero min, (●); 20 min, (○); 40 min (▲); 80 min, (□); and 120 min, (■).

24% eluted between fractions 26 and 32, near the void volume of the column. This material was tentatively identified as predominantly β-(1→6)-linked glucan. Approximately 10% was present in an insoluble residue, which was exmined by brightfield microscopy. It consisted of many small rings approximately the diameter of yeast-bud scars. When incubated with enzyme II, the L_5 product was hydrolyzed completely to tetramer, trimer, dimer, and glucose within 80 min.

Kinetics of enzyme III on yeast glucan. — In order to determine how the release of reducing sugars correlated with depolymerization, alkali-extracted yeast glucan was digested by enzyme preparation III and the rate of the hydrolysis was estimated (*i*) turbidimetrically, (*ii*) by the release of reducing sugars (Nelson–Somogyi[17]), and (*iii*) by the release of total carbohydrate (phenol–sulfuric)[18]. The results are illustrated in Fig. 7. After an initial lag, carbohydrate was released rapidly into solution. Within 10 min, 43 μmol of glucose equivalents per mL (7.3 mg/mL; 73% of the total glucan) was solubilized. Between 10 min and 3 h, total carbohydrate solubilized increased

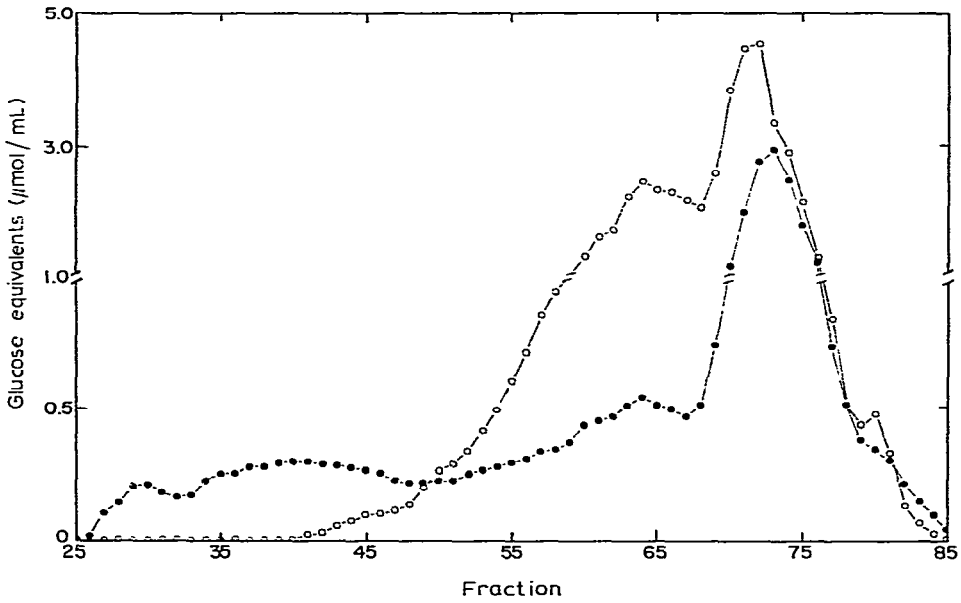

Fig. 6. Comparative enzymolysis of laminaran and yeast glucan by enzyme IIIb. Laminaran (100 mg) and yeast glucan (100 mg) were separately digested with 0.007 laminaranase units of enzyme IIIb at 30° for 24 and 36 h, respectively. The mixtures contained substrate and enzyme in 5.0 mL of 20mM potassium phosphate buffer, pH 6.5, containing 0.005% of sodium azide. The laminaran mixture was heat-inactivated and applied directly to Bio-Gel P-6. The glucan mixture was centrifuged to remove undigested residue (10%) and the supernatant solution was heat-inactivated and applied to Bio-Gel P-6. Fractions (2.5 mL) were collected and analyzed for reducing sugars by the automated alkaline ferricyanide method. Yeast glucan, (●); laminaran, (○).

only slightly to 48 μmol of glucose equivalents per mL (7.8 mg/mL; 78%). The rate of production of reducing groups was lower, but occurred at a more consistent rate. Within 10 min, only 0.75 μmol of glucose reducing-equivalents per mL (0.13 mg/mL; 1.3% of the total glucan) were formed. By 3 h, the total reducing sugar released into the supernatant solution increased to 1.6 μmol of glucose equivalents per mL (0.29 mg/mL; 2.9%). In the first 10 min, the ratio of total carbohydrate to reducing-group equivalents was 57. After 3 h, the ratio was decreased only to 30. The rate of decrease in turbidity at first followed the rate of total carbohydrate release, and then slowed and more nearly followed the rate of reducing-group formation.

DISCUSSION

Obata *et al.*[19] and others[20] have shown that *Oerskovia* produces specialized proteases that contribute to the lytic activity of the enzyme complex. We believe that the high lytic activity of enzymes IIIa and IIIb can be attributed to their affinity for the linear β-(1→3)-linked portion of the yeast-glucan wall.

Synergistic activity was demonstrated among the enzymes produced by *Oerskovia*. The yeast lytic activity observed in assays of recombined fractions was generally greater than the expected sum of the activities, with the exception being recombination

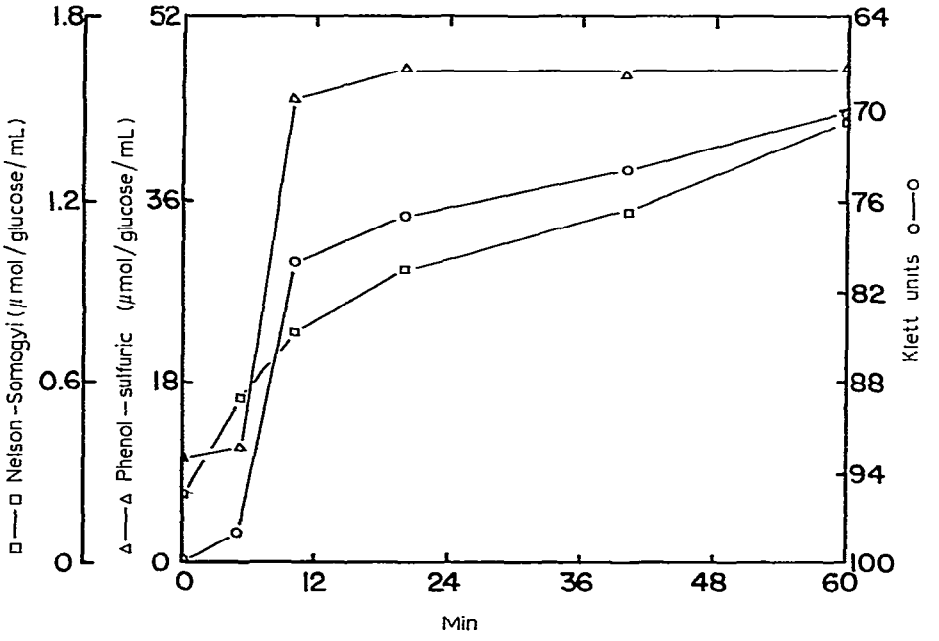

Fig. 7. Release of reducing sugars and total carbohydrate from yeast glucan by enzyme III. Alkali-extracted yeast-glucan (200 mg) was digested with 240 lytic units of enzyme III (Fig. 2) in 20 mL of 25mM potassium phosphate buffer, pH 6.5. The mixture was incubated at 30°. Samples (3.0 mL) were removed periodically. One mL of each sample was diluted to 10 mL with distilled water and read for absorbance (○) in a Klett colorimeter (no. 66 filter). The remaining 2.0 mL of the sample was heat-inactivated, centrifuged, and assayed for reducing groups (Nelson–Somogyi, □) and total carbohydrate (phenol–sulfuric, △).

of enzyme preparations I and III. This synergism could account for some of the apparent losses of enzyme activity that occurred during fractionation. Appreciable loss was also attributable to irreversible denaturation of protein.

Enzyme I attacks laminaran in an endo manner; it also hydrolyzes lichenan. These characteristics establish enzyme I as an endo-glucanase belonging to the class E.C. 3.2.1.6. Although it eventually yields glucose from laminaran or lichenan, the principal product in limited hydrolysis is L_3. It appears to cleave preferentially β-(1→4) linkages adjacent to β-(1→3)-linked glucose residues, because an oligomeric residue was observed as an intermediate product of lichenan hydrolysis[21,22]. Enzyme I also cleaves β-(1→6) linkages that are adjacent to several β-(1→3)-linked glucose residues[23,24], because it reduces yeast glucan to glucose.

Enzyme II also attacked laminaran and lichenan in an endo-manner; the attack on lichenan was limited to a few sites for which it exhibited high specificity. The distribution of hydrolysis products with time showed that enzyme II was more active against L_5 than against L_3, and it possessed little or no ability to hydrolyze L_2. This result could account for the appearance of L_2 as the major hydrolysis product observed upon digestion of viable cells with crude enzyme[5]. The L_5 product from enzymes IIIa and IIIb is probably converted rapidly into L_2 and L_3, and the latter

is hydrolyzed at a lower rate to form L_2 plus glucose. Enzyme II has been classified as an endo-β-(1→3)-glucanase of type E.C. 3.2.1.39.

Enzymes IIIa and IIIb were much more active against live yeast per unit of apparent (1→3)-β-D-glucanase activity than was enzyme II. Enzymes IIIa and IIIb exhibited a ratio of yeast lytic to laminaranase activity of 500, whereas enzyme II exhibited a ratio of 4.8 (estimated from Figs. 1 and 2). Additionally, with enzymes IIIa and IIIb, the activity against yeast glucan is higher than the activity against laminaran; with enzyme II, the opposite is true. The activity of enzyme II against laminaran is higher than the activity against yeast glucan.

The laminaran polymer is a straight-chain (1→3)-β-D-glucan with β-(1→6)-side branches occurring on ~5% of the chain residues[25]. Thus, the polymer has an uninterrupted, β-(1→3) chain-length of ~20. In contrast, the major β-(1→3)-linked glucan of *S. cerevisiae* has only ~3% of the glucose residues involved in interchain linkages[26–28], giving an average uninterrupted β-(1→3) chain-length of ~33. The relatively higher activities exhibited by enzymes IIIa and IIIb against yeast glucan than against laminaran suggests that the highly lytic enzymes more readily attack the longer, linear, β-(1→3) portion of the yeast-glucan structure. Because the microfibrillar (1→3)-β-D-glucan is believed to be the primary structural component of yeast walls[29], these findings are consistent with the rapid disruption of structural integrity exhibited by enzymes IIIa and IIIb. The lytic enzymes IIIa and IIIb rapidly solubilize and decrease the turbidity of alkali-extracted yeast glucan, but release very few reducing groups. This behavior can be accounted for partially by the fact that enzymes IIIa and IIIb form L_5 as the smallest hydrolysis product. However, assays of total carbohydrate released by enzyme preparation III showed that even after 3 h, 30 glucose residues were solubilized for each reducing group produced. Moreover, the initial broadening of the substrate peak during hydrolysis of laminaran by enzyme IIIb indicated that the initial cleavages must yield products much larger than d.p. 5.

The favored substrate for enzymes IIIa and IIIb must have a d.p. $\geqslant 14$ because oligosaccharides of d.p. 9 are not hydrolyzed. No significant quantities of intermediate d.p. 6, 7, and 8 residues, and no residues smaller than d.p. 5 were observed during the hydrolysis of laminaran or yeast glucan by enzymes IIIa and IIIb. It can, therefore, be concluded that at least the final cleavages take place in an exo-fashion. Random, endo-cleavage of an oligosaccharide of d.p. 14 would yield a variety of oligosaccharide end-products. A specific binding-site large enough to encompass 14 glucose residues can be imagined for an enzyme of this size, namely, 27,000 (refs. 30–32). Lysozyme (mol. wt. 12,500) is known to bind six pyranose rings[33]. Hydrolysis of yeast glucan by enzyme IIIb yields residual oligomers that are eluted near the void volume on P-6 and, hence, have a d.p. of ~40, but the hydrolysis of laminaran yields mainly the d.p. 5 plus d.p. 9 residues. Probably the larger oligosaccharides obtained from the hydrolysis of yeast glucan consist of (1→3)-β-D-glucan with occasional β-(1→6) linkages.

On the basis of the action-pattern data, we can offer an explanation for the synergistic activity observed to occur among the (1→3)-β-D-glucanases of *Oerskovia*

during the degradation of yeast walls: enzymes IIIa and IIIb are polysaccharidases[22] that have apparent specificities for high d.p., linear β-(1→3)-linked glucan. Each cleavage produces an oligosaccharide of d.p. 5 or larger, so that solubilization proceeds rapidly. As indicated by the kinetic studies, initial endo cleavages might occur in a linear β-(1→3)-linked region. Enzymes I and II have apparent specificities for β-(1→3)-linked glucan of lower d.p., and are capable of hydrolyzing or bypassing β-(1→6) linkages in predominantly β-(1→3)-linked yeast glucan, but enzyme III is apparently not capable of bypassing β-(1→6) linkages in predominantly β-(1→6)-linked glucan, because significant amounts of residual material of higher molecular-weight is found following extensive hydrolysis of yeast glucan by this enzyme. None of the four enzymes, either separately or together, is capable of degrading the bud-scar region, which contains large amounts of chitin[34].

EXPERIMENTAL

Preparation of enzymes. — Yeast cell-wall-degrading enzymes in a 4% (w/v) solution of lyophilized, extracellular broth solids from *Oerskovia xantineolytica*[3] were precipitated with 20–40% saturated ammonium sulfate at 3° and adsorbed onto alkali-extracted, yeast glucan (1 g glucan/16 g of crude broth-solids)[16,35]. The enzyme-glucan complex was dialyzed against four, 1-L changes of buffer solution (10mM 2-mercaptoethanol, mM calcium chloride, and 50mM potassium phosphate, pH 6.5 plus 0.02% sodium oxide) for 96 h at 30°. The yeast glucan was hydrolyzed, and the enzymes were recovered from the supernatant solution. The volume of the enzyme solution was reduced by evaporation at 5°.

Preparation of enzyme substrates. — Log-phase yeast cells were prepared from *Saccharomyces cerevisiae* C-299 and *Hanseniospora valbyensis* 61-516 (obtained from H. J. Phaff, University of California, Davis) and used as substrates for yeast lytic assays. *S. cerevisiae* was cultivated in yeast nitrogen base (Difco) with 0.5% glucose. *H. valbyensis* was grown in the same medium plus 1.0 g/L of yeast extract. For stock cultures, 15 g/L of agar was added. Log-phase cells for enzyme assays were prepared as described previously[3].

Water-insoluble laminaran (from *Laminaria cloustoni* fronds) was purchased from Pierce Chemical Company, Rockford, IL. For action-pattern studies, laminaran oligosaccharides having d.p. <12 were removed by preparative gel-chromatography on Bio-Gel P-6. Alkali-insoluble yeast glucan was prepared by the method of Manners *et al.*[28]. Yeast mannan was prepared by the method of Peat *et al.*[36]. Crude lichenan (Fluka, AG) from *Cetraria islandica* was obtained as a gift from E. T. Reese (U.S. Army Natick Labs, Natick, MA) and used without further purification.

Reducing-sugar assays. — The arsenomolybdate method[17] was used to determine enzymic activity against laminaran, lichenan, and glucan. An automated alkaline ferricyanide method[37] was used to determine reducing sugars in effluents from either preparative columns of Bio-Gel P-6 or analytical columns of Bio-Gel P-2. Total carbohydrate was determined by the phenol–sulfuric acid method[18].

Measurement of enzymic activities. — Yeast lytic activity was measured by an osmotic-shock assay[3]. A yeast lytic unit is that amount of enzyme producing a change of 1% per min in the turbidity of an osmotically-shocked suspension of log-phase *S. cerevisiae*. *H. valbyensis* cells were sometimes used for assays, because their walls are five times more sensitive to hydrolysis than walls of *S. cerevisiae*. In this eventuality, enzyme activity is expressed as *H. valbyensis* lytic units. A 1% change is equivalent to a change of ~1 Klett unit. With a 30-min reaction period, the assay was linear between 0.5 and 1.5 yeast lytic units; crude enzyme showed less linearity as a function of its concentration than purified enzyme.

Laminaranase, glucanase, and α-D-mannanase were measured by release of reducing groups[17] in mixtures containing 2.0 mg of substrate, appropriate amounts of enzyme, and 50mM potassium phosphate buffer, pH 6.5, in a final volume of 1.0 mL. Reactions were started by the addition of substrate, incubated for 20–200 min at 30° and terminated by the addition of 1.0 mL of alkaline arsenomolybdate reagent. One unit of activity is that amount of enzyme-releasing reducing groups equivalent to 1.0 μmol of glucose or mannose per min under the conditions described. Standard samples of glucose or mannose yielded a linear response from 0.05 to 0.75 μmol of sugar.

The rate of casein hydrolysis was used to assay for proteinase activity[38].

Protein determination. — Protein in column fractions was monitored spectrophotometrically at 280 nm. Protein in enzyme preparations was measured by the method of Lowry *et al.*[39], with bovine serum albumin as a standard.

Molecular-weight determinations. — The molecular weights of each of the purified enzymes was estimated by chromatography on Bio-Gel P-60, by plotting the ratio of the elution volume V_e to the void volume V_o versus the log of the molecular weight of the protein standards.

Gel chromatography for size distribution of hydrolysis products. — Samples (1 mL) were boiled, centrifuged, applied to the top of a 1.15 × 110 cm column of Bio-Gel P-2 (Bio-Rad Laboratories, Richmond, CA.), and eluted with 0.02% sodium azide. The column was standardized by using known oligosaccharides or acid hydrolyzates of laminaran.

Paper chromatography. — Whatman No. 1 paper chromatograms were developed in 54:8:18 isopropylalcohol–acetic acid–water in the descending mode for 18–36 h. Tetra- and higher oligosaccharides up to d.p. 8 were separated by multiple-descent chromatography. Oligosaccharide spots were made visible by the alkaline silver nitrate method[40]. β-(1→3)-Linked oligosaccharides for standards were prepared by acid hydrolysis of laminaran (obtained from D. E. Eveleigh, Rutgers University, New Brunswick, NJ). Other oligosaccharide standards (cellotriose; *O*-β-D-glucosyl-(1→3)-*O*-β-D-glucosyl-(1→4)-D-glucose; and *O*-β-D-glucosyl-(1→4)-*O*-β-D-glucosyl-(1→3)-D-glucose) were obtained as gifts from E. T. Reese (U.S. Army Natick Labs, Natick, MA).[21].

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