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LYSIS OF YEAST CELL-WALLS: NON-LYTIC AND LYTIC (1→6)-β-D-GLUCANASES FROM *Bacillus circulans* WL-12

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ABSTRACT

Two types of extracellular (1→6)-β-D-glucanases are produced by *Bacillus circulans* WL-12, and these enzymes are differentiated by their ability to lyse yeast cell-walls. The non-lytic (1→6)-β-D-glucanase was isolated by a combination of Sephadex G-100, Bio-Gel P-100, and DEAE-Bio-Gel A chromatography. The purified enzyme was electrophoretically homogeneous and had a molecular weight of 52,000. For the substrate pustulan, the enzyme exhibited the following kinetic properties: pH, 5.0; K_m, 0.83 mg of pustulan/ml; V_{max}, 104 microequivalents of D-glucose released/min/mg of protein. Pustulan was hydrolysed by an endo-mechanism, producing D-glucose and gentiobiose as preponderant final products. The non-lytic enzyme was specific for the (1→6)-β-D-glucosidic linkage and did not hydrolyse branched, (1→3)-β-D-linked glucans containing (1→6)-interchain linkages. In contrast, the lytic (1→6)-β-D-glucanase produced D-glucose, gentiobiose, and gentiotriose as the final products of pustulan hydrolysis, and exhibited significant activity on branched (1→3)-β-D-glucans having (1→6)-interchain linkages. In these cases, the major products were gentiobiose and D-glucose, suggesting an ability of the lytic enzyme to cleave some (1→3)-linkages surrounding a (1→6)-branch-point. This latter property may explain the ability of this enzyme to weakly lyse yeast cell-walls.

INTRODUCTION

Isolated preparations of (1→3)-β-D- and (1→6)-β-D-glucanases are powerful tools in analysing the cytology and structure of yeast cell-wall polysaccharides[1,2].

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The success of such applications is dependent upon a convenient source of these enzymes as well as an understanding of their substrate specificities and modes of hydrolytic action.

Bacillus circulans WL-12[3] is a suitable source of (1→3)-β-D- and (1→6)-β-D-glucanases when grown on yeast cell-walls or yeast cell-wall glucan as sole carbon source[4,5,6]. Three types of (1→3)-β-D-glucanases are produced by this micro-organism, and the purification and properties of each of these enzymes have been reported. Two of these enzymes are lytic towards isolated yeast cell-walls[5], whereas the remaining (1→3)-β-D-glucanase is essentially non-lytic[4].

Two (1→6)-β-D-glucanases are also produced by *Bacillus circulans* WL-12. One of these enzymes is lytic upon yeast cell-walls and has been described in detail[6]. However, detailed information on the purification and properties of the other (1→6)-β-D-glucanase, which is non-lytic towards yeast cell-walls, has not been published and forms the basis of the present paper. Furthermore, in an attempt to understand the basis of lytic or non-lytic ability towards yeast cell-walls by enzymes that apparently act very similarly, the substrate specificities and hydrolytic actions of the two types of (1→6)-β-D-glucanase have been examined.

MATERIALS AND METHODS

Micro-organisms and culture conditions. — *Bacillus circulans* WL-12[3] was obtained from the Department of Food Science and Technology, University of California, Davis, U.S.A. For the production of (1→6)-β-D-glucanases, the organism was grown in liquid culture in Yeast Nitrogen Base (Difco. Lab) containing 100mM sodium phosphate buffer (pH 6.5) and either 0.5% of dried, baker's yeast cell-walls[4], or 0.25% of pustulan (Calbiochem) as carbon source. The medium was dispensed as 250-ml volumes in one-litre conical flasks, inoculated with 5.0 ml of starter culture grown in the same medium, and incubated for 46 h at 30° on a rotary shaker (180 r.p.m.).

Enzyme assays. — In standard assays for β-D-glucanase activity, 0.1 ml of enzyme solution was incubated for 20 min at 30° with 0.4 ml of glucan substrate (2.5 mg/ml) in 100mM sodium succinate buffer. A reaction pH of 5.0 was used for (1→3)-β-D-glucanase and non-lytic (1→6)-β-D-glucanase assays, and pH 6.0 for lytic (1→6)-β-D-glucanase assays. Reactions which showed linear rates with time were stopped by the addition of 0.5 ml of alkaline copper reagent and used for the determination of reducing sugars. One unit of β-D-glucanase is defined as that amount of enzyme which releases 1 μmol of reducing-sugar equivalent (expressed as glucose) per minute under the standard assay conditions.

Lytic activity on yeast cell-walls was checked by observing clearing in an agar plate containing suspended baker's yeast cell-walls (cup-plate technique[3]).

The ability of the enzyme preparations to hydrolyse various polysaccharide samples was tested by incubating the substrates at 5 mg/ml with 0.5 unit/ml of enzyme (pH 5.0). At desired intervals, samples were removed from the reaction

mixture for the determination of reducing-sugar release. Insoluble substrates were uniformly suspended with a Potter homogenizer, and reaction mixtures were gently shaken throughout incubation. Sodium azide (0.01%) was added to the reaction mixtures as an antimicrobial agent.

Enzyme preparations. — Lytic (1→6)-β-D-glucanase. This enzyme was purified as described previously[6], or by a modification of this procedure in which the selective adsorption on yeast glucan was omitted and the crude enzyme solution was concentrated by dialysis against polyethylene glycol 6000. This was followed by treatment with protamine sulphate[3] and chromatographic fractionations on CM-cellulose, DEAE-cellulose, hydroxylapatite, and Bio-Gel P-100 (Fleet and Manners, unpublished results). The enzyme exhibited elution behaviours similar to those published by Rombouts and Phaff[6]. Endo-(1→3)-β-D-glucanase and exo-(1→3)-β-D-glucanase were obtained as described previously[4,7].

Substrates. — The polysaccharide substrates used in this study are listed in Table I along with their structures, source, and references.

TABLE I

ORIGIN AND STRUCTURE OF GLUCAN SUBSTRATES

Substrate	*Main β-linkage type*	*Solubility in water*		*Source*	*Reference*
		Hot	*Cold*		
Laminarin	1→3	+	−	Nutritional Biochemicals Corp., U.S.A.	—
Laminarin IL5	1→3	+	−	HWU[a]	10
Laminarin SL5	1→3	+	+	HWU	11
Laminarin 6	1→3	+	+	HWU	10
Pachyman	1→3	−	−	HWU, UC[b]	12
Scleroglucan	1→3; 1/3 substituted with 1→6-linked single residues	+	+	The Pillsbury Co., Minneapolis, Minn., U.S.A.	—
Sclerotan	1→3; 1/3 substituted with 1→6-linked single residues	−	−	Dr. J. S. D. Bacon	13
Pustulan	1→6	+	−	Calbiochem, U.S.A.	—
Luteose	1→6	+	−	HWU	14
Baker's yeast, cell-wall (1→3)-β-D-glucan	1→3	−	−	HWU	15
Baker's yeast, cell-wall (1→6)-β-D-glucan	1→6	+	+	HWU	16
Saccharomyces cerevisiae, alkali-soluble glucan	1→3 1→6	−	−	HWU	17

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Analytical procedures. — Reducing sugars were determined by the Nelson–Somogyi method[8]. Total carbohydrate was measured by the phenol–sulphuric acid assay[9]. D-Glucose was used for the construction of standard curves. Protein was estimated by the method of Lowry *et al.*[18], with crystallized bovine serum albumin as standard. Total and partial hydrolyses with acid were performed as described previously[15].

Chromatography. — Descending paper chromatography was performed on Whatman No. 1 paper at 25° with ethyl acetate–pyridine–water (12:5:4 or 10:4:3). Non-reducing sugars were detected with an alkaline silver nitrate reagent[19], and reducing sugars with aniline oxalate[20]. Oligosaccharides of the laminari- and gentio-series were used as markers.

Chromatography on Sephadex G-100 (Pharmacia), Bio-Gel P-100, and DEAE-Bio-Gel A (Bio-Rad, Richmond, California) was performed according to the recommendations of the manufacturers.

Gel electrophoresis. — Dodecylsulphate electrophoresis was performed by the procedure of Ames[21] with a slab-gel apparatus (Bio-Rad, Richmond, California). The final acrylamide concentration in the separating gel was 12%. The discontinuous buffer system of Laemmli[22] was used. Protein standards for molecular weight determination were trypsinogen (Boehringer, Mannheim; mol. wt. 23,500), chymotrypsinogen A (Worthington, mol. wt. 25,700), ovalbumin (Boehringer, mol. wt. 45,000), beef-liver catalase (Boehringer, mol. wt. 60,000), and bovine serum albumin (Boehringer, mol. wt. 67,000).

Isoelectric focusing was performed with the same electrophoresis apparatus, following the recommendations of Vesterberg[23]. Carrier ampholytes (LKB-Produkter, Stockholm) with pH ranges of 3 to 11, 7 to 9, and 9 to 11 were used. Electrophoresis was also performed with the weakly acidic, discontinuous, cacodylic acid–imidazole buffer-system described by Richards *et al.*[24]. The final acrylamide concentration in the separating gel was 7%.

RESULTS

Purification of the non-lytic (1→6)-β-D-glucanase

Bacillus circulans WL-12 produced operational yields of (1→6)-β-D-glucanases when grown in a synthetic-based medium containing either isolated cell-walls of baker's yeast, cell-wall glucan, or pustulan as carbon source. Although highest levels of enzymes were produced by growth on cell walls or preparations of cell-wall glucan, growth on pustulan avoided the formation of excessive amounts of (1→3)-β-D-glucanases which made the purification procedure more difficult. In this study, the enzyme was produced by growth of the bacterium on pustulan, although it is stressed that the basic purification procedure described works equally well irrespective of the growth substrate.

Culture supernatant solutions containing (1→6)-β-D-glucanase activity were dialysed against mM sodium phosphate buffer (pH 7.0) for 24 h at 2° and concentrated

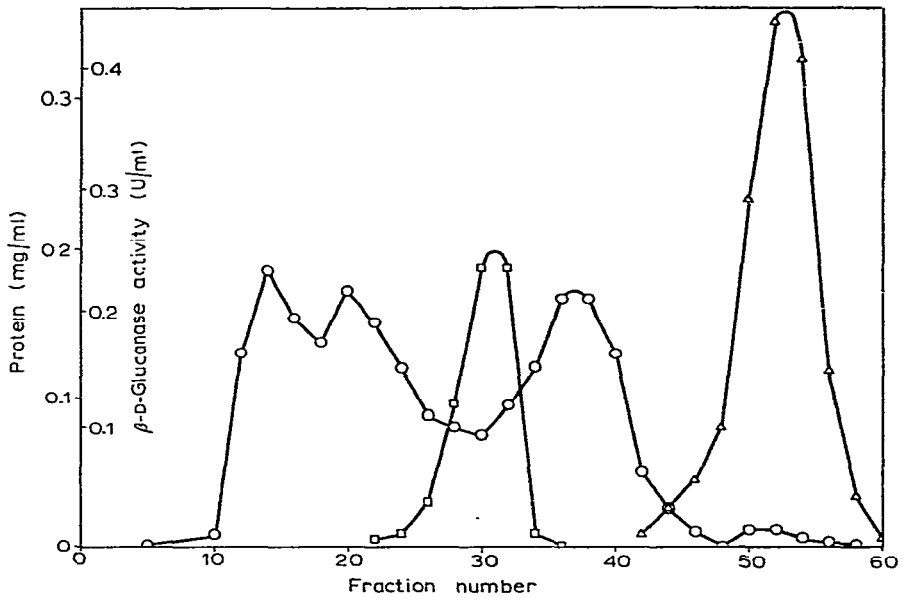

Fig. 1. Sephadex G-100 chromatography of β-D-glucanases from *B. circulans* WL-12. Concentrated enzyme solution (13.7 ml) was applied to a column (2.2 × 101 cm; void volume, 140 ml) of Sephadex G-100 equilibrated with 50mM sodium phosphate buffer (pH 7.0). Elution was effected with the same buffer at a flow rate of 6 ml per h, and fractions of 10 ml were collected: protein (—○—), (1→6)-β-D-glucanase (—△—), and (1→3)-β-D-glucanase (—□—).

by freeze-drying to a white powder; 1 g of enzyme powder (corresponding to ~1 litre of culture liquid) was reconstituted in 12.0 ml of distilled water, and centrifuged for 15 min at 10,000 *g* to remove inert, insoluble material. The supernatant enzyme-solution was treated with 3.5 ml of 5% protamine sulphate solution[3], and again centrifuged at 10,000 *g* to remove a rather voluminous precipitate. The supernatant solution was dialysed against 50mM sodium phosphate buffer as above, and fractionated by Sephadex G-100 chromatography (Fig. 1). As has been shown previously[4], (1→6)-β-D-glucanase activity has some affinity for the column matrix and is eluted late, well-separated from (1→3)-β-D-glucanase activity. Furthermore, this (1→6)-β-D-glucanase activity consists largely of the non-lytic form, exhibiting only a trace of lytic activity as measured by the cup-plate test. Fractions 47–57 were combined, dialysed against distilled water, and concentrated to 10.0 ml by rotary vacuum-evaporation at 25°. No loss in activity was experienced by this step. Attempts to concentrate this enzyme by ultra-filtration with Amicon (Lexington Corp., U.S.A.) UM-10 or PM-10 membranes invariably resulted in leakage of the enzyme through these membranes.

The concentrated (1→6)-β-D-glucanase was next fractionated on a column of Bio-Gel P-100 (2.2 × 103 cm, V_0 = 110 ml) equilibrated with 50mM sodium phosphate buffer (pH 7.0). The enzyme was eluted in a sharp peak of activity (V_e = 225 ml). Active fractions were combined, dialysed against 10mM sodium phosphate

TABLE II

SUMMARY OF THE STEPS INVOLVED IN PURIFICATION OF NON-LYTIC (1→6)-β-D-GLUCANASE

Purification step	*Volume* (*ml*)	*(1→6)-β-D-Glucanase* (*U/ml*)	*Protein concentration* (*mg/ml*)	*Specific activity* (*U/mg of protein*)	*Purification* (*fold*)	*Yield* (%)
Dialysed culture liquid	960	0.091	0.098	0.93		100
Freeze-dried and redissolved	12.3	5.60	3.52	1.59	1.71	79
Protamine sulphate precipitation and dialysis	13.7	4.95	3.15	1.57	1.69	78
Sephadex G-100 chromatography	109.2	0.24	0.0045	53.3	57.3	30
Concentration	9.9	2.66	0.052	51.2	55.0	30
Bio-Gel P-100 chromatography	39.7	0.58	0.0078	74.4	80.0	26
DEAE-Bio-Gel A chromatography	59.5	0.36	0.0047	76.6	82.4	25

buffer (pH 7.0), and applied to a column (1.3 × 10.7 cm) of DEAE-Bio-Gel A. The column was equilibrated and eluted with 10mM sodium phosphate buffer (pH 7.0). Non-lytic (1→6)-β-D-glucanase activity did not adsorb onto the column under these conditions, and was eluted in the initial fractions. Lytic (1→6)-β-D-glucanase[6] and non-lytic (1→3)-β-D-glucanase[4] are bound to this column and are effectively removed from non-lytic (1→6)-β-D-glucanase preparations if small residual concentrations are still present.

Table II presents a summary of the steps involved in the purification of the non-lytic (1→6)-β-D-glucanase. The loss of (1→6)-β-D-glucanase activity during passage over Sephadex G-100 is due largely to an almost completely irreversible binding of the lytic (1→6)-β-D-glucanase component to this column material[6].

Properties of the non-lytic (1→6)-β-D-glucanase

Electrophoretic properties, molecular weight, and isoelectric point. — Quantities (6 μg) of enzyme protein were applied to single wells of a slab gel for dodecylsulphate electrophoresis. Only a single band of protein was observed after subsequent staining. On the basis of its mobility in the gel, the enzyme was assigned a molecular weight of 52,000 (*cf.* 54,000 found for the lytic (1→6)-β-D-glucanase[6]).

In isoelectric-focusing experiments, the enzyme was made to migrate from both the low and high pH side of the gel by using carrier ampholytes with pH ranges from 7 to 11. The gels were either stained[21] or segmented and extracted for pH measurements[23] and for activity determination. Only one band of protein was detected, and this proved to be the active (1→6)-β-D-glucanase. The isoelectric point was at pH 8.2–8.3.

Disc electrophoresis of the active enzyme preparation with a weakly acidic buffer-system also revealed a single protein band which corresponded to active enzyme.

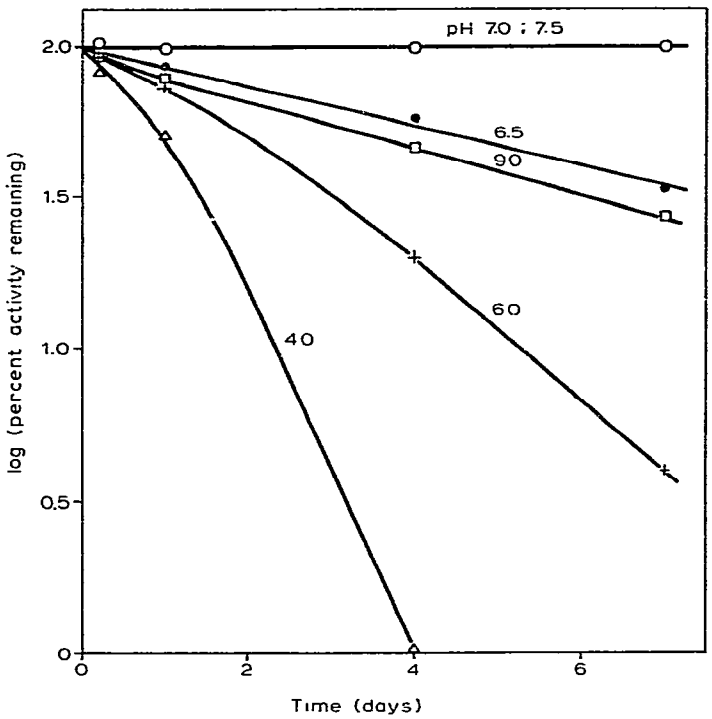

Fig. 2. Stability of non-lytic (1→6)-β-D-glucanase at various pH values. Enzyme (0.05 U per ml) was incubated at 30° in 100mM sodium phosphate buffers (pH 6.0 to 7.5), 100mM sodium succinate buffer (pH 4.0), or 100mM glycine–NaOH buffer (pH 9.0), all containing 0.01% of sodium azide. At intervals, the residual activity was measured as described in Materials and Methods.

Stability and inhibition. — The non-lytic (1→6)-β-D-glucanase was stable for at least 2 months when stored at 2° in 10mM sodium phosphate buffer (pH 7.0). Optimum stability was at pH ~7.0 (Fig. 2). Neither Ca^{2+} nor EDTA affected enzyme stability at pH 7.0. Freezing of the pure enzyme caused a 30% loss of activity, and the remainder of the activity was lost after 4 months of storage at −20°.

Enzyme activity was inhibited by Hg^{2+} (mM) and iodoacetamide (10mM), the latter indicating the presence of sulphydryl groups in the enzyme. The divalent cations Co^{2+}, Ca^{2+}, Mg^{2+}, and Zn^{2+} exhibited no effect on enzyme activity. The enzyme was insensitive to sodium azide (0.01%), which was used as an antimicrobial agent.

Kinetics. — The rate of hydrolysis of pustulan was linear for 1 h (2 mg/ml of substrate, 0.01 enzyme U/ml, pH 5.0). The optimum pH, as determined with a series of Tris–succinate buffers, was 5.0. At this pH, the activity was 40% higher in 80mM succinate buffer and 30% higher in 80mM sodium phosphate buffer.

The enzyme followed Michaelis–Menten kinetics, as determined by the Lineweaver–Burk and Hill equation procedures[25]. A K_m of 0.83 mg of pustulan/ml and a V_{max} of 104 microequivalents of glucose released/min/mg of protein were calculated.

TABLE III

HYDROLYSIS OF VARIOUS GLUCAN PREPARATIONS WITH THE NON-LYTIC AND LYTIC (1→6)-β-D-GLUCANASE ENZYMES[a]

Glucan substrate	*Non-lytic enzyme Reaction time (h)*					*Lytic enzyme Reaction time (h)*				
	2	*5*	*8*	*12*	*24*	*2*	*5*	*8*	*12*	*24*
Laminarin IL5	1.4	1.2	1.2	1.2	—	51	84	126	130	—
Laminarin SL5	1.2	1.0	1.0	0.9	—	141	183	286	300	—
Laminarin 6	1.4	1.4	1.4	1.2	—	180	225	286	290	287
Laminarin 6 (Smith-degraded)	—	—	—	—	—	46	—	90	—	—
Pachyman	0	0	0	0	—	10	12	15	15	—
Sclerotan	0	0	0	0	—	0	0	0	0	—
Baker's yeast, insoluble (1→3)-β-D-glucan	0	0	0	0	—	38	—	48	50	52 (77.3)[b]
Baker's yeast (1→6)-β-D-glucan	600	700	800	920	923	750	920	1015	1115	1110
Sacch. cerevisiae, alkali-soluble glucan	63	75	88	95	102	160	220	—	300	340

[a]The values in the Table represent reducing-sugar release, expressed as μg D-glucose equivalents/ml; the enzyme concentration was 0.5 U/ml in both cases; the substrate concentration was 5 mg/ml. [b]Represents total carbohydrate solubilization after 24 h of hydrolysis.

Substrate specificities and action patterns of the non-lytic and lytic (1→6)-β-D-glucanases. — The (1→6)-β-D-glucans pustulan and luteose were extensively degraded by both enzyme types, producing, initially, a series of gentiosaccharides. For the non-lytic enzyme, these were eventually degraded to D-glucose and gentiobiose (with only traces of gentiotriose), whereas the lytic enzyme gave D-glucose, gentiobiose, and gentiotriose as major products. This result was consistent with the inability of the latter enzyme to cleave gentiotriose when tested separately. On the basis of these products of hydrolytic action, both enzymes can be classified as endo-(1→6)-β-D-glucanases.

The highly branched (1→6)-β-D-glucan from baker's yeast cell-walls[16] and (1→6)-β-D-glucosidic linkages in the alkali-soluble glucan from *Saccharomyces cerevisiae* NCYC 1109[17] were readily hydrolysed by both enzyme types. The lytic enzyme exhibited a rather higher activity towards these two substrates (Table III). The initial products for both enzymes were gentiosaccharides, and smaller amounts of unidentified, mixed-linkage oligosaccharides. For the non-lytic enzyme, the preponderant final products of hydrolysis were D-glucose, gentiobiose (R_{Glc} 0.44), mixed-linkage oligosaccharide (R_{Glc} 0.31), gentiotriose (trace only, R_{Glc} 0.18), and mixed-linkage oligosaccharide (R_{Glc} 0.12). Both glucan substrates yielded similar soluble products of hydrolysis, except that gentiotriose was a major product for the lytic enzyme. The mixed-linkage oligosaccharide (R_{Glc} 0.31) was isolated by preparative paper chromatography and characterized as 3^2-β-D-glucosylgentiobiose[2].

The non-lytic enzyme was highly specific for the (1→6)-β-D-glucosidic linkage and exhibited no hydrolytic action when tested on a wide variety of other polysaccharide substrates, including both linear and branched (1→3)-β-D-linked glucans (Table III). Although the lytic enzyme showed the highest affinity towards (1→6)-β-D-linked substrates, this enzyme also exhibited small, but significant, activity on branched (1→3)-β-D-glucans containing (1→6)-interchain linkages (laminarin IL5, laminarin SL5, and laminarin 6, Table III). The degree of laminarin hydrolysis by the lytic enzyme increased with the extent of branching of this substrate. Laminarin substrates IL5, SL5, and 6 have degrees of branching 0.08, 1.6, and 1.7, respectively[10,11]. No activity was noted towards periodate-oxidized samples of laminarin 6 or IL5, although a Smith-degraded sample of laminarin was weakly hydrolysed (Table III).

After concentration, the products of laminarin hydrolysis by the lytic enzyme were shown by paper chromatography to consist mainly of gentiobiose and glucose, together with traces of higher mixed-linkage oligosaccharides (R_{Glc} 0.31 and 0.24) and gentiotriose. The products were isolated by preparative paper chromatography and further examined to confirm their identities. The product corresponding to gentiobiose yielded only D-glucose on total hydrolysis with acid, and gentiobiose and D-glucose on partial hydrolysis with acid. It was not hydrolysed by samples of exo-(1→3)-β-D- and endo-(1→3)-β-D-glucanases, as expected. On partial hydrolyses with acid, the oligosaccharide having R_{Glc} 0.31 yielded D-glucose, gentiobiose, and laminaribiose. After borohydride reduction and partial hydrolysis with acid, a mixture of D-glucose, laminaribiose, D-glucitol, and gentiobiitol was obtained. On incubation with an exo-(1→3)-β-D-glucanase, a mixture of glucose and gentiobiose was liberated. Collectively these results characterize this oligosaccharide as 3^2-β-D-glucosylgentiobiose [Glc-(1→3)-Glc-(1→6)Glc]. Insufficient amounts of the higher products of laminarin hydrolysis were obtained for a detailed analysis. However, the product whose mobility corresponded to that of gentiotriose was resistant to (1→3)-β-D-glucanase hydrolysis, but was degraded to D-glucose and gentiobiose on treatment with the non-lytic (1→6)-β-D-glucanase. The oligosaccharide having R_{Glc} 0.24 was not hydrolysed by the non-lytic (1→6)-β-D-glucanase, but gave gentiobiose and laminaribiose on hydrolysis with the endo-(1→3)-β-D-glucanase, and D-glucose and 3^2-β-D-glucosylgentiobiose on hydrolysis with the exo-(1→3)-β-D-glucanase. These results suggest a possible structure for this oligosaccharide as 3^2-β-D-laminaribiosylgentiobiose [Glc-(1→3)-Glc-(1→3)-Glc-(1→6)-Glc].

Pachyman, an insoluble, slightly branched (1→3)-β-D-glucan, was only very weakly hydrolysed by the lytic enzyme. Sclerotan and scleroglucan were resistant to hydrolytic cleavage by the lytic enzyme.

The very insoluble, yeast cell-wall (1→3)-β-D-glucan[15], which contains ~3% of branching, was weakly hydrolysed by the lytic (1→6)-β-D-glucanase, giving mainly gentiobiose and smaller amounts of glucose.

DISCUSSION

There are only a few reports on the occurrence, isolation, and properties of enzymes that are capable of hydrolysing the (1→6)-β-D-glucosidic linkage[26–29]. So far, only endo-(1→6)-β-D-glucanases have been described, although the exo-(1→3)-β-D-glucanases of yeasts also exhibit exo-(1→6)-β-D-glucanase activity[30,31].

The non-lytic (1→6)-β-D-glucanase of *B. circulans* readily hydrolyses the (1→6)-β-D-linked trisaccharide gentiotriose and, in this respect, differs from the other endo-(1→6)-β-D-glucanases described[28,29], including the lytic (1→6)-β-D-glucanase from *B. circulans*[6]. Also, unlike other (1→6)-β-D-glucanases, the non-lytic enzyme is highly specific for the (1→6)-β-D-glucosidic linkage and is not able to cleave the (1→3)-β-D-glucosidic linkage when it occurs in association with the (1→6) linkage. The latter property has been well-documented for the (1→6)-glucanase of *Gibberella fujikuroi*, where the (1→3)-β-D-glucosidic linkages of the substituted glucosyl residues in the non-branched laminarin from *Eisenia bicyclis* are hydrolysed. The well-defined specificity of the non-lytic (1→6)-β-D-glucanase of *B. circulans* makes this enzyme very useful in structural analyses of polysaccharides.

The difference in substrate specificity between the lytic and non-lytic (1→6)-β-D-glucanases from *B. circulans* suggested a possible explanation for their contrasting behaviours towards yeast cell-walls. The ability of the lytic enzyme to hydrolyse laminarin appears to be related to the degree of branching of this substrate through the (1→6)-β-D-glucosidic linkage. Previous[10,11] interpretations of laminarin structure proposed a molecule of ~20 D-glucose residues, with a low degree of branching which may vary from 0.05 to 3.0 branch-points per molecule, depending on the source and solubility of the polysaccharide. More recent work[33] suggests that the inter-chain linkages may attach single D-glucosyl groups to the main chains of (1→3)-linked D-glucose residues. The laminarins used in this study were not known to contain inter-residue (1→6)-β-D-glucosidic linkages, such as occur in the laminarin from *Eisenia bicyclis*[32]. The activity of the lytic enzyme on laminarin was higher for the more highly branched laminarin samples, and the main products, in all cases, were gentiobiose and D-glucose. No (1→3)-β-D-linked oligosaccharides were produced. The formation of gentiobiose could only occur through cleavage of the (1→3)-β-D linkages as shown in Fig. 3*a*, although such hydrolytic action would not explain the formation of D-glucose. The production of small proportions of gentiotriose and 3^2-β-D-glucosylgentiobiose would be consistent with this mechanism of action if a very small percentage of the laminarin molecules were branched as shown in Figs. 3*b* and 3*c*. The fact that Smith degradation of laminarin decreases the susceptibility to the lytic enzyme is further confirmation of the presence of side chains containing single D-glucosyl groups in the original polysaccharide[33], and of the validity of the deductions concerning the specificity of the lytic enzyme. Smith degradation would remove the side chains as shown in Figs. 3*a* and 3*b*, but would not eliminate the side chain seen in Fig. 3*c*[34]. Side chains of the latter type have not been reported previously in laminarin structures.

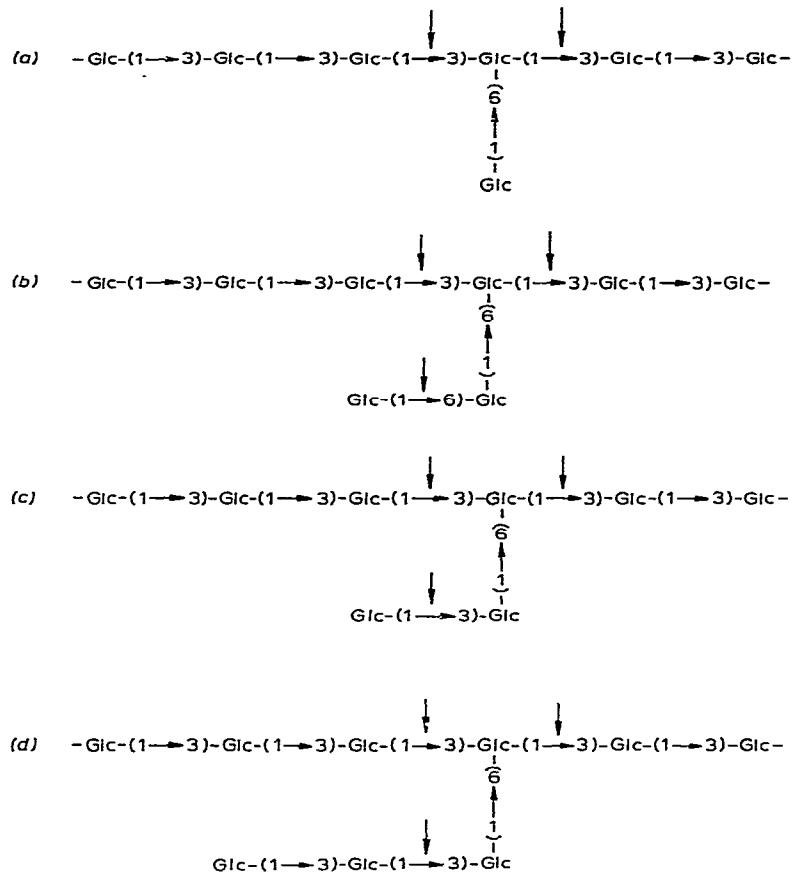

Fig. 3. Action of the lytic (1→6)-β-D-glucanase on (1→3)-β-D-linked glucans: the arrows indicate some possible points of hydrolysis.

The presence of D-glucose in the lytic-enzyme hydrolysates of laminarin might be explained by (*i*) a weak action of the enzyme on gentiobiose over long periods of incubation and at high concentrations of enzyme, (*ii*) an occasional splitting of the linkages shown by the side-chain in Figs. 3*b* and 3*c*, and (*iii*) cases where the branch points are situated one D-glucose residue from the ends of the molecule.

The inability of the lytic enzyme to hydrolyse sclerotan or scleroglucan might be explained by the relatively high degree of branching of this substrate and therefore possible steric hindrance of enzyme attack. Every fourth residue of sclerotan is branched through position 6 with a single D-glucosyl group[13].

Since the lytic enzyme causes a weak lysis of yeast cell-walls, it was expected that this enzyme would hydrolyse isolated preparations of the wall rigid-glucan component. This very insoluble glucan consists exclusively of (1→3)-β-D-linked residues, with only 3% of branching through the (1→6) linkage. Because of the low level of branching, this glucan was only weakly attacked by the lytic (1→6)-β-D-

glucanase and was not visibly solubilized. The formation of gentiobiose as a major product is consistent with the ability of this enzyme to cleave adjacent (1→3)-β-D-glucosidic linkages, as shown in Figs. 3*a* or 3*d*. Such action is likely to cause some loss of cell-wall integrity, and hence lysis of intact cell-walls, although a visible solubilization of the isolated glucan was not observed. This result is not entirely unexpected for the isolated glucan preparation, as the products of the "debranching" action would be linear (1→3)-β-D-glucans of large molecular weight; these polysaccharides are not known for their ease of solubility. Interestingly, the degree of solubilization of this glucan by the lytic enzyme (77 μg/ml, Table III) is ~1.5–2.0% which, given the uncertainties of underestimation with enzyme assays using insoluble substrates, is not a major discrepancy from the 5–6% of residues expected to be (1→6)-linked[15]. The non-lytic (1→6)-β-D-glucanase had no hydrolytic action on the insoluble (1→3)-β-D-glucan of the yeast cell-wall. Therefore, it seems that the peculiar "lytic property" of the lytic (1→6)-β-D-glucanase resides in its ability to hydrolyse certain (1→3)-β-D linkages surrounding a (1→6)-interchain linkage in the substrate.

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