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PURIFICATION, CHARACTERIZATION, AND ACTION-PATTERN STUDIES ON THE ENDO-(1 → 3)-β-D-GLUCANASE FROM *Rhizopus arrhizus* QM 1032*

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ABSTRACT

The extracellular (1 → 3)-β-D-glucanase [(1 → 3)-β-D-glucan glucanohydrolase, EC 3.2.1.6] produced by *Rhizopus arrhizus* QM 1032 was purified 305-fold in 70% overall yield. This preparation was found to be homogeneous by ultracentrifugation (sedimentation velocity and equilibrium studies), electrophoresis on acrylamide gel with normal, sodium dodecyl sulfate, and urea–acetic acid gels, and upon isoelectric focusing. The amino acid composition of the enzyme has been determined and it possesses a carbohydrate moiety composed of mannose and galactose (in the ratio ~5:1) that is linked to the protein through a 2-acetamido-2-deoxyglucose residue. The molecular weight, as determined by equilibrium sedimentation, is 28,800 and this number was confirmed by electrophoresis on gels of sodium dodecyl sulfate. The enzyme does not possess subunit structure. It hydrolyzes its substrates with retention of configuration and possesses transglycosylating ability. The rates of hydrolysis of a wide variety of substrates were determined, and its action pattern on a series of oligosaccharides containing mixed (1 → 3)-, (1 → 4)-, and (1 → 6)-β-D-glucopyranosyl residues was investigated. The enzyme favors stretches of β-D-(1 → 3) linkages, but it can hydrolyze β-D-(1 → 4) linkages that are flanked on the non-reducing side with stretches of β-D-(1 → 3) links. The enzyme will not act on (1 → 6)-β-D-glucosyl linkages located in stretches of β-D-(1 → 3) and will not act on (1 → 3) β-D-glycosidic linkages involving sugars other than D-glucose.

INTRODUCTION

The extracellular, endo-acting (1 → 3)-β-D-glucanase [(1 → 3)-β-D-glucan glucanohydrolase, EC 3.2.1.6] from *Rhizopus arrhizus* QM 1032, originally discovered by Reese and Mandels[1], is of interest because of its use in the structural analysis of polysaccharides[2], and because of its unusual action-pattern. Perlin and his co-

*Dedicated to Professor Dexter French on the occasion of his 60th birthday. The authors thank him for his contributions, which have been of much help to everyone in the field. One of us (D.R.C.) would like to express especial thanks to Dexter for his inspiration and continued faith.

workers[3-5] have shown that, although it is classed as a (1 → 3)-β-D-glucanase, crude preparations appear to split certain (1 → 4)-β-D-glucosidic linkages in oat and barley glucans and in lichenan. There have been three purification schemes for this enzyme reported in the literature. Those of Garcia-Ballesta[6] and Moore and Stone[7] are partial purifications. The former gave a product that was still contaminated with amylase and with an exo-attacking enzyme, and the latter is stated by the authors to result in only partial purification. The purification reported by Marshall[8] is more extensive, and more data are given on the purest preparation, but no claim to having achieved purification to homogeneity is made. The purification scheme reported here produces material of considerably higher specific activity, and in considerably higher yield, than any previously reported and the material produced is homogeneous, as judged by a number of modern criteria. We have investigated the activity of this highly purified enzyme on a number of polysaccharides and oligosaccharides and, in addition, have examined its action-pattern on a number of oligosaccharides.

It should be noted that endo-(1 → 3)-β-D-glucanases have been isolated from other organisms. Yamamoto and Nagasaki, for instance, have recently isolated a crystalline enzyme of this type from *Rhizopus chinensis* R-69 whose properties are significantly different from those of the *Rhizopus arrhizus* enzyme[9]. Moore and Stone have isolated an endo-(1 → 3)-β-D-glucanase from the higher plant *Nicotiana glutinosa* and compared it with the enzyme from *Rhizopus arrhizus*[7].

RESULTS

Purification of the glucanase. — The purification scheme was designed to handle 10-liter batches of culture filtrate produced in a New Brunswick fermentor. All workers who have reported purifications of this glucanase have used a culture medium containing cellobiose, with the exception of Garcia-Ballesta[6]. It has been our experience that, as claimed by Reese and Mandels[1], the presence of cellobiose in the medium is necessary for the production of high enzyme-levels. We have carried out extensive studies on the factors that lead to maximum enzyme concentration in

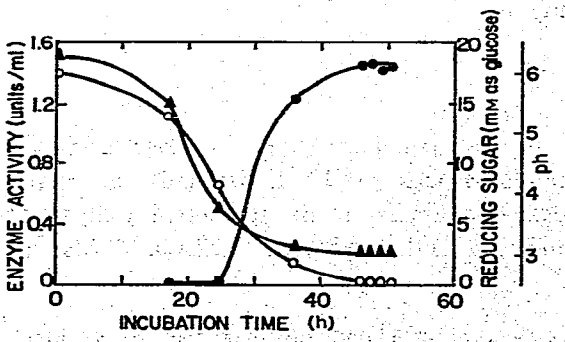

Fig. 1. Variation of glucanase activity (●), reducing sugar (○), and pH (▲) in the culture medium during growth of *Rhizopus arrhizus*. The culture conditions are given in the Experimental section.

the culture filtrate. Nothing is gained by using concentrations of cellobiose higher than that given in Materials and Methods, and at this level of carbohydrate, the maximum concentration of enzyme occurs at the point of carbohydrate exhaustion in the medium. It also correlates with a drop in the pH of the medium to the region of 3.0. The factor most closely correlated with maximum concentration of enzyme is this drop in pH, as it signals the maximum enzyme level, regardless of the amount of residual cellobiose (Fig. 1).

The procedure, as may be seen in Table I, involves concentration and dialysis, fractionation with ammonium sulfate, and column chromatography employing CM-cellulose and Sephadex G-100. The concentration and dialysis is performed with an Amicon ultrafiltration system equipped with a PM-10 membrane, and is an important step in the procedure. It gives a quantitative recovery of the enzyme, with a 7.5-fold purification that is undoubtedly largely due to the removal of contaminating protein existing as small peptides in the culture filtrate.

As may be seen in Table I, the method gives very high yields, and the resulting material is very pure, as will be seen later. It can be used routinely to produce 70% overall yields and final specific activities above 550 units/mg of protein, with more than 7500 units being isolated as completely pure material from 10 liters of culture filtrate.

Criteria of purity. — The enzyme produced by this procedure was pure, as judged by ultracentrifuge studies (both sedimentation velocity and equilibrium studies). It was also homogeneous with respect to electrophoresis on acrylamide gels, with normal sodium dodecyl sulfate, and urea–acetic acid gels, and upon isoelectric focusing.

Amino acid composition. — The amino acid composition of the enzyme is shown in Table II, and presents no unusual features. The quality of this analysis is indicated by the fact that the minimum molecular weight calculated from the amino acid

TABLE I

SUMMARY OF GLUCANASE PURIFICATION

Stage of Purification	*Volume at end*	*Activity units/ml*	*Total units*	*Recovery this step*	*(%) Total*	*Protein (mg/ml)*	*Spec. act. units/mg*	*Purification (fold)*
Culture filtrate	9310 ml	1.18	10986	—	100	0.636	1.85	—
Concentration–dialysis	261 ml	42.8	11171	102	102	2.08	13.9	7.5
Ammonium sulfate fractionation (35–75%)	47.2 ml	216	10195	91.3	92.8	6.38	33.9	18.3
CM-Cellulose column	35.4 ml	257	9098	89.2	82.8	0.566	454	245
Sephadex G-100 column	83.3 ml	91.6	7630	83.9	69.5	0.162	565	304

TABLE II

AMINO ACID COMPOSITION[a]

Amino acid	*Residues/mol*	*Integral values*	*Mol %*
Asx	40.7	41	14.49
Asp		9	3.18
Asn		32[b]	11.31
Thr	19.7	20	7.07
Ser	24.0	24	8.48
Glx	19.6	20	7.07
Glu		10	3.53
Gln		10[b]	3.53
Pro	16.9	17	6.01
Gly	39.9	40	14.13
Ala	25.4	25	8.83
Cys/2	4.2[c]	4 (2 Cys)	1.41
Val	15.3	15	5.30
Met	4.6[c]	5	1.77
Ile	10.4	10	3.53
Leu	10.9	11	3.89
Tyr	9.0	9	3.18
Phe	13.9	14	4.95
Lys	9.7	10	3.53
His	3.7	4	1.41
Arg	9.0	9	3.18
Trp	5.0	5	1.77

[a]Total residues: 283. Calculated mol.wt.: 29769. Calculated V: 0.710 cm^3/g; this is the partial specific volume used in the calculation of the sedimentation-equilibrium value for mol. wt. [b]Asn and Gln are arbitrary assignments for purposes of V calculation. [c]Cys/2 and Met results from peroxyformic acid-oxidized sample; determined as cysteic acid and methionine sulfone, respectively.

composition is in very good agreement with that determined by equilibrium sedimentation, and the $E^{280}_{1\%}$ calculated from the tyrosine and tryptophan analyses, using the molar absorptivity of 1197 for tyrosine[10] and 5559 for tryptophan[10], is in remarkable agreement with the observed value (calculated 12.96, observed 12.98 cm^{-1}). As the enzyme gives no detectable reaction with Ellman's reagent, it may be assumed that the four cysteine residues exist in the form of two cystine components, and that the enzyme possesses no free sulfhydryl groups.

Molecular-weight determinations. — Molecular weights were determined by using equilibrium sedimentation by the Yphantis technique[11], electrophoresis on sodium dodecyl sulfate gels, and gel-permeation chromatography on Sephadex G-100. Equilibrium sedimentation gave a value of 28,800, sodium dodecyl sulfate gel-electrophoresis 32,100, and gel permeation 42,700. The sodium dodecyl sulfate gel-electrophoresis was conducted at 7.5, 10, and 12.5% gel concentrations and there was no effect on the apparent molecular weight, indicating that the carbohydrate moiety on the enzyme (to be discussed later) did not interfere with the binding of sodium dodecyl sulfate. If sodium dodecyl sulfate gel electrophoresis was done

without pre-reduction of the enzyme, the apparent molecular weight was 29,800, indicating that reduction of the disulfide bonds significantly alters the shape of the protein–sodium dodecyl sulfate complex. The larger apparent molecular-weight given by gel-permeation studies probably indicates the influence of a shape factor. Gel permeation, unlike equilibrium sedimentation and sodium dodecyl sulfate-gel techniques, is sensitive to molecular shape. Given the limitations of the technique, the molecular weight by sodium dodecyl sulfate gel is in satisfactory agreement with that determined by equilibrium sedimentation, and this value is in remarkably good agreement with that determined from the amino acid analysis.

Carbohydrate moiety. — Analysis of the glucanase preparation for carbohydrate by the phenol–sulfuric acid procedure[12] gave a value of 3.9%, expressed as glucose. 2-Amino-2-deoxyglucose was one of the components appearing in the amino acid analysis, amounting to 0.82 mol per mol of enzyme. Thus, each enzyme molecule contains a single 2-amino-2-deoxyglucose residue and it would appear, from our present knowledge of glycoprotein structure, that this residue is *N*-acetylated in the native enzyme and is the point of attachment of the neutral carbohydrate to be discussed later[13]. Investigation of the monosaccharides occurring in an acid hydrolyzate of the enzyme, by thin-layer chromatography on kieselguhr, showed the occurrence of a small proportion of galactose and a considerably larger proportion of mannose. Rough quantitation of the amounts of these two monosaccharides by comparison of intensity of the spots compared with those of standards, showed that the molar ratio of mannose:galactose was 5:1. One six-unit oligosaccharide chain per enzyme molecule would give a neutral-sugar analysis of 3.4%. If the neutral carbohydrate analysis just given, which was based on a glucose standard, is corrected for the 5:1 mannose–galactose ratio, using the correction factors of DuBois *et al.*[12], the corrected value is 3.3%, which is in good agreement with that expected from a six-unit chain of neutral-sugar residues.

Other properties of the enzyme. — The $E_{1\%}^{280}$ value for the enzyme is 12.48 cm^{-1} and when this is corrected for the carbohydrate content, the value is 12.98 cm^{-1} for the protein portion alone. The isoelectric point of the enzyme, as determined by isoelectric focusing, is 7.5, and the isoionic[14] pH is 7.35. The Arrhenius activation-energy for the hydrolysis of laminaran by the enzyme was found to be 11,800 cal mol^{-1}. The effect of temperature on the kinetic parameter K_m (the Michaelis constant) was determined and it was found to be insensitive to temperature over the range 10–37° and to have the value of 0.314 mg/ml. This would suggest that, at temperatures near the freezing point, it might be possible to carry out experiments on the enzyme–substrate complex with little or no interference from substrate turnover.

Stereochemistry of the hydrolysis. — The stereochemistry of the enzymic hydrolysis was determined by monitoring the mutarotation of the hydrolysis products. After the initial time required for completion of the enzymic hydrolysis (5 min under the conditions chosen), the rate of change of optical rotation with time followed precisely the kinetics expected of a mutarotation process[15], and as the observed change

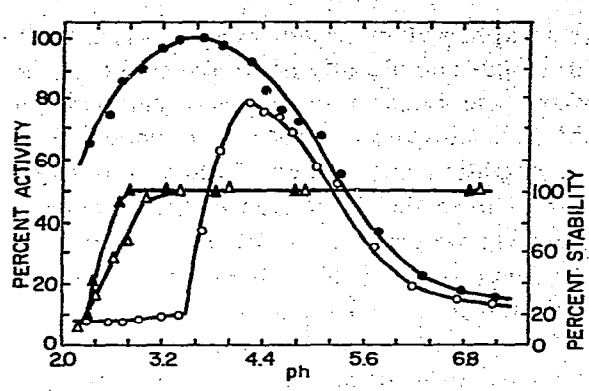

Fig. 2. Effect of pH on the rate of action and stability of the enzyme. Solid symbols, 0.25 mg/ml gelatin; open symbols, no gelatin. (●, ○) Activity; (▲,△) stability on exposure to various pH conditions for 5 min, which corresponds to the incubation time used in the pH–rate study.

in rotation was upward, the hydrolytic mechanism proceeds with retention of configuration.

The dependence of enzyme activity and stability on pH. — Reese and Mandels, in their original report on this enzyme[1], stated that its pH optimum was 4.8, and it has been usual to conduct assays at this pH, as was done in the present work. Both Garcia-Ballesta[6] and Marshall[8] have observed a shift in the pH optimum of the enzyme to lower values in the presence of an extraneous protein added to stabilize the enzyme. We have studied the effect of the addition of gelatin on the pH optimum and pH stability of the enzyme at constant ionic strength of the buffer. The results of these experiments are shown in Fig. 2.

Demonstration of transglycosylase activity. — It would appear from the literature that only configuration-retaining, glycoside hydrolases catalyze transglycosylation reactions. As one of the major importances of the present enzyme is its use in structural studies on polysaccharides, it was obviously important to determine whether the enzyme can bring about transglycosylation. This would lead to erroneous interpretation of structure, if it were significant and if it were ignored. We have investigated the products formed by the enzyme when it acts on all members of the homologous series from laminarabiose to laminaraheptaose. These results show that, when the enzyme acts on laminarapentaose it produces, in addition to the expected products of lower molecular weight, oligosaccharides that appear upon paper chromatography in the positions for hexa- and hepta-saccharides, with possibly some octa- and nonasaccharide as well. The linkage nature of these higher oligosaccharides, which are clearly the result of transglycosylation reactions, was investigated by submitting them to the action of the exo-(1 → 3)-D-glucanase from the Basidiomycete QM 806. This enzyme's specificity has been carefully investigated[16] and it is known that it removes β-(1 → 3)-linked D-glucosyl groups from the non-reducing terminal of a glucan chain. Its action is blocked by anomalous linkages, with the exception of those of the β-D-(1 → 6) type, which are by-passed, resulting in the production of gentiobiose

TABLE III

RELATIVE RATES[a] OF HYDROLYSIS OF SOME POLYSACCHARIDES AND OLIGOSACCHARIDES BY *Rhizopus arrhizus* ENDO-(1 → 3)-β-D-GLUCANASE

Substrate	*Relative rate*
Soluble laminaran *(L. cloustoni)*	1000
Insoluble laminaran *(L. cloustoni)*	994
L. digitata lamanaran	881
Ecklonia laminaran	76
Sclerotium rolfsii glucan	65
Barley β-D-glucan	440
Oat β-D-glucan	425
Yeast D-glucan	105
Pachyman	71
Laminaraheptaitol	722
Laminarahexaitol	560
Laminarapentaitol	405
Laminaratetraitol	111
Laminaratriitol	31
Laminarabiitol	17
4-*O*-β-Laminarabiosyl-D-glucitol	22
4-*O*-β-Laminaratriosyl-D-glucitol	85
4-*O*-β-Laminaratetraosyl-D-glucitol	306
4-*O*-β-Laminarapentaosyl-D-glucitol	536
6-*O*-β-Laminarabiosyl-D-glucitol	19
6-*O*-β-Laminaratriosyl-D-glucitol	83
6-*O*-β-Laminaratetraosyl-D-glucitol	280
6-*O*-β-Laminarapentaosyl-D-glucitol	492

[a]On the rate scale used, the following substances gave zero rate of reaction: 3-*O*-β-cellotriosyl-D-glucitol, cellobiitol, gentiobiitol, 3-*O*-β-D-galactosyl-D-glucitol, 6-*O*-β-D-galactosyl-D-glucitol, corn amylose, corn amylopectin, pustulan, crown-gall polysaccharide, cello-oligosaccharides, clam glycogen, calf-liver glycogen, yeast mannan, nigeran, chondroitin sulfate, rhamnogalactan, sugar beet arabinan, guar gum, locust gum, (1 → 4)-β-D-xylan, and (1 → 3)-β-D-xylan.

from this structural feature. It is evident that, if the higher oligosaccharides produced by the endo-glucanase as the result of transglycosylation are entirely β-D-(1 → 3)-linked, then the Basidiomycete enzyme should produce only D-glucose and laminarabiose when it acts upon them. On the other hand, if linkages other than β-D-(1 → 3) are present, other oligosaccharides will be present in the mixture. The mixture of higher oligosaccharides resulting from the action of the endo-glucanase on laminarapentaose was submitted to the action of the Basidiomycete glucanase, and D-glucose and laminarabiose were the only products observed. This indicates that the linkages produced by the transglycosylation process are all β-D-(1 → 3).

Relative rates of hydrolysis of poly- and oligo-saccharides. — The relative rates of hydrolysis of a broad range of glycosidically linked materials are given in Table III. These data are of obvious interest in connection with possible structural studies with the enzyme, and are also useful for the purpose of gaining a general knowledge of its action pattern.

Action pattern of the endo-glucanase on a series of oligosaccharides. — Studies were undertaken to obtain information on the action pattern of the enzyme on a series of oligosaccharides of known structure. Moles of each of the hydrolysis products, and that of the starting material, were obtained by quantitative paper-chromatographic analysis. Such data allow a number of significant conclusions to be drawn concerning the mode of action of the enzyme upon the substrate. These data are given in Table IV.

DISCUSSION

The purification procedure described here gives enzyme in considerably higher yield and of considerably greater specific activity than the purification schemes previously described in the literature[6,8]. In addition, we present extensive data on purity, which establish that the product of the final step in the procedure is homogeneous. This material is eminently suitable for use in structural studies on poly- and oligo-saccharides and for studies on the mechanism of action of the enzyme, and the procedure lends itself to the production of enzyme on a large scale suitable for structural studies on the enzyme itself (amino acid sequence, and so on).

In our hands, the properties of the enzyme are essentially identical to those observed by other workers, with one notable exception. Garcia-Ballesta reported a molecular weight in excess of 100,000 as judged by gel-filtration[6] on Sephadex G-200. Marshall gave a molecular weight of 10,200 from gel filtration[8] on Bio-gel P-60. We report here a molecular weight of 28,800 on the basis of equilibrium-centrifugation studies. We have confirmed the molecular weight of our preparation by sodium dodecyl sulfate gel-electrophoresis and by gel filtration. These differences are puzzling. It should be observed that the preparation reported by Garcia-Ballesta is much less pure than either that of Marshall or that described here, as judged by specific activity. The preparation of Garcia-Ballesta has a specific activity of 1.4 IU/mg, whereas that of Marshall is 106 and ours 565. We have investigated the possibility that the molecular weight of this extracellular enzyme could vary with different isolates of the organism. All of our work on the development of the isolation procedure was performed on isolates from a culture provided by Dr. Reese fourteen years ago. However, we have investigated enzyme produced by a culture of *Rhizopus arrhizus* QM 1032 provided in 1974 by Dr. E. G. Simmons, Dept. of Botany, University of Massachusetts, and have obtained material having molecular weight identical to that reported here. It is possible that the Marshall preparation was the result of the action of proteolytic enzymes in the culture medium, but we are not aware of a case of a decrease of over 50% in the molecular weight of an enzyme without drastic action on its catalytic activity. In very extensive work on this enzyme over a period of more than ten years, we have never observed variation, from the given molecular weight, that was beyond experimental error. Another possible explanation for this discrepancy is that the enzyme possesses subunit structure and that Marshall was dealing with the monomer whereas we are dealing with a trimer. However, we have

subjected our preparation to electrophoresis on sodium dodecyl sulfate gels and can find no evidence of dissociation.

The presence of covalently bound carbohydrate in the enzyme molecule is interesting, but as yet there is no evidence as to whether it is involved in the catalytic mechanism. It is common to find comparable amounts of carbohydrate in many proteins secreted by microorganisms, and the chemical composition in the present example is typical.

Catalysis without inversion is a significant aspect of the enzyme's mechanism and indicates that it acts by a double displacement or by some other mode that preserves configuration, such as that proposed by Thoma *et al.*[17]. Thus this enzyme is added to the list of endo-attacking enzymes that hydrolyze with retention of configuration. Insofar as we can determine, there is no known case of such an enzyme that hydrolyzes with inversion. The demonstration that the enzyme has significant transglycosylase activity is consistent with the foregoing. The mechanism proposed by Thoma *et al.*[17] has two salient features, a relatively long transition-state lifetime and compulsory departure of the aglycon from the enzyme surface prior to resolution of the transition state. Both of these conditions would be expected to favor transglycosylation.

The data on the relative rates of hydrolysis given in Table III expose some features of the enzyme's action. It plainly shows a preference for stretches of β-(1 → 3)-linked D-glucosyl residues. The rate of action on reduced laminara-oligosaccharides increases almost linearly over the range 4–7 residues and reaches 70% of that for soluble laminaran at laminaraheptaitol. The introduction of β-D-(1 → 4) linkages into the chain decreases the rate (oat and barley glucan), but these compounds are still good substrates. Perlin[3–5] and his associates have produced evidence that crude preparations of *Rhizopus* enzyme are capable of hydrolyzing β-D-(1 → 4) linkages in oat and barley glucan, and we show here that our highly purified preparation hydrolyzes β-D-(1 → 4) linkages in oligosaccharides of known structure (see later). The introduction of β-D-(1 → 6) linkages (*Ecklonia* laminaran, yeast glucan, *Sclerotium rolfsii* glucan), however, markedly slows the action of the enzyme, indicating, possibly, that the enzyme cannot attack the β-D-(1 → 6) linkages interspersed among β-D-(1 → 3) linkages. Furthermore, the presence of β-D-(1 → 6) linkages may sterically hinder action of the enzyme on nearby β-D-(1 → 3) linkages.

A surprising amount of information concerning the pattern of attack of the *Rhizopus* enzyme may be obtained by quantitation of the hydrolysis products produced from oligosaccharides of known structure. Data bearing on this point are presented in Table IV. As an example, considering the structure of laminaratetraose:

$$\beta\text{-D-Glc-(1} \rightarrow 3)\text{-}\beta\text{-D-Glc-(1} \rightarrow 3)\text{-}\beta\text{-D-Glc-(1} \rightarrow 3)\text{-D-Glc}$$
$$\uparrow \qquad\qquad \uparrow \qquad\qquad \uparrow$$
$$a \qquad\qquad b \qquad\qquad c$$

there are obviously three possible points of hydrolytic attack, *a*, *b*, and *c*. If the sole point of attack were at point *b*, then this would be immediately evident from the analysis of the products, which would consist only of laminarabiose, and if no glucose

were produced, one could conclude that there was no secondary attack on the hydrolysis products. On the other hand, if the enzyme initiated primary attack at "*a*", "*b*" and "*c*", then one would observe a mixture of glucose and laminaratriose ("*a*" and "*c*" attack) and laminarabiose ("*b*" attack). If the ratio of glucose and

TABLE IV

ACTION PATTERN OF *Rhizopus arrhizus* ENDO-(1 → 3)-β-D-GLUCANASE

Substrate[a]	*Relative [E]*[b]	*Products*	*Mol %*
Laminaratetraose	1	D-Glucose	21.7
		Laminarabiose	60.4
		Laminaratriose	18.9
4[3]-*O*-β-Laminarabiosyl-laminarabiose	2	D-Glucose	13.3
		Laminarabiose	82.4
		Branched trisaccharides	4.3
4-*O*-β-Laminarabiosyl-D-glucose	5	D-Glucose	25.6
		Laminarabiose	24.8
		Cellobiose	2.4
		Starting material	47.2
4-*O*-β-Laminaratriosyl-D-glucose	2	D-Glucose	40.2
		Laminarabiose	34.0
		Cellobiose	25.8
4-*O*-β-Laminaratetraosyl-D-glucose	1	D-Glucose	7.0
		Laminarabiose	18.0
		Cellobiose	30.4
		Laminaratriose	31.4
		4-*O*-β-Laminarabiosyl-D-glucose	13.2
4-*O*-β-Laminarapentaosyl-D-glucose	1	D-Glucose	13.1
		Laminarabiose	22.8
		Cellobiose	9.1
		Laminaratriose	32.0
		4-*O*-β-Laminarabiosyl-D-glucose	23.0
6-*O*-β-Laminarabiosyl-D-glucose	5	D-Glucose	21.4
		Laminarabiose	2.3
		Gentiobiose	22.3
		Starting material	54.0
6-*O*-β-Laminaratriosyl-D-glucose	2	D-Glucose	8.6
		Laminarabiose	39.0
		Gentiobiose	49.0
		6-*O*-β-Laminarabiosyl-D-glucose	2.5
		Starting material	0.9
6-*O*-β-Laminaratetraosyl-D-glucose	1	D-Glucose	5.8
		Laminarabiose	25.5
		Gentiobiose	21.3
		Laminaratriose	28.2
		6-*O*-β-Laminarabiosyl-D-glucose	19.2
6-*O*-β-Laminarapentaosyl-D-glucose	1	D-Glucose	5.7
		Laminarabiose	22.2
		Gentiobiose	11.1
		Laminaratriose	30.8
		6-*O*-β-Laminarabiosyl-D-glucose	30.2

[a]Nomenclature used for oligosaccharides is that of Whelan[40]. [b]A relative [E] of 5 is equivalent to 1 IU/ml.

laminaratriose were the same, within experimental error, one would be justified in the conclusion that only primary attack had occurred and it would be possible to compute the relative amount of attack on "*a*" plus "*c*" in comparison to "*b*", from the ratio of products in the mixture. Should there be secondary attack on the laminaratriose of laminarabiose produced, then this will become immediately evident by the ratio of glucose:laminaratriose shifting away from 1:1. In an actual experiment (Table IV), the mol% of products found was glucose, 21.7; laminarabiose, 60.4; and laminaratriose, 18.9. Half of the difference between the glucose and laminaratriose figures must represent the laminaratriose that was further degraded to glucose and laminarabiose by secondary attack, and this allows assignment of reasonably accurate values to the different points of attack. Thus, 59% of the primary attack is at point "*b*" and 41% at points "*a*" and "*c*" combined. Further, it may be asserted that secondary attack on the hydrolysis products is relatively unimportant. When these data are combined with the rate data in Table III, which indicate that the enzyme requires a stretch of three β-D-(1 → 3)-linked residues before it can achieve reasonable rates of attack, then the tentative conclusion may be reached that the primary attack is actually 59% at "*b*" and 41% at "*c*".

This type of analysis becomes particularly interesting in the case of the oligosaccharides having mixed linkages. There can be no doubt that in the case of 4^2-*O*-β-laminarabiosyl-laminarabiose* that the major point of primary attack and, in fact the major attack, is at the β-D-(1 → 4) linkage. Perlin and his associates[3-5] have shown that crude enzyme in the form of culture filtrates attacks β-D-(1 → 4) linkages in oat and barley glucans and in lichenan. It is evident from the foregoing observation that highly purified enzyme will do the same and, at least in some structures, the β-D-(1 → 4) linkage is favored over the β-D-(1 → 3). In the case of 4-*O*-β-laminarabiosyl-D-glucose, it is evident that the β-D-(1 → 4) linkage is still the favored point of attack, although the action of the enzyme on this material is sluggish as it takes five times the concentration of enzyme to obtain reasonable amounts of hydrolysis product and about 50% of the starting material is recovered unaltered. As the number of (1 → 3)-β-D-glucosyl residues toward the non-reducing side of the β-D-(1 → 4) bond increases, the rate of enzyme action increases and β-D-(1 → 3) bonds again become the favored point of attack. The enzyme's action toward oligosaccharides having β-D-(1 → 6) linkages is also interesting. It is evident that, in the case of 6-*O*-β-laminarabiosyl-D-glucose, the enzyme has a very low rate of attack and the close correspondence between the amount of D-glucose and gentiobiose produced leaves no doubt that the favored point of attack is the β-D-(1 → 3) linkage. It would appear, from the small amount of laminarabiose produced, that the enzyme has a very limited ability to attack β-D-(1 → 6) linkages. However, as we shall point out later, we consider that the enzyme is incapable of attacking these linkages, and that the production of laminarabiose is due to its transglycosylating ability that has already

*The nomenclature used here, and throughout this paper, for oligosaccharides is that proposed by Whelan (see ref. 40).

been mentioned. Thus there is a marked contrast between the action on β-D-(1 → 4) and β-D-(1 → 6) linkages.

We consider that the foregoing observations, and others reported in the literature, may be rationalized on the basis of the structures shown in Fig. 3. Computer-simulation studies have shown that the most stable conformation for (1 → 3)-β-D-glucans is a flexible helix, whereas that of (1 → 4)-β-D-glucans is an extended ribbon[18]. It is evident from an inspection of the perspective drawings in the Reese and Scott paper[18], that the insertion of a β-(1 → 4)-linked D-glucose residue in a (1 → 3)-β-D-glucan chain does not seriously disrupt the flexible helix, but that the similar insertion of a β-(1 → 6)-linked D-glucosyl residue does. The situation for β-D-(1 → 3)-linked and mixed β-D-(1 → 3)- and β-D-(1 → 4)-linked material is not unlike that shown in Fig. 3, but the reader should consult the perspective drawings in the Reese and Scott paper for a completely accurate presentation of the conformations[18]. In any event, the data presented in this paper indicate that the active site of the *Rhizopus* enzyme is analogous to that of lysozyme[19] in that it contains a number of binding sites designed to fit the glucosyl residues in the β-D-(1 → 3)-flexible helix and these binding sites extend on both sides of the location of the catalytic mechanism. It would appear that those on the non-reducing terminal side of the catalytic site exert most control over specificity, but those on the right also have an effect, as the enzyme has a slow but readily measurable action on laminarabiose and laminarabiitol, but no action whatever on cellobiose, cellobiitol, gentiobiose, and gentobiitol. Perlin and his associates[3-5] have postulated that the enzyme is specific for the glycosidic linkage on the reducing-terminal side of a laminarabiosyl residue and is not specific for the residue to which it is attached. It was upon this basis that they rationalized the enzyme's attack on oat and barley glucans and upon lichenan. It is evident from the work presented here that this picture was too simple, and that the binding extends over a considerable stretch of the glucan chain toward the non-reducing terminal side of the catalytic site (the rate of action is still increasing at the level of a 7-unit chain; see Table III). However, the general shape of the chain is not sufficiently altered by the insertion of isolated β-D-(1 → 4) linkages as to render the β-D-(1 → 4)-glycosidic linkage unavailable to the active site (Fig. 3). In the case of an inserted β-D-(1 → 6)-linkage, the orientation of the glycosidic bond is radically altered, and it is no longer located at the catalytic site[18]. The data on 6-*O*-β-laminarabiosyl-D-glucose presented in Table IV would appear to indicate a very limited attack on the β-D-(1 → 6) linkage. However, as we have just shown, the enzyme has some transglycosylating capacity at the extremely high levels of enzyme used in this experiment; it is not unlikely that limited transfer of a D-glucosyl residue to the substrate occurred, resulting in the formation of 6-*O*-β-laminaratriosyl-D-glucose. The action pattern on this compound results in the formation of laminarabiose as a major product (Table IV).

The work reported here, then, constitutes an increase in our fundamental knowledge of the action of polysaccharides of the endo-type, and it also increases the usefulness of the *Rhizopus* endo-glucanase as a structural tool.

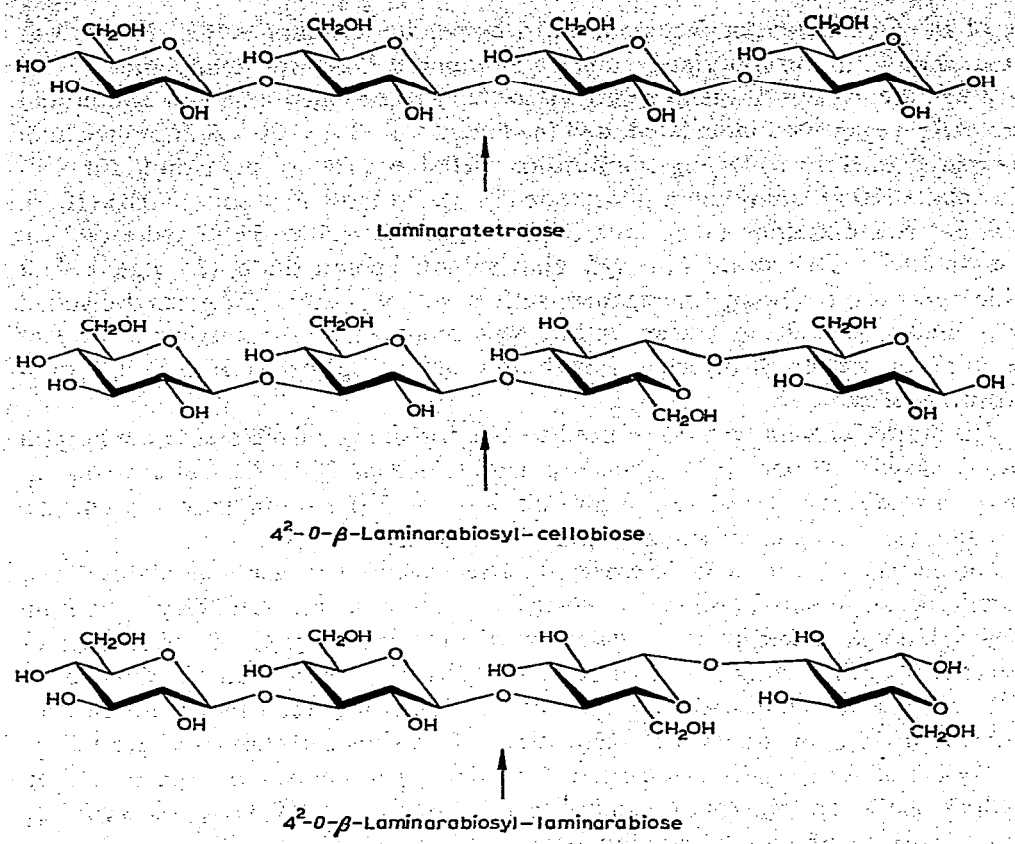

Fig. 3. Rationalization of the susceptibility of certain β-D-(1 → 4)-linkages to hydrolysis by the *Rhizopus* enzyme. The vertical arrows indicate the bond hydrolyzed. The nomenclature used for the oligosaccharides is that of Whelan[40].

EXPERIMENTAL

General methods. — Enzyme assay. D-Glucanase activity was estimated by the ability of the enzyme to hydrolyze sodium borohydride-reduced, cold-water-soluble laminaran (3 mg/ml) at 37° in 50 mM acetate buffer (pH 4.8) containing 0.25 mg/ml of gelatin. Borohydride-reduced material was used to increase the sensitivity of the reducing-power assay. The resulting increase in the reducing power of the incubation medium was determined by the alkaline copper method of Nelson[20] and Somogyi[21]. One unit of enzyme activity is defined as the amount of enzyme required to increase the reducing power equivalent to one μmol of glucose per min under these conditions.

Estimation of protein. Protein was estimated by the Hartree[22] modification of the Folin–Lowry procedure, with crystalline, bovine-plasma albumin (Pentex, Inc., Kankakee, Ill.) as the standard.

Growth of the organism. — Rhizopus arrhizus QM 1032, obtained from Dr. E. T.

Reese of the Quartermaster Research and Engineering Center, Natick, Mass., was maintained at 4° in the sporulated state on Sabouraud Dextrose Agar slants.

When the organism was to be grown for enzyme production, a new Sabouraud Dextrose Agar slant was inoculated, and incubated for 38 h at 30°. A liquid medium was prepared with the following composition: cellobiose (60 g), potassium dihydrogenphosphate (20 g), ammonium phosphate (14 g), Proteose Peptone (Difco) (10 g), calcium chloride dihydrate (4 g), urea (3 g), magnesium sulfate heptahydrate (3 g), ferrous sulfate heptahydrate (50 μg), zinc sulfate heptahydrate (35 μg), cobalt chloride hexahydrate (30 μg), manganese sulfate monohydrate (15 μg), and distilled water to make 10 liters. A starter culture was prepared by inoculating 250 ml of sterile medium with fresh mycelial growth and incubating for 30–36 h at 30° on a reciprocating shaker.

The starter culture was transferred to the remainder of the sterile medium in a New Brunswick Model F-14 fermentor maintained at 30°. The culture was incubated with stirring at 180 r.p.m. and air flow of 2 liters/min until the enzyme activity reached a maximum (usually after 48–52 h; see Fig. 1).

Polyacrylamide-gel electrophoresis. — Urea–acetic acid gels containing 7.5% acrylamide were prepared and run as described by Panyim and Chalkley[23]. Sodium dodecyl sulfate-gel electrophoresis was carried out according to the procedure of Weber *et al.*[24] Protein bands were detected on both types of gel with the Coomassie Blue R 250 stain as described by Weber *et al.*; destaining was carried out electrophoretically in 18% methanol–9% acetic acid with a Canalco Quick Gel Destainer.

Isoelectric focusing. — Isoelectric focusing experiments were done with an LKB model 8101 apparatus with Ampholine specified for the pH range 5–8 according to directions supplied by the manufacturer.

Sedimentation velocity. — Sedimentation-velocity studies were carried out at a protein concentration of 1.42 mg/ml and at an ionic strength of 0.15, in a Spinco Model E ultracentrifuge. The sample was rotated at 59,840 r.p.m. in an An-D rotor at 24°.

Purification of the enzyme. — The culture medium was filtered through glass wool and immediately transferred to the 12-liter reservoir of the Amicon ultrafiltration system (Amicon Corp., Lexington, Mass.). The solution was concentrated at 4° by pressure filtration in a Type 402 Amicon cell fitted with a PM-10 Diaflo membrane until the volume had been decreased to about 250 ml. Pressure dialysis was then carried out by passing 1.5 liters of 50 mM acetate buffer (pH 4.8) through the cell.

Fractionation with ammonium sulfate. — The material precipitating between 35 and 75% saturation with solid ammonium sulfate (Schwarz–Mann special enzyme grade) at 0° was taken up in 100 ml of cold, 5 mM sodium phosphate buffer (pH 7.0) containing 0.2% (w/v) of sodium chloride and pressure dialyzed in the Amicon cell by passage of 1 liter of the same buffer through the cell. The dialyzed solution was then concentrated to about 50 ml.

Chromatography on O-(carboxymethyl)cellulose. — A 2.5 by 45 cm column of

Whatman CM-11 was equilibrated with 5mM phosphate buffer (pH 7.0) containing 0.2% sodium chloride at 4°. The enzyme solution was added to the column and washing was continued with the equilibration buffer until a large band of non-adsorbed protein was eluted; the enzyme was then eluted with a linear gradient from 0.2–2.0% sodium chloride (in 5 mM phosphate buffer, pH 7.0; total volume 500 ml). The enzyme-containing eluate was collected in 6-ml fractions. The column was operated at a hydrostatic pressure of 75–85 cm.

Fractions containing enzyme activity were pooled and concentrated to about 30 ml in a Type 52 Amicon cell equipped with a PM-10 membrane. Buffer exchange was carried out by passage of 200 ml of 50 mM acetate buffer (pH 4.8) through the cell, and then the solution was concentrated to about 8 ml.

Sephadex-gel chromatography. — The product from the *O*-(carboxymethyl)cellulose column was applied to a 2.6 by 80 cm column of Sephadex G-100 equilibrated with 50mM acetate buffer (pH 4.8) at 4°. The column was operated at a hydrostatic pressure of 35 cm in the upflow mode, and 6-ml fractions were collected. Following identification of the fractions containing (1 → 3)-β-D-glucanase activity, those demonstrating homogeneity on urea–acetic acid gel electrophoresis were pooled and concentrated by ultrafiltration to a final protein concentration of about 1 mg/ml.

Generally, the purified enzyme-preparations were stored at 4° in 50 mM acetate buffer (pH 4.8), conditions under which they appeared to be stable indefinitely. Some preparations were subsequently freed of buffer salts by pressure dialysis with double-distilled, deionized water or by passage through a 1.5 by 90 cm column of Bio-Gel P-2. The resulting solutions were lyophilized, then stored over anhydrous calcium sulfate at 4°.

Characterization of the enzyme. — Estimation of E(1%) at 280 nm. A solution of the purified enzyme was freed of buffer salts by pressure dialysis and concentrated to about 1 mg/ml. The absorbance at 280 nm was accurately determined with a Hitachi–Coleman 124 double-beam spectrophotometer, and then triplicate aliquots of the solution were dried by lyophilization, followed by heating in a vacuum oven at 38° until they reached constant weight. All weights were determined with a Mettler microbalance under conditions of temperature equilibrium.

Amino acid analysis. Portions of salt-free enzyme solution to which norleucine had been added as an internal standard were made 6 M in hydrochloric acid and hydrolyzed at 110° under oxygen-free conditions for 18, 24, and 35 h, respectively. Analysis of the amino acids in the hydrolyzates was accomplished by a Beckman Model 119 amino acid analyzer operated in a single-column mode. Results were quantitated by an automatic, computing integrator.

For the estimation of cysteine and methionine, the enzyme was subjected to oxidation by peroxyformic acid as described by Hirs[25], followed by hydrolysis in 6M hydrochloric acid for 21 h and amino acid analysis as before.

The tryptophan content of the enzyme was estimated by the colorimetric procedure of Messineo and Musarra[26]. As suggested by these authors, results were

corrected for a control that contained enzyme but no fructose, to compensate for the effect of carbohydrate in the sample.

Sulfhydryl groups. The attempt to identify free sulfhydryl groups in the enzyme was made by the method of Robyt *et al.*[27]. Determinations were carried out on both native enzyme and enzyme which had been incubated for 2 h at 37° in 1% sodium dodecyl sulfate solution.

Molecular weight estimation. (*1*) *Sedimentation equilibrium ultracentrifugation.* Molecular weight of the enzyme was estimated by the high-speed equilibrium method of Yphantis[11] with a Spinco Model E analytical ultracentrifuge. A 0.4 mg/ml solution of the enzyme in 50mM acetate buffer (pH 4.8) containing 0.1M sodium chloride (total ionic strength 0.125) was placed over a fluorocarbon layer in one sector, and buffer was placed in the outer sector, of a double-sector, synthetic boundary-cell having sapphire windows. The cell was placed in the An-D rotor and accelerated to 30,000 r.p.m. at a temperature of 21.4°. Exposures of the Rayleigh interference-fringes were made for analysis at 56.25 h after the start of the run, well after equilibrium had been attained. Data for blank correction were obtained by mixing the cell contents and then again accelerating the rotor to 30,000 r.p.m. Exposures were made immediately, before significant sedimentation had taken place.

(*2*) *Gel electrophoresis on sodium dodecyl sulfate.* The procedure of Weber *et al.*[24] was used, at acrylamide concentrations of 7.5, 10, and 12.5%. The following molecular-weight standards were obtained from Schwarz–Mann, Orangeburg, N.Y.: horse heart cytochrome c, bovine chymotrypsinogen A, ovalbumin, and bovine serum albumin.

(*3*) *Gel filtration.* A 2.6 by 80 cm column of Sephadex G-100 operated in the upflow mode (the same column used for enzyme purification) was calibrated with 2 ml each of 2.5 mg/ml solutions of chymotrypsinogen A, ovalbumin, and bovine serum albumin, respectively, in 50mM acetate buffer (pH 4.8). The void volume was determined with a similar solution of Blue Dextran 2000.

Examination of the carbohydrate moiety. Neutral carbohydrate was estimated by the phenol–sulfuric acid procedure[12]; hexosamine content was estimated with the amino acid analyzer following hydrolysis for 6 h in 4M hydrochloric acid at 110°, as suggested by Spiro[28].

To determine whether the results of the foregoing procedures corresponded to covalently bound carbohydrate or a free contaminant, samples of the purified enzyme were applied to urea–acetic acid gels as described previously. Duplicate gels were stained for protein with Coomassie Blue R250 and by the periodic acid–Schiff (PAS) procedure of Zacharius *et al.*[29]. The PAS-stained gels were destained electrophoretically in 7% acetic acid. For identification of the neutral sugars making up the carbohydrate moiety, a sample of the purified enzyme, desalted on a Bio-Gel P2 column, was hydrolyzed in M sulfuric acid for 6 h at 100°. The hydrolyzate was diluted to 0.2M with water, and then deionized by successive passage through Bio-Rad AG50W-X8 cation-exchange resin (H^+ form) and AG1-X8 anion-exchange resin (formate form) as suggested by Spiro[30], and finally concentrated in a rotary vacuum evaporator.

Glass plates coated with a 250-μm layer of Kieselguhr G were purchased from Analtech, Inc., Newark, Delaware. To approximate the conditions described by Waldi[31], each of the 20 by 20 cm plates was sprayed with 8 ml of a solution prepared by combining equal volumes of 0.1M phosphoric acid and 0.1M disodium hydrogenphosphate solutions. The plates were dried overnight in an oven at 100° before use. Following application of samples of the sulfuric acid hydrolyzate, and known sugar solutions, chromatography was carried out in a Brinkmann sandwich-type chamber by irrigation with 4:5:1 1-butanol–acetone–phosphate buffer (the same phosphate solution used to spray the plates originally). Following irrigation, plates were dried in an oven at 100° and the sugar-containing spots were detected by spraying with the anisaldehyde–sulfuric acid reagent of Stahl and Kaltenbach[32] and heating in an oven at 100°.

Stereochemistry of hydrolysis products. — A mixture of β-D-(1 → 3)-linked oligosaccharides containing D-glucose through laminaraheptaose was dissolved in 50mM acetate buffer (pH 4.8) to a concentration of 38.4 mg/ml and kept to mutarotate for 2 h at room temperature. This solution was introduced into a 6-ml, side-arm cell of 20.004 mm pathlength, which was placed in the sample chamber of a Bendix Type 143A automatic polarimeter (The Bendix Corporation, Cincinnati Division, Cincinnati, Ohio) equipped with a 5893 Å (sodium D) interference filter. The instrument had previously been zeroed with distilled water in the sample cell.

After it had been determined that the optical rotation of the oligosaccharide solution remained constant, approximately 400 units of enzyme in 1 ml of acetate buffer was added to the polarimeter cell and mixed. The optical rotation of the solution was recorded on an attached strip-chart recorder until it reached a constant value. The temperature of the solution was 26.5°.

Effect of pH on activity and stability of the enzyme. — Buffer solutions were prepared to cover the pH range 2–8 which, under assay conditions, contained citric acid and aminotris(hydroxymethyl)methane at concentrations of 15 mM each and sodium chloride to bring the ionic strength to 50 mM in each case. Incubations were conducted at 37° both with and without added gelatin, and aliquots were taken for analysis at 1-min intervals to assure that initial reaction-rates were recorded. Corrections were made for the influence of the pH conditions on the response of the alkaline copper analytical-procedure. There was no detectable hydrolysis of substrate in the absence of enzyme.

To evaluate the influence of pH on the stability of the enzyme, a constant amount of enzyme was incubated at 37° in the absence of substrate under each of the pH conditions employed in the elucidation of the pH–rate profile, both with and without added gelatin. Aliquots of each incubation mixture were removed at various times and immediately assayed by the standard assay procedure.

Estimation of K_m. — Solutions of reduced, cold-water-soluble laminaran in 50mM acetate buffer (pH 4.8) were prepared in the general concentration range of 0.08–1.20 mg/ml. Enzyme was added, and incubations were carried out at both 37

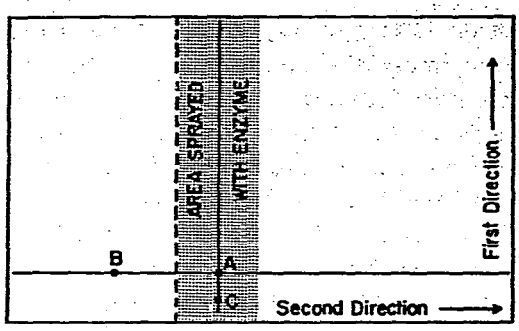

Fig. 4. Diagram of the chromatogram used to investigate the transferase activity of the enzyme. See Experimental section.

and 10°. Aliquots were removed at 1-min intervals, and assayed as described for the standard assay procedure. Gelatin was not employed in this study.

Transferase activity. — The action of the enzyme on oligosaccharide substrates was examined by a two-dimensional, paper-chromatographic technique similar to that described by French *et al.*[33]. A sheet of Whatman 3MM chromatography paper was arranged as shown in Fig. 4. A solution containing approximately equimolar amounts of laminarabiose through laminaraheptaose, plus D-glucose and gentiobiose, was applied at points "A" and "B". The shorter edges of the chromatogram were stapled together to form a cylinder, which was given[34] three ascending irrigations in 6:1:2 1-propanol–ethyl acetate–water at 70°.

Following the initial irrigations, the first-dimension guide strip containing point "B" was cut off and set aside temporarily. The shaded area indicated in Fig. 4 was then sprayed with a solution of about 1 unit of enzyme in 3 ml of 50mM pyridinium acetate buffer (pH 4.8). The chromatogram was incubated for one h at 37° in a closed chamber saturated with water vapor, and then dried in a 70° forced-draft oven. Another application of the oligosaccharide solution was made at point "C", and the chromatogram was again irrigated, exactly as before but in the perpendicular direction. The compounds on the chromatogram and the first-dimension guide strip were detected by the silver nitrate dipping procedure[35].

To elucidate further the action of the enzyme indicated by the preceding experiment, the hydrolysis of laminarapentaose was studied in greater detail. Laminarapentaose (L_5) was purified from a partial acid hydrolyzate of cold-water-insoluble laminaran by descending paper-chromatography in 68% (v/v) 1-propanol. The purity of the L_5 was confirmed by ascending paper-chromatography in 6:1:2 1-propanol–ethyl acetate–water and by thin-layer chromatography on Kieselguhr G in 14:3:3 1-butanol–pyridine–water[36].

A mixture was prepared in which the L_5 concentration was 10 mg/ml (12 mM) and the enzyme concentration was 0.2 unit/ml in 50 mM pyridinium acetate buffer (pH 4.8). Following incubation for 1 h at 37°, 20-µl portions of the mixture were applied to a two-dimensional chromatogram at the points "A" and "B" indicated in Fig. 4. A known mixture of oligosaccharides was also applied to the guide strip,

and the chromatogram was given four ascending irrigations at 70° in 6:1:2 1-propanol–ethyl acetate–water. The guide strip was cut off as before, and then the area indicated in Fig. 4 was sprayed with 1 ml of a solution of 90 IU of exo-(1→3)-β-D-glucanase (from Basidiomycete QM 806)[37] in 50 mM pyridinium acetate buffer (pH 4.8). The chromatogram was incubated for 1 h in a moist chamber at 37° and then dried in an oven at 100°. Following application of the known mixture of oligosaccharides at position "C", the chromatogram was given three ascending irrigations in the second direction, as before.

Action of the enzyme on selected oligo- and poly-saccharides. — β-D-(1→3)-Linked gluco-oligosaccharides were prepared by partial acid hydrolysis of pachyman (from *Poria cocos*). They were purified by column chromatography on coconut charcoal[38], followed by preparative paper-chromatography in 7:1:2 1-propanol–ethyl acetate–water.

Branched oligosaccharides were synthesized by the action of *Euglena gracilis* laminarabiose phosphorylase. *Euglena gracilis* strain Z cells were grown and harvested as described by Goldemberg and Marechal[39]. The cells were disrupted by sonication, the cellular debris was removed by brief centrifugation at 5000*g*, and the supernatant clarified by centrifugation for 1 h at 100,000*g*. The laminarabiose phosphorylase was partially purified by (*1*) selecting the protein fraction that precipitated from the 100,000*g* supernatant between 30 and 70% saturation with ammonium sulfate, and (*2*) passage of the ammonium sulfate fraction through a column of DEAE-cellulose at pH 6.5. The material not adsorbed on the ion-exchange column contained most of the laminarabiose phosphorylase activity, but was essentially free from α-D-glucosyl phosphatase activity.

Three homologous series of branched oligosaccharides resulted from the incubation of α-D-glucosyl phosphate and the *Euglena* enzyme with cellobiose, gentiobiose, and 4^2-*O*-β-D-glucosyl-laminarabiose, respectively. The latter acceptor was obtained from the Fred Smith Carbohydrate Collection in this laboratory, as were all of the other oligo- and poly-saccharides used that were not commercially available and whose sources are not otherwise identified.

The oligosaccharides in each of the three laminarabiose phosphorylase incubation-mixtures were purified by paper chromatography in 7:1:2 1-propanol–ethyl acetate–water, as previously described. The exo-(1→3)-β-D-glucanase[16] from Basidiomycete QM 806 converted each of these oligosaccharides into a mixture of D-glucose and the original acceptor, indicating that the added glucosidic linkages were all β-D-(1→3).

The ability of the *Rhizopus arrhizus* endo-(1→3)-β-D-glucanase to hydrolyze the several oligosaccharides prepared, and a series of polysaccharides (Table III) was examined under standard assay-conditions, with exclusion of gelatin. Each of the oligosaccharides was reduced with sodium borohydride to prevent its initial reducing power from overwhelming the assay system, and the substrate concentration in each case was set at 1.5 mM. The rate of hydrolysis of each substrate was compared with that of soluble laminaran at the same concentration (soluble laminaran was

assumed to have an average d.p. of 15). The hydrolysis of polysaccharides was examined at a substrate concentration of 0.5 mg/ml, and again the rate was compared with that of soluble laminaran at the same concentration. It was necessary first to dissolve several of the polysaccharides in M sodium hydroxide, and then titrate to pH 4.8 with acetic acid, and finally dialyze against the assay buffer to prepare them for this study. In the case of the *Ecklonia* laminaran, yeast glucan, pachyman, and *Sclerotium rolfsii* polysaccharide, significant amounts of material precipitated on neutralization and dialysis; thus, the actual substrate concentrations in those instances were less than 0.5 mg/ml.

The action pattern of the *Rhizopus arrhizus* enzyme on several of the oligosaccharide substrates was investigated. Incubations were carried out at 37° in the assay buffer (excluding gelatin) for up to 48 h at enzyme concentrations up to 1 IU/ml, depending on substrate susceptibility. The substrate concentration in each case was 5 mg/ml. At the end of each incubation period, the reaction was stopped by heating the incubation mixture in a boiling-water bath. An aliquot of each mixture was immediately withdrawn and applied to a paper chromatogram for qualitative examination. The remainder of each incubation mixture was deionized on a mixed-bed, ion-exchange resin and taken to dryness on a rotary vacuum evaporator (Rotary Evapo-Mix, Buchler Instruments, New York). A portion of the residue was taken up in water and applied in a 20-cm streak to a paper chromatogram. The 57 cm-long chromatogram was irrigated for 36 h with 7:1:2 1-propanol–ethyl acetate–water. The saccharide-containing bands, identified by guide strips stained with the silver nitrate dip-reagents, were eluted with water and quantitated by the phenol–sulfuric acid procedure. As gentiobiose and laminaratriose were not well separated by this solvent system, eluted bands suspected of containing these two components were further subjected to paper electrophoresis in 0.1M borate buffer (pH 9.2) for 3–4 h at 600 V.

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