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1,3-β-D-GLUCANASES FROM *PISUM SATIVUM* SEEDLINGS

II. SUBSTRATE SPECIFICITIES AND ENZYMIC ACTION PATTERNS

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Summary

Two purified pea 1,3-β-D-glucanases (EC 3.2.1.6) hydrolyse laminarin (degree of polymerization 20), laminaridextrins (degree of polymerization 3—7), and their reduced ^{3}H-derivatives, 1,3-β-D-glucans which are partially substituted (carboxymethyl-pachyman) or crystalline (curdlan), and mixed-linkage β-glucans. Enzyme kinetics and product-formation indicate endo-hydrolase activity with weak transglycosylase capacity. The enzymes do not hydrolyse β-glucosides, the 1,3 linkage adjacent to the reducing end of chains, or cellulose and its derivatives. They degrade mixed-linkage β-glucans, in a manner similar to *Rhizopus arrhizus* endo-1,3-β-D-glucanase, to form the products expected from hydrolysis of linkages adjacent to 1,3-β linkages.

With respect to action patterns, glucanase I (from apical growing tissue) differs from glucanase II (from basal maturing tissue) in several respects: (a) on a molar basis, I generates reducing groups from all substances more rapidly than II; (b) lower laminaridextrins are hydrolysed by I at the non-reducing terminal linkage, while II preferentially hydrolyses internal linkages; (c) laminarin is hydrolysed to lower laminaridextrins by I more rapidly than II, but I takes longer than II to completely degrade laminarin chains; (d) the enzymes are differentially sensitive to different classes of non-competitive inhibitors. It is concluded that these β-glucanases differ in such a way that I preferentially continues to degrade fragments produced by endo-hydrolytic attack on long

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Abbreviations: CM-pachyman, *O*-carboxymethylpachyman; G_1 to G_6, cellodextrins (glucose to cellohexaose); L_2 to L_8, laminaridextrins (laminaribiose to laminarioctaose); L_{2H} to L_{7H}, ^{3}H-reduced laminaridextrins (laminaribiitol to laminariheptaitol).

chains ('multiple attack' action pattern), while II hydrolyses internal linkages of the longest chains available ('multi-chain attack').

Introduction

Two 1,3-β-D-glucanase activities from pea seedlings were purified to homogeneity and shown to possess markedly different physical and basic enzymic properties [1]. Glucanase I, M_r 22 000, is concentrated in apical growing regions and is most effectively assayed reductometrically (as laminarinase), whereas glucanase II, M_r 38 000, from basal mature tissue, is relatively more active in viscometric assays (as CM-pachymanase). Speculations about possible function(s) for such 1,3-β-D-glucanase activities in plant cell growth and differentiation cannot be evaluated until more detailed information on action patterns and other biochemical properties of the enzymes is available. The work reported here concerns measurements of substrate specificities, the mode of attack towards laminaridextrins and other potential substrates, and inhibitor sensitivities of the two purified pea glucanases. It is concluded that both enzymes are endo-hydrolases (EC 3.2.1.6) which act specifically on poly- or oligosaccharides containing 1,3-β-D-glucosidic linkages. Glucanase I appears to act via 'multiple attack' and II via 'multi-chain attack', which are terms hitherto applied to different modes of hydrolysis by α-amylases [2].

Materials and Methods

Enzyme sources and assays

Pea 1,3-β-D-glucanases (I and II) were assayed and purified to homogeneity from extracts of apical and basal stem tissue, respectively, as described in the previous paper. They were stored at −20°C in 20 mM sodium acetate buffer (pH 5.5) containing 0.25% (w/v) bovine serum albumin. Endo-1,3-β-glucanase (sample S176L) from *Rhizopus arrhizus* and endo-1,4-β-glucanase (sample S199A) from *Streptomyces* QM B194 were gifts of Dr. E.T. Reese, U.S. Army, Natick, MA [2]. The *Rhizopus* preparation, as assayed by methods described here, was free of detectable 1,4-β-glucanase and laminaribiase activity, but the *Streptomyces* enzyme contained some 1,3-β-glucanase activity. The *Rhizopus* glucanase hydrolyses either a 1,4- or 1,3-β linkage in mixed-linkage glucans, provided it is adjacent to a 1,3-β linkage [4,6], while the *Streptomyces* glucanase cleaves only 1,4 linkages [3,5].

Substrates

A number of potential substrates were gifts from the sources indicated: insoluble pachyman [6], Dr. B.A. Stone. La Trobe University, Melbourne; highly crystalline curdlan [7], Dr. T. Harada, Institute of Scientific and Industrial Research, Osaka University; barley β-glucan, containing 68%, 1,4 and 32% 1,3-β linkages, Dr. G.B. Fincher, La Trobe University, Melbourne; ivory nut mannan (1,4-β-linked), Dr. T.E. Timell, S.U.N.Y., Syracuse; pustulan (1,6-β-linked), Dr. E.T. Reese, U.S. Army, Natick Lab., MA. Commercial sources included: laminarin, chitin and wheat straw xylan from Sigma, St. Louis,

MO; lichenan from Koch-Light Lab., Colnbrook, Bucks., U.K. All polymeric substrates were dialysed against distilled water for three days at room temperature before use.

Water-soluble 1,3-β-linked oligosaccharides from laminaribiose to laminariheptaose (L_2 to L_7) were prepared by a method similar to that used for generating cellodextrins by fractionation on a charcoal/celite column of partial hydrolysates of laminarin [8]. The products cochromatographed with laminaridextrins prepared elsewhere (gift of B.A. Stone), and yielded R_G values consistent with a homologous series. Measurements of optical rotation density (22°C, 589 nm) showed that the oligosaccharides were optically pure and linearly related in a Freudenburg plot. Reduced [^{3}H]laminaridextrins were prepared by treating unlabelled laminaridextrins with NaB^3H_4 (New England Nuclear Corp., Boston, Mass.) as described by Cole and King [9]. Specific activities of the products were approximately 1.85 Ci/mol dextrin.

Reduced forms of laminariheptaose (L_7), laminarin and curdlan were prepared by treating aqueous solutions with sodium borohydride overnight at room temperature [9]. Excess borohydride was removed by addition of glacial acetic acid and dialysis. Periodate (Smith) oxidation of the non-reducing termini followed by reduction was carried out on samples of 10 mg oligosaccharides or polysaccharides [10]. Samples were incubated for 96 h at 4°C with 10 ml of 50 mM sodium metaperiodate, followed by 10 ml of 0.1 M $NaBH_4$ over a 30 min period and finally conc. HCl to a concentration of 2 M. Hydrolysis was at 100°C for 2 h in sealed tubes, and acid was removed by evaporation over KOH at room temperature in vacuo. The periodate-oxidized and reduced samples were desalted with a bicarbonate form of mixed-bed resin and lyophilized. Carboxymethylation of pachyman was carried out as described [11] to form a soluble product, degree of solubilization 0.32.

Chromatography

Descending paper chromatography was used to characterize hydrolysis products of reduced laminaridextrins and barley β-glucan. Whatman No. 1 paper was irrigated for 26 h with *n*-propanol/ethyl acetate/water (6 : 1 : 3, v/v/v) at room temperature. Papers were cut into 0.5—1 cm sections and either counted in Aquasol (New England Nuclear), or reducing groups were visualized with $AgNO_3$ or potassium periodocuprate [12]. Spots were delineated and eluted for estimation of reducing power.

In order to fractionate higher oligosaccharides by gel chromatography, Sephadex G-15 (Pharmacia, Uppsala, Sweden) was suspended in 20 mM sodium acetate buffer (pH 5.5) containing 0.05% sodium azide and packed in columns (35 × 0.7 cm). Laminarin which had been incubated with glucanases for various times and then boiled, was loaded onto the column and eluted with the same buffer at room temperature with a constant flow rate (8 ml/h, 0.2 ml/fraction). The fractions were assayed for carbohydrate by the phenol-H_2SO_4 method [13].

Inhibitors

Reagents used in inhibition studies were commercial analytical grade and were obtained as follows: *p*-chloromercuribenzoate, iodoacetamide, succinic

anhydride, (Sigma, St. Louis, MO); *n*-bromosuccinimide (Aldrich, Milwaukee, WI).

Results

Substrate specificities

Table I shows relative initial rates at which glucanase I and II generate reducing groups from various polysaccharides and laminaridextrins. Both glucanases act only on products containing continuous 1,3-β-D-glucosidic linkages. Glucanase I hydrolyses all substrates at a greater rate per mole of enzyme (or per unit weight) than glucanase II. The rates of hydrolysis of soluble laminarin are higher than those for insoluble, crystalline or substituted 1,3-β-D-glucans. Mixed-linkage β-glucans that contain runs of consecutive 1,3-β linkages are also hydrolysed. Both glucanases hydrolyse laminaridextrins at rates which increase with the degree of polymerization (at least up to L_8), as expected for endo-hydrolases. Glucanase II possesses relatively very weak ability to hydrolyse the lower laminaridextrins (up to L_5), indicating that it is more specifically restricted to action on long chains than glucanase I.

Whether intact terminal glucose units are required in substrates to render them susceptible to hydrolysis by the two glucanases was also tested. As shown in Table II, when the reducing terminal is converted to an alcoholic group by borohydride treatment, the rates of hydrolysis are only about 10% less than those of the unaltered substrates. When, in addition, the non-reduced terminal is oxidized by periodate, the hydrolysis rates with glucanase I are lowered to

TABLE I

SUBSTRATE SPECIFICITIES OF PEA 1,3-β-D-GLUCANASES

Purified glucanases I and II (0.3 μmol protein/ml) were incubated with polymeric substrates (10 mg/ml) or laminaridextrins (1.0 mg/ml) at 35°C in 20 mM sodium acetate buffer (pH 5.5) containing 0.03% sodium azide. Initial rates of production of reducing groups were measured. Under such conditions, no activity was detected towards phenolic glucosides, cellulose, cellodextrins (G_2 to G_6), starch, wheat straw xylan, ivory nut mannan, chitin, arabinogalactan or pustulan.

Substrate	Relative rate of hydrolysis (μmol glucose equiv./μmol enzyme per min)	
	Glucanase I	Glucanase II
Soluble 1,3-β-D-glucan		
Laminarin	455.0	104.0
CM-pachyman	127.0	20.0
Insoluble 1,3-β-D-glucan		
Pachyman	94.5	21.4
Curdlan	34.2	13.2
Mixed-linkages glucan		
Barley β-glucan	87.5	63.4
Lichenan	35.7	28.6
Laminaridextrin		
L_2	2.4	0.0
L_3	23.2	0.7
L_4	33.9	1.3
L_5	35.7	2.0
L_6	45.6	12.5
L_6-L_8	60.2	44.6

TABLE II

RELATIVE HYDROLYSIS RATES OF SUBSTRATES WITH MODIFIED TERMINAL GLUCOSE UNITS

Substrates were subjected to a Smith oxidation at their non-reducing terminal and reduction at the reducing terminal with sodium borohydride. These products (5 mg/ml) were incubated with purified glucanases and initial rates of hydrolysis were assayed reductometrically as in Fig. 1.

Substrate	Rate of hydrolysis (μmol glucose equiv./μmol enzyme per min)	
	Glucanase I	Glucanase II
Laminariheptaose	108	37
Reduced	97	34
Oxidized	34	30
Laminarin	420	144
Reduced	352	135
Oxidized	160	110
Curdlan	64	52
Reduced	57	48
Oxidized	20	39

approximately one-third of those with the unaltered substrate, while rates with glucanase II are lowered only about 20%. Thus, it appears that neither enzyme activity is dependent on the reducing terminal of substrates, but glucanase I shows a preference or affinity for linkages close to the non-reducing terminal. This difference is also reflected in the observation (Table I) that glucanase I hydrolyses lower laminaridextrins much more rapidly than does glucanase II.

Products formed from β-glucans

Equimolar quantities of glucanase I and II were incubated with soluble laminarin and hydrolysates were fractionated on Sephadex columns at intervals as shown in Fig. 1. Lower laminaridextrins are formed relatively rapidly (within minutes) in the presence of glucanase I, but only slowly (after hours) with glucanase II. During the first few minutes of hydrolysis with glucanase I, while discrete lower dextrins are generated, the peak of laminarin (V_0) remains the predominant component of the reaction mixture. This suggests that after initial endohydrolysis, glucanase I preferentially continues to hydrolyse fragments to a lower degree of polymerization. At no time (up to 6 h) does glucose or laminaribiose accumulate as the major hydrolysis product. Even after hours of incubation, laminarin remains in the reaction mixture as a distinct component. In contrast, with glucanase II almost all (85%) of the laminarin is hydrolysed internally within 10 min to products which distribute broadly over a relatively high polymerization degree range. In a few hours, essentially no laminarin remains in the reaction mixture. It is clear that both enzymes are endohydrolases, but glucanase I has a much greater capacity for hydrolysing lower laminaridextrins than II.

Taken together, the above results (Tables I and II, Fig. 1) imply that neither glucanase readily hydrolyses 1,3-β-glucan near the reducing terminal. This interpretation is confirmed more directly by examining the products generated from reduced (tritiated) laminarin (Fig. 2). During a brief incubation (30 min),

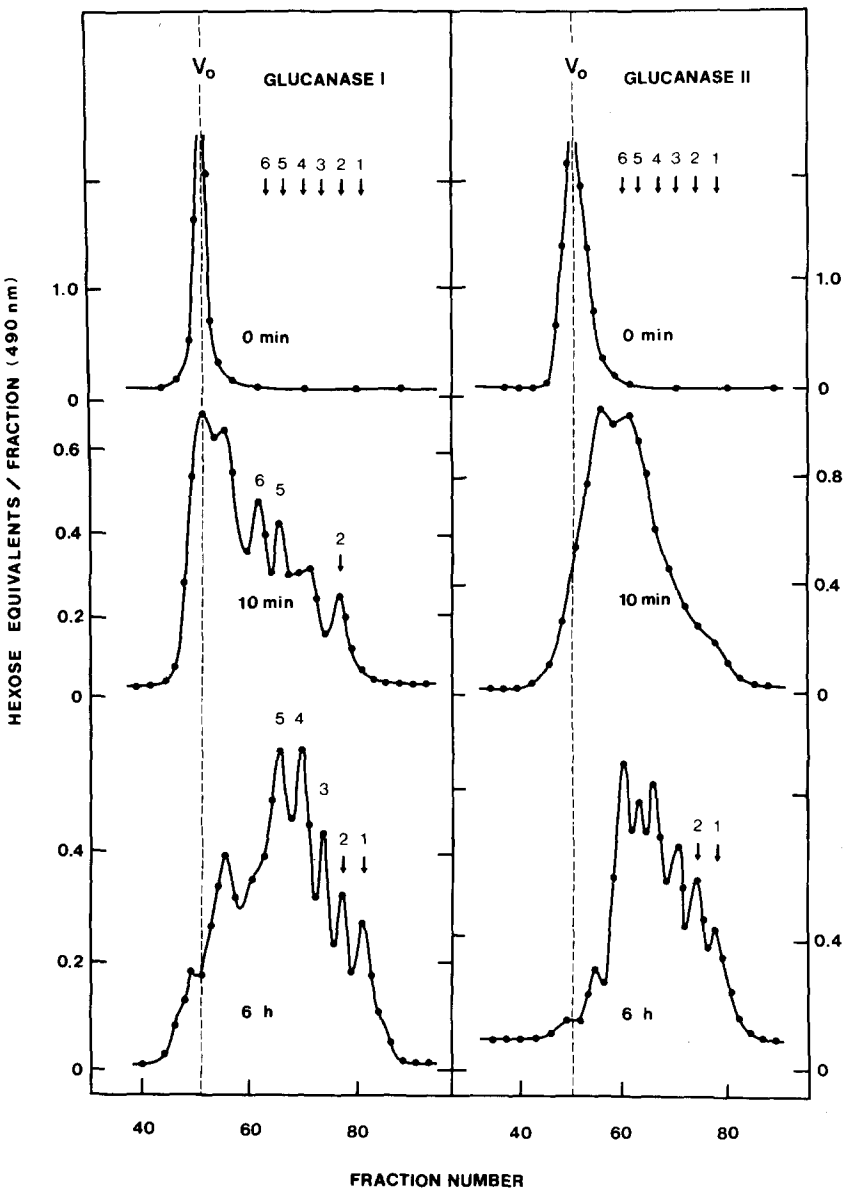

Fig. 1. Gel chromatography of hydrolysates of laminarin. Purified glucanase I and II (2.0 μmol/ml) were incubated with laminarin (5 mg/ml) at 35°C. Aliquots (0.2 ml) were removed at time intervals, reactions were stopped by boiling, and mixtures were applied to a Sephadex G-15 column. Fractions (0.15 ml) were assayed for total carbohydrate, V_0 was determined with blue dextran, and the elution volumes of authentic laminaridextrins (G_1 to L_6) are indicated by numbered arrows.

neither glucanase attacks linkages up to the fourth glycosidic bond from the reduced terminal, i.e., there are produced no radioactive oligosaccharides with a degree of polymerization lower than 5. Glucanase I forms relatively more of radioactive oligosaccharides with a higher degree of polymerization and barely

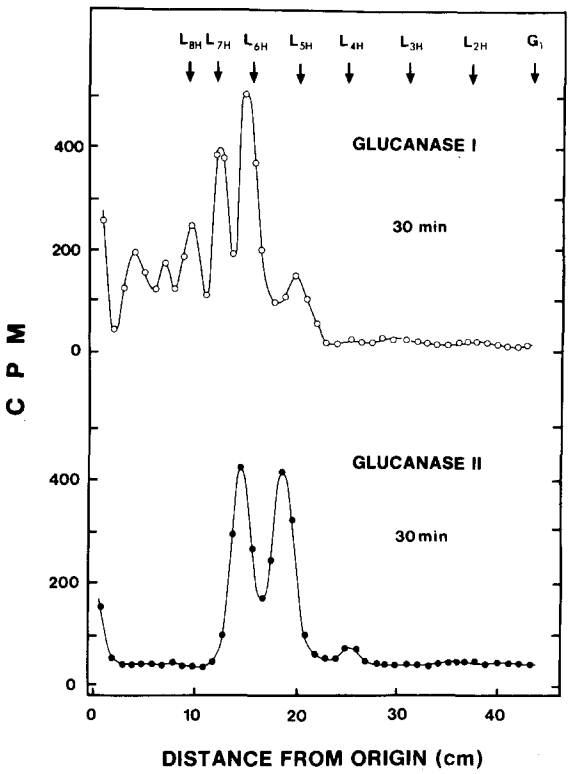

Fig. 2. Paper chromatography of hydrolysates of reduced [^{3}H]laminarin. Purified glucanases I and II (1.0 μmol/ml) were incubated with [^{3}H]laminarin (2 mg/ml, $1.5 \cdot 10^6$ cpm/ml) as described in Fig. 1. Aliquots (0.1 ml) were chromatographed along with purified reduced [^{3}H]laminaridextrin standards, (L_{2H} to L_{8H}).

any of degree of polymerization 5, suggesting that this enzyme randomly degrades linkages between the 6th glucose unit and the non-reducing end. Glucanase II forms only two prominent peaks corresponding to reduced laminaripentaose and laminarihexaose (L_{5H} and L_{6H}) and almost no undegraded higher laminaridextrins remain in the mixture (see also Fig. 1). This would result if glucanase II preferentially cleaves the most internal linkages of the longest available chains so that D.P. * 20 → D.P. 10 → D.P. 5. In time, e.g. 6 h, both glucanases degrade the intermediate dextrins further, with tritiated L_{2H} and L_{4H} as the main products (data not included).

The products formed when fungal and pea glucanases act for 24 h on barley mixed-linkage β-glucan are shown in Fig. 3. Both pea glucanases generate glucose, laminaribiose and laminaritriose in progessively lower yields. The latter components presumably derive from increasingly uncommon runs of three or four 1,3-β linkages in the glucan. Both glucanases also generate, as a major constituent, a trisaccharide possessing chromatographic mobility similar to 3-*O*-β-D-cellobiosyl-D-glucose. This is the product to be expected from hydrol-

* Degree of polymerization.

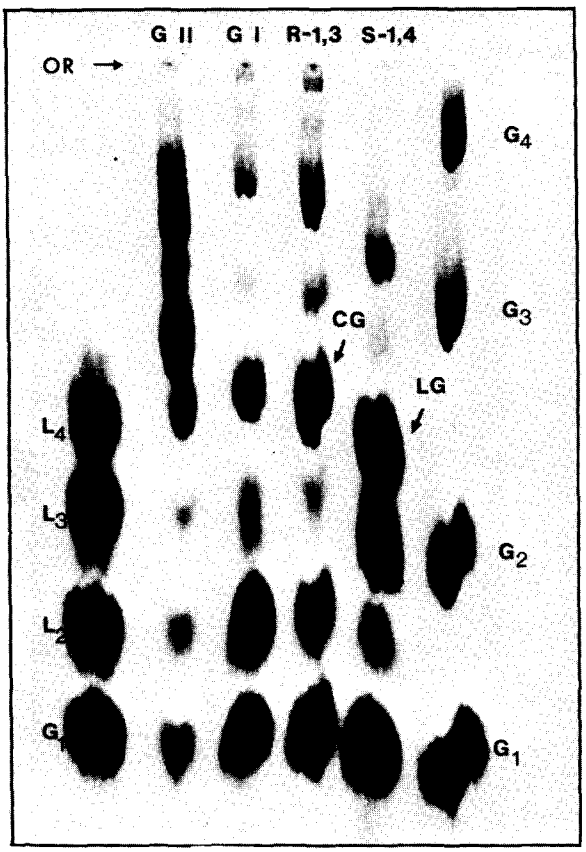

Fig. 3. Products formed from barley β-glucan by pea glucanases I and II and fungal glucanases. Reaction mixtures (1 ml) contained barley β-glucan (10 mg) plus 0.4 μmol of purified pea glucanase or 1.5 mg of fungal β-glucanase. These were incubated for 24 h at 35° C in acetate buffer, pH 5.5, 0.03% sodium azide. Aliquots (100 μl) of hydrolysates were chromatographed by descending paper chromatography. Abbreviations are: G_1—L_4, laminaridextrin standards; G_1—G_4, cellodextrin standards; R-1,3, *Rhizopus* endo-1,3-β-glucanase; S-1,4, *Streptomyces* endo-1,4-β-glucanase (cellulase); G I and G II, pea glucanase I and II; LG, 4-*O*-β-laminaribiosyl-D-glucose; CG, 3-*O*-β-cellobiosyl-D-glucose. Note: untreated barley β-glucan is not mobile in this system and remains entirely at the origin (OR).

ysis of linkages adjacent to 1,3 bonds, even if these are 1,4-β linkages [5]. These four products are also formed in similar relative amounts by action of *Rhizopus* endo-1,3-β-D-glucanase (see also Refs. 4 and 5). Cellobiose and 4-*O*-β-D-laminaribiosyl-D-glucose are formed by hydrolysis of linkages adjacent to 1,4 bonds by pea cellulases [8] and *Streptomyces* cellulase (Fig. 3) but not by pea 1,3-β-D-glucanases. It is also evident that neither pea glucanase leaves any unhydrolysed barley glucan (origin material) at the end of the incubation. Nevertheless, glucanase I shows a greater capacity than II for completing the degradation of oligosaccharides to degree of polymerization 3 or less, implying that I hydrolyses 1,4 linkages in this substrate more readily than does II.

Products formed from laminaridextrins

Fig. 4 shows progress curves for production of labelled products from

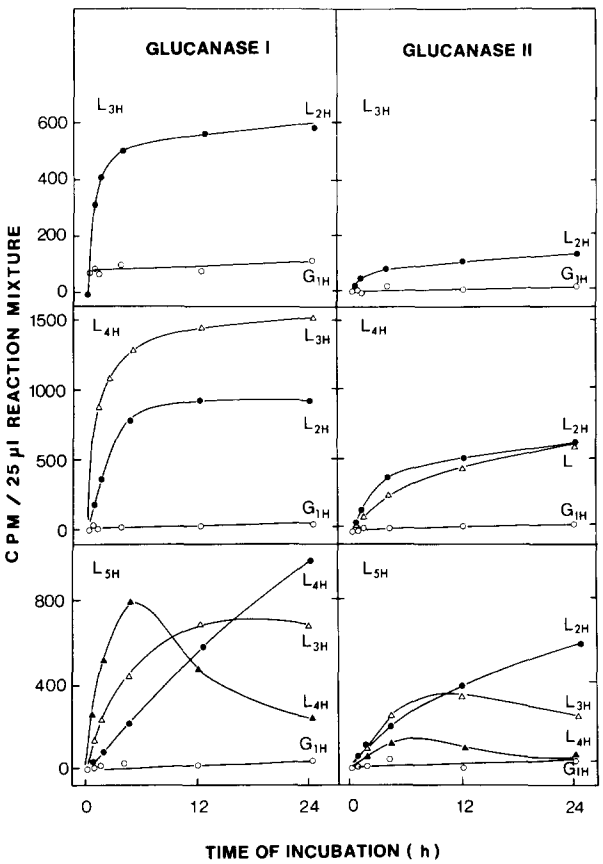

Fig. 4. Products formed from reduced [^{3}H]laminaridextrins by glucanases I and II. Reaction mixtures (0.2 ml) contained [^{3}H]laminaridextrins (L_{3H}—L_{5H}, 1 mg) and other ingredients under conditions as in Fig. 2.

reduced [^{3}H]laminaridextrins (L_{3H}—L_{5H}) by the two glucanases. Neither enzyme generates labelled sorbitol from any of these substrates, just as neither hydrolyses laminaribiitol, indicating that the linkage adjacent to the reduced terminal is not cleaved. However, all of the other linkages are susceptible to hydrolysis by both enzymes. The main differences between the actions of glucanase I and II are that (a) the rate of hydrolysis is higher with I than II and (b) I shows preferential hydrolysis of the linkage adjacent to the non-reduced terminal, whereas II preferentially hydrolyses more internal linkages. Thus, for example, with L_{5H} as substrate, glucanase I generates L_{4H} first and only eventually forms lower degree of polymerization products, whereas II generates L_{2H} and L_{3H} first and forms hardly any L_{4H}. The pattern shown by glucanase II with these substrates is essentially identical to that shown by pea endocellulases acting on [^{3}H]cellodextrins [8], whereas glucanase I behaves with such substrates as if it were an exo-hydrolase.

Limited transglycosylation may occur in these reaction mixtures and slightly alter the proportions of oligodextrins which are generated. Thus, when purified

glucanases are incubated with relatively high concentrations of [^{14}C]glucose (7.5 mM) plus unlabelled laminaritetraose (1.5 mM), a small fraction of initial radioactivity is incorporated into dextrins with degree of polymerization up to 4 (Fig. 5). Shorter or longer incubation than that illustrated does not increase the yield of these dextrins. The most prominent product formed by glucanase I has a mobility equivalent to L_4, while glucanase II forms mainly L_3. Presumably these products are generated by transglucosylation of [^{14}C]glucose to L_3 and L_2, respectively, since the latter are the main saccharides generated from L_4 by glucanases I and II (cf. Fig. 4).

Inhibition

Competitive inhibition of the action of these glucanases on CM-pachyman is demonstrated by effects of added laminaridextrins during viscometric assays (Fig. 6). The degree of inhibition of CM-pachymanase activity increases with the degree of polymerization of added laminaridextrins (up to L_6) in a linear manner using glucanase I, and exponentially using glucanase II. This reflects the fact that glucanase II shows a particularly marked preference for longer-chain laminaridextrins (Table I, Fig. 4). At any one degree of polymerization it requires more laminaridextrin to achieve a given percentage inhibition of CM-pachymanase activity using glucanase I than using II, as indicated by the relative *K* values (see inset, Fig. 6).

Glucono-β-lactone and nojirimycin (5-amino-5-deoxy-D-glucopyranose), which are known to act as competitive inhibitors of some glucosidases and exoglucanases but not endo-glucanases [14], did not interfere with the CM-pachymanase activities of pea glucanases I or II at concentrations up to 0.1 M.

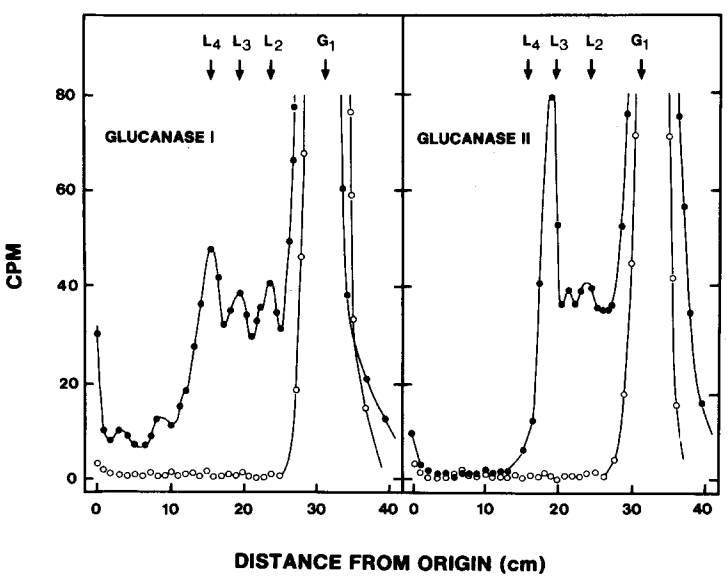

Fig. 5. Transglucosylation by purified pea 1,3-β-D-glucanases. Glucanases I and II (0.1 ml, 0.5 μmol) were incubated with a mixture of 10 mM D-[^{14}C]glucose (0.75 ml, spec. act. 313 mCi/mol) plus 10 mM unlabelled laminaritetraose (0.15 ml) at 35°C. Aliquots (50 μl) of reaction mixtures were chromatographed on paper. Control, zero time (○- - - - -○); after 4 h incubation (●- - - - -●).

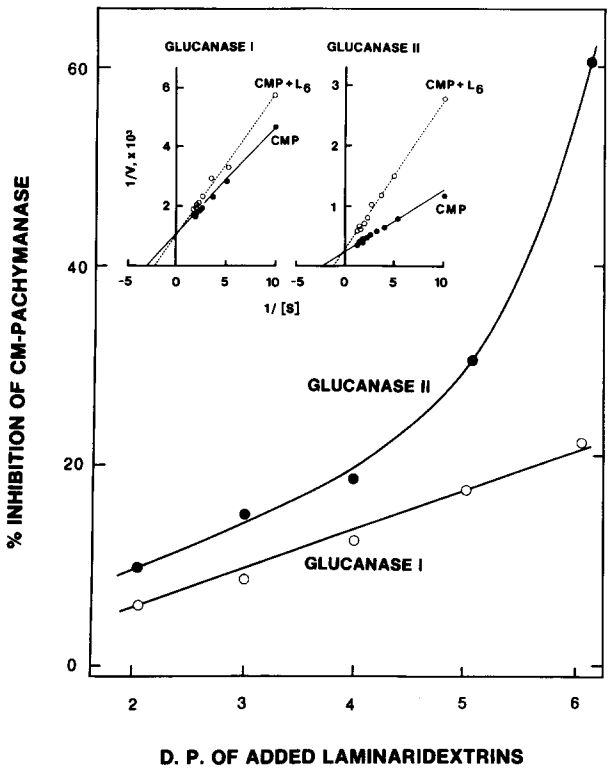

Fig. 6. Inhibition of CM-pachymanase by laminaridextrins. Purified glucanases, I and II (0.05 ml, 0.1 μmol) were added to CM-pachyman (0.5 ml, 1% w/v) in the presence of laminaridextrins (0.1 ml, 5 mM) and incubated for 10 min at 35° C. Inhibition was competitive with K values for glucanase I and II of 18.3 mg/ml (0.39 mM) and 3.4 mg/ml (0.07 mM), respectively. Glucose does not inhibit CM-pachymanase activity. D.P., degree of polymerization.

With respect to non-competitive inhibitors, a brief survey (not shown here) indicates that glucanases I and II possess differential susceptibilities to different types of inhibitor. While both glucanases are inhibited by sulfhydryl agents (*para*-chloromercuribenzoate, iodoacetamide) and heavy metal ions (Cu^{2+}, Hg^{2+}), albeit to different degrees, glucanase I is more sensitive than II to reagents that interact with tryptophane and lysine residues (e.g., *n*-bromosuccinimide), while the reverse is true of hydroxyl-binding reagents (e.g., succinic anhydride). Thus, selective use of these classes of inhibitor could be employed to assay one glucanase in the presence of the other.

Discussion

Pea 1,3-β-D-glucanases hydrolyse poly- or oligosaccharides and their derivatives containing 1,3-β linkages between anhydroglucose residues. The highest polymerization degree products are degraded most rapidly unless they are crystalline (Tables I and II), as expected from endo-hydrolase action. These glucanases do not hydrolyse cellulose or cellodextrins, but both are capable of

relatively rapid hydrolysis of mixed-linkage β-glucans. As shown in Fig. 3, the reaction products generated from barley β-glucan are identical to those formed by *Rhizopus* 1,3-β-D-glucanase, and the trisaccharide product containing both linkages is different from that formed by *Streptomyces* [3,5] endo-cellulase. It is probable that, with this substrate, the pea 1,3-β-D-glucanases are in fact hydrolysing some 1,4-β linkages, as illustrated in Fig. 7; the controlling feature being contiguity of a 1,3-β linkage at the non-reducing side [5]. The fact that glucanase I completely hydrolyses barley β-glucan to lower laminaridextrins (degree of polymerization <4) while glucanase II does not (Fig. 3), may indicate that II requires a series of contiguous 1,3-β linkages before it will bind to the substrate and hydrolyse it, since such sequences are likely to be relatively uncommon in this substrate.

Neither glucanase hydrolyses β-glucans with other linkages, or polysaccharides containing other sugars, and neither possesses β-glucosidase or significant laminaribiase activity (Table I). Moreover, glucose and laminaribiose do not appear amongst the reaction products in the early stages of attack on substrates of higher degree of polymerization (Figs. 1 and 2), which argues against any tendency towards an exohydrolase mode of action with long-chain substrates. In general, the range of substrate specificities as described is similar to that recorded for the other purified plant endo-1,3-β-D-glucanases (EC 3.2.1.6) and those from fungi of the *Rhizopus* type [4,5].

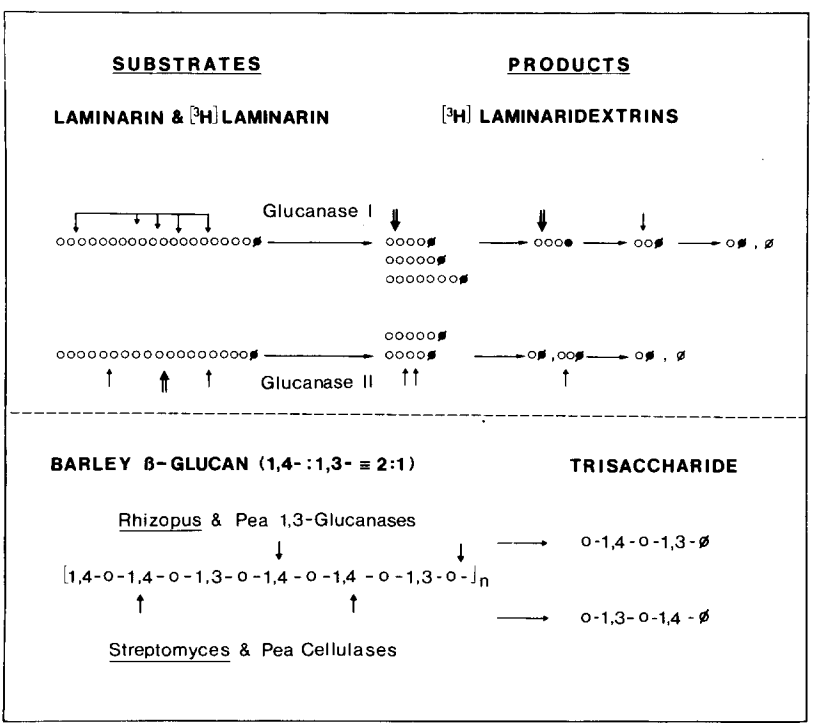

Fig. 7. Action patterns of β-glucanases on various substrates. Symbols used are: ○, glucosyl unit; ⊙, reducing glucosyl unit; ●, reduced glucosyl unit; ⇓, preferential hydrolytic attack; ↓, slower or later hydrolytic attack.

The degradation profiles and relative reaction rates observed with several substrates in this study confirm the conclusion that the pea 1,3-β-D-glucanases differ in the rapidity with which they reduce the initial chain lengths of a population of substrate molecules. Thus, for example, glucanase I lowers the viscosity of CM-pachyman solutions relatively slowly, just as I takes a longer period than II to deplete the medium of all long laminarin chains (Fig. 1). Nevertheless, I generates reducing power from CM-pachyman and laminaridextrins from laminarin much more rapidly than II (Table I). Thus, the sites at which glucanase I hydrolyses substrates with a high degree of polymerization do not appear to be as stringently confined to the most internal linkages as is the case with glucanase II.

Neither glucanase is capable of hydrolysing the linkage adjacent to the reducing terminal of a substrate, since laminaribiose and laminaribiitol are not attacked (Table I, Fig. 4) and [^{3}H]sorbitol is not formed from [^{3}H]laminaridextrins (Fig. 4) or [^{3}H]laminarin (Fig. 2). Reduction of this terminal has little effect on reaction rates (Table II). Nevertheless, both enzymes appear to avoid hydrolysing long chains at linkages closer than five or six glucose units from the reducing or reduced terminal, i.e., the main products formed in brief incubations are clustered around polymerization degree 6 (Figs. 1 and 2). The importance of an intact non-reducing terminal for the action of glucanase I is clear from the inhibition which results when that terminal is oxidized. Presumably it is required for binding, though the terminal linkage is not hydrolysed until relatively short laminaridextrins are generated (Fig. 1). The non-reducing terminal is then cleaved preferentially by glucanase I which appears to begin acting as an exo-hydrolase, whereas II continues to hydrolyse internal linkages at random and relatively slowly (Fig. 4).

These conclusions are summarized diagramatically by the action patterns depicted in Fig. 7. Glucanase I is shown as hydrolysing laminarin and [^{3}H]-laminarin (degree of polymerization 20) at points between about five glucosyl units from the reducing end and two glucosyl units from the non-reducing end of a given chain. This accounts for the rapid appearance of [^{3}H]dextrins with degree of polymerization 5 to 6 (Fig. 2), as well as some laminaribiose, even while most long chains in the preparation remain unhydrolysed (Fig. 1). Eventually, free glucose appears (Fig. 1) due to rapid hydrolysis of the terminal at the nonreducing end of dextrins of low polymerization degree (Fig. 4). Glucanase II is shown as preferentially hydrolysing the most internal linkages of the longest chains in the preparation, in order to account for the relatively rapid disappearance of products of high polymerization degree (Figs. 1 and 2) and the fact that this enzyme clearly avoids hydrolysing terminal linkages of laminaridextrins (Fig. 4).

Thus, these two endo-glucanases appear to differ primarily in their relative rates of attack on chains with high vs. low degree of polymerization. On analogy with studies on the α-amylases [2], glucanase I generates the degradation profiles to be expected from a 'multiple attack' action pattern, wherein the enzyme preferentially continues for a time to hydrolyse fragments produced by an initial endohydrolytic encounter with long chains. However, glucanase II appears to act via 'multi-chain attack', wherein it hydrolyses the longest available chains internally before attacking fragments of low degree of polymeriza-

tion. To our knowledge, this is the first occasion on which evidence has been obtained to show that action patterns which are well established with α-amylases may also apply to endo-β-glucanases. Such modes of action may prove to be important in regulating the distribution of specific substrates containing 1,3-β linkages in differentiating tissues, particularly since the two pea glucanases are localized at opposite ends of the growing stem.

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