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

I. ISOLATION AND PURIFICATION

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Summary

Two buffer-soluble endo-1,3-β-D-glucanases (EC 3.2.1.6) have been purified to within 1% of electrophoretic homogeneity from etiolated *Pisum sativum* stem tissues. Purified glucanase I and II differ in physical properties, such as electrophoretic mobility in sodium dodecyl sulfate polyacrylamide gels (M_r values were 22 000 and 37 000, respectively) and isoelectric focusing, (p*I* values were 5.4 and 6.8, respectively). Although the enzymes have similar pH optima (5.5—6.0), K_m values for various substrates (0.6—7.4 mg/ml) and thermal inactivation profiles, they are localized in different tissues and they differ markedly in the rates with which they attack the internal linkages of long- vs. short-chain substrates. Glucanase I is concentrated in apical regions of the stem and is most effectively assayed reductometrically (as laminarinase), while glucanase II is localized in mature regions and is relatively more active in viscometric assays (as carboxymethyl-pachymanase).

Introduction

Glucanase and glucosidase activities which hydrolyse β-linked substrates have been extracted from many higher plants where their potential functions have been the subject of much speculation. Roles have been suggested, for example, in regulation of cell-wall expansion [1—3], localized wall digestion [4,5], β-glucan synthesis [6,7], rate of translocation [4], and substrate mobilization

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Abbreviations: CM-pachyman, *O*-carboxymethylpachyman; SDS, sodium dodecyl sulfate.

in nutrition [5]. In order to establish that these hydrolases can and do perform such diverse functions, it is necessary to have precise information on their substrate affinities and distribution in relation to the properties of endogenous substrates. The present study of 1,3-β-D-glucanases is part of a program to purify such enzymes and substrates in order to provide this information for a growing plant tissue.

Previous studies with pea seedlings have demonstrated that growing regions contain relatively high levels of β-glucosidase activity as assayed against β-nitrophenylglucoside or cellobiose [2,8], plus two endo-1,4-β-D-glucanases (EC 3.2.1.4) assayed against CM-cellulose or cellodextrins [9,10] and an unknown number of 1,3-β-D-glucanases as assayed against CM-pachyman or laminarin [1]. All of these hydrolase activities are subject to marked fluctuations in response to treatments of pea tissue with growth-altering concentrations of the auxin type of growth hormone or inhibitors of protein synthesis [1,8]. Such observations have been taken to mean that these enzymes catalyse important events in the sequence of reactions leading to plant growth. However, to date, only the cellulases have been purified to homogeneity [9] and characterized enzymically [10], and only one of these was shown to be truly hormone-induced [11]. The other cellulase was localized in the wall in close association with its substrate, and perhaps with cellulose synthetase [6,7], for which it may provide the primer. Properties of the other glycosidases are largely unknown.

This paper reports on purification and physical properties of two pea endo-1,3-β-D-glucanases (EC 3.2.1.6). A subsequent paper in this journal provides details of their substrate specificities and modes of action.

Materials and Methods

Enzyme sources and assays

Pea seedlings (*Pisum sativum*, L., var. Alaska) were grown in darkness for 7—8 days and harvested as previously described [1,8]. Particular care was taken to ensure that the tissue was free of detectable fungal contamination before extraction. Preliminary tests established that the capacity of buffer-soluble extracts of the tissue to generate recuding power from soluble laminarin (laminarinase activity) was highest in growing apical regions of the seedling, while the ability to reduce the viscosity of solutions of CM-pachyman (CM-pachymanase activity) was concentrated in maturing basal regions. It was tentatively concluded that these assays reflected the distribution of two 1,3-β-D-glucanases (I and II, respectively). Accordingly, 10-mm segments of elongating tissue from the sub-apical regions of seedlings were excised and used as the source of glucanase I, which was assayed as laminarinase, and 10-mm segments of mature tissue from the middle of the first internode of the basal regions were used as source of glucanase II, assayed as CM-pachymanase. Very little (less than 5%) of either activity was detected in buffer-insoluble residues by these assay procedures [1].

For standard reductometric assays, 0.45 ml of 0.1% (w/v) dialysed laminarin (Sigma, St. Louis, MO) in 20 mM sodium acetate buffer (pH 5.5) was incubated with 0.05 ml enzyme preparation at 35°C. Aliquots were withdrawn at

intervals for estimation of reducing power [12]. Enzyme activities were expressed as the amounts of glucose equivalents produced during the initial stages of the reaction when the activity was a linear function of time. The particular stock of laminarin which was used throughout these tests yielded only glucose upon complete hydrolysis, and reducing power estimation indicated an average degree of polymerization of 20. Partial acid hydrolysis with fuming HCl, and chromatography on a charcoal-celite column [10], resulted in production of an unbroken series of laminaridextrins and glucose as a minor component of the reaction mixture. Accordingly, this laminarin appeared to be unbranched pure 1,3-β-D-glucan.

Other substrates were assayed reductometrically in the same way as laminarinase. These included laminarihexaose, prepared from laminarin by partial acid hydrolysis and chromatography [10], and curdlan [13], a partially crystalline and insoluble bacterial 1,3-β-D-glucan which was supplied by Dr. T. Harada, Osaka University, Japan.

CM-pachyman with a degree of substitution of 0.32 was prepared by the method of Stone [14] from water-insoluble pachyman (a fungal 1,3-β-D-glucan, degree of polymerization, 255). CM-pachymanase activity was assayed viscometrically [1] at 35°C, pH 5.5, using 0.1 ml of enzyme preparation plus 0.9 ml of CM-pachyman solution (0.8%, w/v, sodium acetate buffer containing 0.2 M NaF) in Cannon-Manning semimicroviscometers. The rate of viscosity loss was proportional to the amount of enzyme added, up to approximately 20% loss, a limitation which was observed in all assays. One unit of CM-pachymanase activity is defined as the amount of enzyme required to cause 1% loss in viscosity in 2 h under these conditions.

Enzyme extraction

All extraction and purification procedures were performed at 2°C. Approximately 12 000 apical or basal segments (200 and 250 g fresh weight, yielding 0.42 and 0.17 g soluble protein, respectively) were homogenized in a blender in 2 vols. of buffer containing 20 mM sodium acetate (pH 5.5), 5% glycerol and 0.05% sodium azide. The brei was squeezed through nylon cloth and centrifuged at 13 000 × *g* for 10 min. Crude glucanase I (apical) and II (basal) extracts were precipitated with ammonium sulfate (Swartz-Mann, New York), and fractions containing most of the enzyme activities were dissolved in buffer and processed for further purification.

Glucanase purification

Microgranular DEAE-cellulose (Whatman DE-52) was desalted, washed and equilibrated with 20 mM sodium acetate buffer (pH 5.5) in a column (30 × 2.5 cm). The ammonium sulfate fraction containing glucanase activity (70 ml) was applied to the column and eluted (35 ml/h) with a linear salt gradient (200 ml of 0—2.0 M NaCl in the same buffer). Fractions (2 ml) containing high glucanase specific activity were pooled and concentrated by ultrafiltration (Amicon stirred cells, models 2000 and 200, fitted with UM-10 membranes).

Glucanases partially purified by DEAE-cellulose chromatography were further purified by gel filtration in a column (90 × 1.5 cm) of Sephadex G-50 (fine grade, Pharmacia Co., Uppsala, Sweden), equilibrated in 20 mM acetate

buffer (pH 5.5). They were eluted with the same buffer (23 ml/h) and fractions (2 ml) with high specific glucanase activity were pooled and concentrated by ultrafiltration. Yields of approx. 170 μg of glucanase I and 200 μg of glucanase II were obtained from the original 200—250 g fresh weight of tissue.

Gel electrophoresis

Purified glucanase I and II preparations (10 μg) were subjected to discontinuous electrophoresis [15] on polyacrylamide (7%) gels (50 × 5 mm tubes) under non-denaturing conditions. Electrophoresis was performed in duplicate gels for 2.5 h at 2 mA per gel at 2°C. Protein bands were visualized by staining one gel for 15 min with 1% Coomassie blue in 50% methanol/7% acetic acid, and destaining with 30% methanol/7% acetic acid. The other gel was frozen on solid CO_2 and sliced into 1 mm sections from which glucanase activity was eluted for assay by incubating for 40 min at 35°C in acetate buffer.

SDS gel electrophoresis [16] was conducted using purified glucanase I and II (10 μg) and samples of enzyme preparations at various stages of purification (approx. 100 μg protein). The preparations were lyophilized and suspended in 80 mM Tris-HCl (pH 6.8)/2% SDS/5% mercaptoethanol/10% glycerol with bromophenol blue (as marker). Samples were boiled for 3 min, cooled at room temperature, and applied to a slab gel (Bio-Rad, 1.5 mm, 12.5% polyacrylamide). Electrophoresis was carried out at 30 mA for 3 h, after which the gel was stained for 1 h with Coomassie blue and destained overnight. Marker proteins of known molecular weights were processed similarly.

Isoelectric points of purified glucanases I and II were determined [17] by electrofocusing proteins at room temperature in 4% polyacrylamide gels (130 × 2.5 mm tubes) containing 2% ampholines (comprising 1.6% pH range 5 to 7, plus 0.4% pH range 3—10) dissolved in 'lysis' buffer, i.e. 9.5 M urea/20% (w/v) Nonidet P-40/5% mercaptoethanol. The polymerized gels were overlayered with lysis buffer for 2 h. The lower reservoir was filled with 0.01 M H_3PO_4 and the upper reservoir with 0.02 M NaOH (degassed). A pH gradient was established by subjecting gels to a gradual increase in voltage (200—400 V for 1.5 h). Lysis buffer and NaOH was then removed from the surface of the gels and the samples were applied. The tubes were subjected to 400 V for 12 h and then 800 V for 1 h. Gels were placed in 30% methanol/7% acetic acid (v/v) for 2—4 h, stained with 0.1% Coomassie blue in 50% methanol/10% acetic acid (v/v), and destained overnight.

Results

Distribution of 1,3-β-D-glucanase activities

The data in Table I show that the crude enzyme preparation extracted from apical regions of pea seedlings contains laminarinase at a specific activity which is about three times that in extracts from basal regions, whereas CM-pachymanase specific activity is only about one-sixth as great in the apex as in the base. Detailed studies on the distribution of these two activities (data not shown here) indicate gradients with maximum laminarinase located in the apical region which has just ceased growing, and maximum CM-pachymanase in the oldest parts of the stem. Thus separate β-glucanase activities in pea seedlings appear to be developed at opposite ends of the plant.

TABLE I

DISTRIBUTION OF 1,3-β-D-GLUCANASE ACTIVITIES IN PEA STEMS

Glc, glucose.

Assay	Units	Apical region	Basal region
Laminarinase (reductometric)	μg Glc equiv./10 mm segment per h	63	19
	μg Glc/mg protein	604	182
CM-pachymanase (viscometric)	units/10 mm segment	11	67
	units/mg protein	105	650
Reducing power / Viscosity loss	μg Glc equiv./unit viscosity loss	5.7	0.28

The ratio of reducing power generated from laminarin to viscosity-loss of CM-pachyman is about 20 times greater in crude extracts from apical than basal segments (Table I). This represents a minimal value for a basic difference in the degree of 'randomness' with which the glucanases from growing and mature parts of the pea stem appear to attack their substrates.

In ammonium sulfate solutions, most (75%) CM-pachymanase activity sediments at between 20 and 45% saturation, whereas most laminarinase activity precipitates at between 50 and 70% saturation. The higher saturation range was employed as a first step for purification of the laminarinase from apical segments (henceforth called glucanase I) and the lower saturation range to purify the CM-pachymanase from basal segments (glucanase II).

Chromatography

In fractions eluted from DE-52 cellulose columns with a continuous gradient of NaCl, most pea protein is removed at a salt concentration below 1.0 M, and the two β-glucanase activities elute at different loci between 1.0 and 2.0 M (Fig. 1). When the pooled glucanase fractions after ion exchange chromatography are concentrated by ultrafiltration, the specific activity of glucanase I (1.2—1.8 M NaCl) increased about 6-fold with 46% recovery in total activity, and that of glucanase II (0.9 — 1.2 M NaCl) increased 120-fold with 82% recovery (Table II).

After fractionation on Sephadex G-50 columns, protein elutes mostly in void volumes and glucanase I and II fractionate (Fig. 2) at relatively high specific activities. The K_{av} value was lowest for glucanase II, suggesting that it possessed the higher molecular weight. Final yields of glucanase I and II were 13 and 10%. with 88- and 240-fold purification, respectively (Table II). Approximately 0.4% (w/w) of apical and 1.2% (w/w) of basal protein in the original crude extracts was 1,3-β-D-glucanase.

Electrophoresis

Fig. 3 shows the distribution of laminarinase and CM-pachymanase activities and stainable protein following non-denaturing polyacrylamide gel electrophoresis. Glucanase I and II migrated in a standard anionic system (pH 8.0) with R_F values of 0.36 and 0.15 (vs. bromophenol blue), respectively.

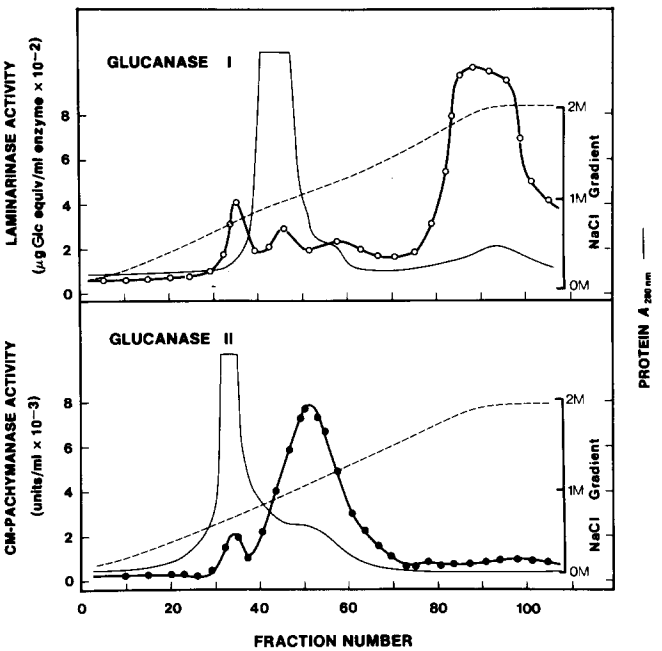

Fig. 1. Chromatography on DEAE-cellulose. Ammonium sulfate (50—70% saturation) precipitate of crude extract of apical segments (glucanase I) was loaded onto Whatman DE-52, eluted with a salt gradient, and assayed for laminarinase (○———○). Ammonium sulfate (20—45% saturation) precipitate from basal segments (glucanase II) was chromatographed and assayed for CM-pachymanase (●———●). Glc, glucose.

Glucanase I is the more anionic of the two, i.e., the numbering system proposed for these enzymes corresponds to the standard naming of isozymes. Both purified preparations contain major bands of protein which coincide with their catalytic activities.

Fig. 4 shows SDS gel-electrophoresis profiles of enzyme preparations during

TABLE II

PURIFICATION OF PEA 1,3-β-D-GLUCANASES FROM APICAL (I) AND BASAL (II) REGIONS OF PEA STEMS

Values are calculated for total extracts derived from 200—250 g of tissue. Glc, glucose.

Fractionation sequence	Glucanase I (reductometric)		Glucanase II (viscometric)	
	Total units (mg Glc/2 h)	Specific activity (mg Glc/mg protein)	Total units (units × 10^{-3})	Specific activity (units × 10^{-3}/mg protein)
Crude extracts	254	2.2	1110	2.8
$(NH_4)_2SO_4$ precipitate	250	2.5	585	7.9
DE-52, UM-10	82	14.5	296	507
Sephadex G-50, UM-10	33	194	106	677

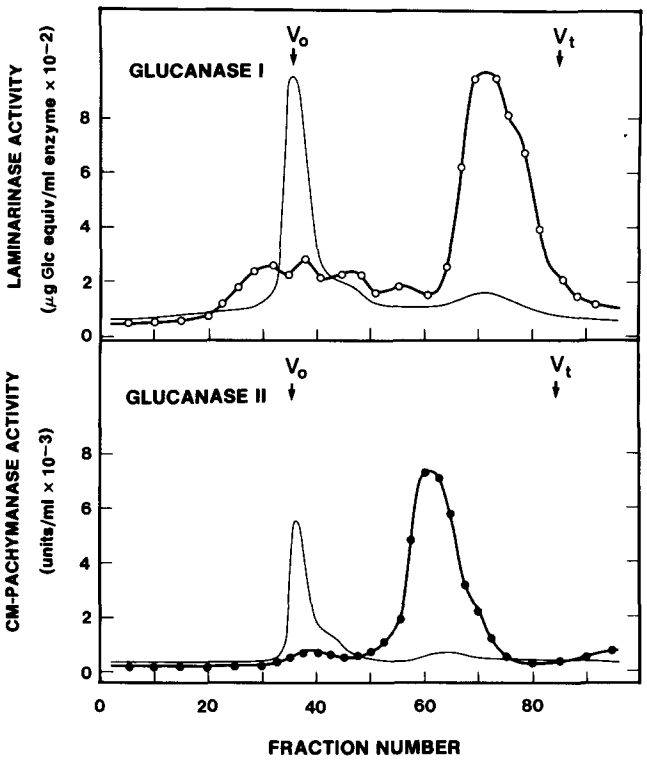

Fig. 2. Chromatography on Sephadex G-50. Enzyme preparations of glucanase I (apical) or II (basal) after Whatman DE-52 fractionation (Fig. 1) were concentrated and applied to a Sephadex G-50 column previously equilibrated with acetate buffer containing 5% glycerol and 0.05% sodium azide. Symbols are as in Fig. 1.

various stages of purification, together with marker enzyme loci. Crude extracts of apical tissue contain a large number of distinct proteins, particularly in the relatively high-molecular-weight range, compared to extracts from basal tissue. However, even in crude extracts, and certainly in ammonium-sulfate precipitates, the glucanases are visible as distinct protein components. Both purified glucanases electrophorese as major proteins with less than 1% contamination by other proteins. The molecular weights of glucanase I and II corresponded to 22 000 and 37 000, respectively. These values are considered to be more accurate than those obtained from K_{av} values on Sephadex G-50 (14 000 and 29 000, respectively) because of the likelihood that these enzymes bind to the gel. Crude pea extracts also chromatographed on this gel with elution volumes of laminarinase and CM-pachymanase activities corresponding to those of purified glucanase I and II, indicating that the glucanases were not altered in molecular weight during purification.

When subjected to isoelectric focusing in polyacrylamide gels, purified glucanase I and II display isoelectric points of 5.4 and 6.8 respectively (data not shown). Neither purified glucanase contains significant amounts of carbohydrate (less than 1% by weight, phenol-sulfuric acid test).

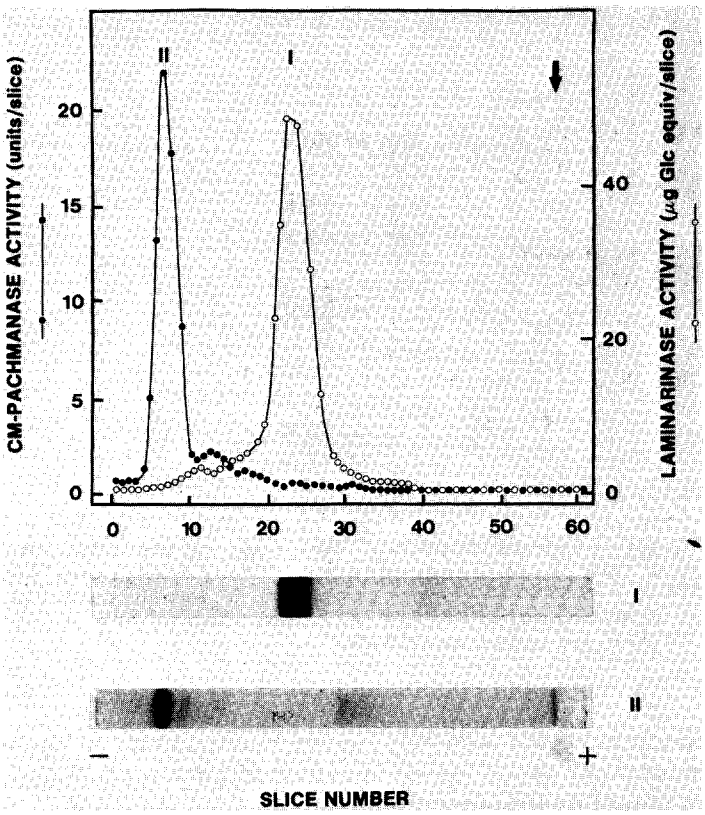

Fig. 3. Discontinuous polyacrylamide gel-electrophoresis of purified 1,3-β-D-glucanase I and II. Glucanases (approx. 10—20 μg) were electrophoresed in a standard anionic system (pH 8.0) and mobilities were determined relative to bromophenol blue. Major bands of protein which were present in glucanase I and II preparations coincided with activity as measured reductometrically (○———○) and viscometrically, (●———●), respectively.

Enzymic properties

After purification, both glucanase preparations could still be assayed either reductometrically or viscometrically. On a weight basis, glucanase I is approximately 14 times more active than II in the laminarinase assay, but II is 15 times more active than I in the CM-pachymanase assay (see Discussion).

The pH optimum for both glucanase I and II is close to 6.0 when assayed reductometrically against laminarin and 5.5 when assayed viscometrically against CM-pachyman. The pH optimum for hydrolysis of CM-pachyman is also 5.5 when assayed reductometrically. The implication is that the reductometric and viscometric assays are both measuring products of the same active sites on each enzyme, though the pH optimum is shifted slightly towards basic values when the substrate is negatively charged.

Table III summarizes V and K_m values for the purified glucanases, using assays of activity with laminarihexaose, laminarin, CM-pachyman and curdlan. Generally, it requires only a few mg/ml of substrate to half-saturate both enzymes. Glucanase I shows lowest apparent affinities towards CM-pachyman

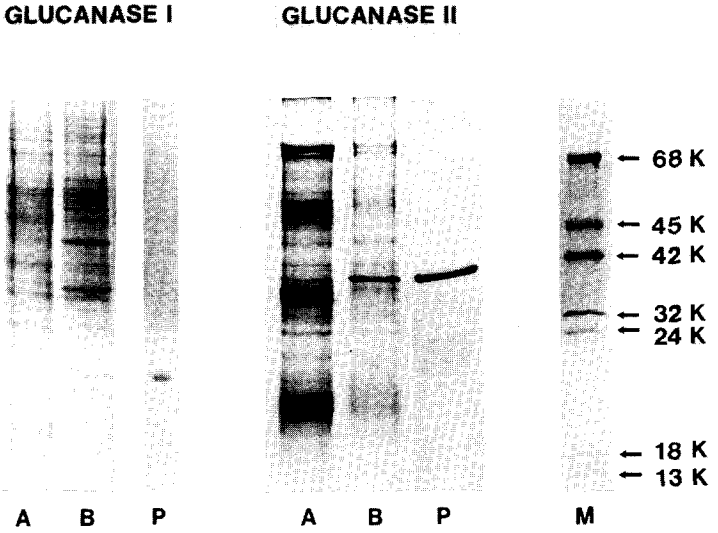

Fig. 4. SDS gel-electrophoresis at various stages of purification. A, crude extracts; B, ammonium sulfate precipitates; P, purified enzymes. Molecular weights of approximately 22 000 for glucanase I and 37 000 for glucanase II were extrapolated from the positions of marker proteins (M), i.e., from the anode (top): bovine serum albumin (68 K, i.e., 68 000 daltons), ovalbumin (45 K), carbonic anhydrase (32 K), chymotrypsinogen (24 K), β-lactoglobulin (18 K) and cytochrome c (13 K).

and curdlan, probably because of the relatively reduced accessibility of these substrates. However, glucanase II shows lowest apparent affinity for laminarihexaose, suggesting that it prefers longer chains. $1/K_m$ and V values for both enzymes are highest for laminarin, which combines substantial chain length (degree of polymerization, 20) and maximum accessibility (solubility) as a substrate.

With respect to stability of the purified glucanases, both show similar sensitivities to thermal inactivation (Fig. 5). At high dilution, pH 5.5, they lose all activity within 10 min at 70°C, and 50% of activity within 30 min at about 55°C. They may be stored in acetate buffer (pH 5.5) for about 3 months at 2°C

TABLE III

KINETIC CONSTANTS OF PURIFIED 1,3-β-D-GLUCANASE I AND II

Substrates (up to 2%, w/v) were incubated (total volume 1.0 ml) with purified glucanase I or II (0.05 μmol) in 20 mM sodium acetate buffer (pH 5.5) at 35°C and initial velocities were determined. All assays were reductometric and values were calculated from linear Lineweaver-Burk plots. D.P., degree of polymerization.

Substrate	D.P.	V (μmol Glc equiv./μmol enzyme per min)		K_m (mg/ml)	
		I	II	I	II
Laminarihexaose	6	98	24	2.2	7.4
Laminarin	20	570	127	1.5	0.6
CM-pachyman	255	182	34	3.3	4.5
Curdlan	450	75	28	4.2	2.8

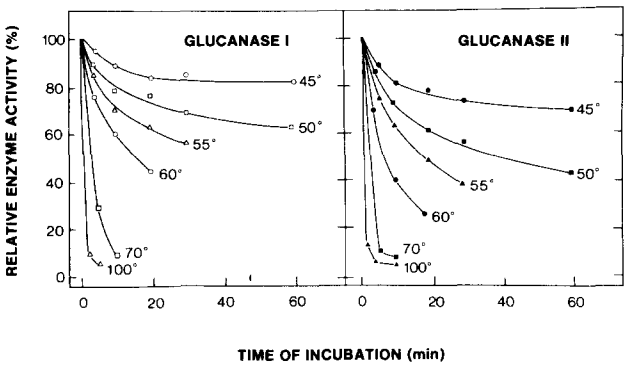

Fig. 5. Decay of pea 1,3-β-D-glucanase activities at various temperature. Samples (0.2 μmol in 0.5 ml buffer, pH 5.5) of purified glucanases were incubated at different temperatures and aliquots were withdrawn at time intervals for CM-pachymanase assays.

without loss of activity, but thereafter they both decay to approximately 50% of initial activity within 5 months. The addition of glycerol (2.5%, v/v) helps to stabilize both enzymes, and bovine serum albumin (0.25%, v/v) prevents inactivation for at least 6 months. These measurements were made with CM-pachyman as substrate, but checks of stability with the laminarinase assay show that both activities decay in parallel, as expected for enzymes with single active sites for these two substrates.

Discussion

Two pea 1,3-β-D-glucanases were purified by ammonium sulfate fractionation and chromatography and physical and enzymic properties were determined. In most respects, these glucanases resemble those that have been examined from various other higher plant sources, all of which possess relatively low molecular weights, acidic pH optima, and K_m values of a few mg/ml with either laminarin or CM-pachyman as substrates.

Other higher plants have not been reported to contain more than one endo-1,3-β-D-glucanase, though this may be due to the fact that tissue distribution has not been studied. In pea stems, it is clear that apical and basal tissues contain separate glucanases (Table I) which differ not only in physical properties but also in relative K_m and V values for various substrates (Table III). Thus, the apical enzyme, glucanase I, is more readily assayed reductometrically as a laminarinase than viscometrically as CM-pachymanase, and the basal enzyme, glucanase II, shows reverse properties (Table IV). This does not mean that glucanase I is an exo-glucanase and II and endo-glucanase. More detailed kinetic studies (subsequent paper) show that both are properly considered as endo-glucanases (EC 3.2.1.6) but, following endohydrolysis, glucanase I tends to continue cleaving lower-molecular-weight fragments whereas II preferentially attacks the longest chains first and at random. Even though K_m values for CM-pachyman are similar for the two enzymes, glucanase I is much less effective than II in reducing the viscosity of a large population of CM-pachyman chains (Table IV). However, I is much more effective than II in hydrolysing

TABLE IV

SUMMARY OF PROPERTIES OF PURIFIED PEA 1,3-β-GLUCANASES

Property	Glucanase I	Glucanase II
Primary tissue locus	Subapical	Basal
Molecular weight	22 000	37 000
Isoelectric point (p*I*)	5.4	6.8
Carbohydrate content (%, w/w)	0.5	0.8
pH optimum, Laminarin	6.0	6.0
pH optimum, CM-pachyman	5.5	5.5
K_m, various substrates (mg/ml)	1.5—4.2	0.6—7.4
Relative hydrolase activities *		
Laminarinase (1% laminarin) (mg Glc/mg protein per h)	87	6.4
CM-pachymanase (0.8% CM-pachyman) (units × 10^{-3} per mg protein)	45	680
Laminarinase/CM-pachymanase (ng Glc per unit viscosity loss)	1 930	9.4

* These values cannot be compared readily to *V* values (Table III) since assays were not conducted with saturated levels of substrates, and CM-pachymanase was measured here by the viscometric method in order to estimate endohydrolytic activity only.

laminaridextrins (Table III). Thus the differences between the enzymes can be accounted for by differences in substrate affinity and randomness of attack, rather than by any fundamental distinction in mechanism of hydrolysis.

With respect to the question of possible precursor-product relationships between the two pea 1,3-β-D-glucanases, the larger II cannot be a dimer of I since physical properties that depend on amino acid composition (e.g., p*I*) are different for the two enzymes, and electrophoresis under strongly dissociating conditions (Fig. 4) does not reduce the size of II. Nor is it likely that glucanase I is processed or metabolized into II during development of young apical to older basal tissues, since such changes would require a doubling of molecular weight. This might conceivably occur through extensive glycosylation, but II is not a glycoprotein (Table IV). The distribution of glucanase I in the pea stem resembles that of many other glycosidases and phosphatases [8,18], however the near absence of II in the apex and its concentration in mature tissues (Table I) is unique. Thus, the two glucanases are almost certainly regulated by very different controls and, by implication, they may have different functions and/or substrates during plant growth and development.

The two endo-1,4-β-D-glucanases (EC 3.2.1.4) which have been purified from pea stems [9] differ from the 1,3-β-D-glucanases in a number of respects besides substrate specificity [10]. Buffer-soluble cellulase is generated by rough endoplasmic reticulum [6,11] and is confined within vesicles in vivo, while buffer-insoluble cellulase is secreted and bound firmly to cellulose in the wall [6]. The 1,3-β-D-glucanases, in contrast, are both buffer-soluble and show little apparent concentration in or affinity for cell organelles or wall material [19]. Also, while the 1,3-β-D-glucanases differ from one another in degree of randomness with which they hydrolyse substrates, the cellulases show kinetic properties which are so similar [10] that they may possess the same active sites. K_m values for standard substrates (degree of polymerization 6 dextrins and CM-polysaccharide) of the four enzymes are all very close (a few mg/ml), but *V* values are higher for the cellulases [10], suggesting that the latter are more efficient

catalysts. Finally, after testing several substrates, the pea cellulases were found to degrade cellohexaose most rapidly, as do many fungal cellulases, which has been interpreted as indicating a binding site for substrates that accomodates six glucose units. In contrast, the pea 1,3-β-D-glucanases degrade laminarin about 5 times more effectively than laminarihexaose (Table III) suggesting, on analogy, that these enzymes possess a relatively larger binding site which distinguishes between chains that are much longer than a polymerization degree of 6.

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