STUDIES ON ENDO-1,3-β-D-GLUCANASES FROM PISUM SATIVUM: PURIFICATION, DEVELOPMENT AND ENZYMIC PROPERTIES.

by

YUK-SHAN WONG

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Department of Biology,
McGill University,
Montreal, Quebec,
Canada.

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ABSTRACT

Two buffer-soluble endo-1,3-β-D-glucanases (E.C. 3.2.1.6) have been purified to electrophoretic homogeneity from etiolated Pisum sativum stem tissues. Glucanase I and II differ in their mobility in SDS polyacrylamide gels (MW = 22K and 37K, respectively) and in isoelectric focusing (pI = 5.4 and 6.8, respectively) but are similar in pH optimum (5.5 - 6.0), in K_m values for various substrates (0.6 - 7.4 mg/ml) as well as in thermal inactivation profiles. Endogenous glucanase I is concentrated in tissues just below the elongating region (apex) of the stem, whereas II is mainly located in basal mature tissues. This pattern parallels the distribution of endogenous substrates for the two enzymes, as investigated with biochemical and histochemical techniques (using fluorescence in aniline blue). The enzymes and substrates may exist in vivo in separate cell compartments. Auxin treatment evokes an increase in specific glucanase II but not I activity. Ethylene suppresses both activities.

Both purified glucanases degrade laminarin, laminaridextrins, and their reduced [^{3}H] derivatives, partially substituted 1,3-β-D-glucan, (O-carboxymethyl-pachyman), crystalline 1,3-β-D-glucans (curdlan), and mixed-linkage β-glucans. Kinetic data and product formation indicate endo-hydrolytic activity with weak transglycosylase capacity. The enzymes do not cleave β-glucosides, laminaribiose, 1,3-linkage adjacent to the reducing end of chains, or cellulose and its derivatives. They hydrolyse mixed-linkage β-glucans in a manner similar to Rhizopus endo-1,3-β-D-glucanase, to form the products expected from hydrolysis of linkages adjacent to 1,3-β-linkages.

Glucanase I generates reducing groups from all substrates more rapidly than II, and it hydrolyses lower laminaridextrins at non-reducing terminal linkages while II preferentially degrades internal linkages. Laminarin is hydrolysed to lower laminaridextrins by I more rapidly than II, but I takes longer than II to completely degrade laminarin chains. Glucanase I is more sensitive to inhibitors that bind tryptophane and lysine residues, whereas II is inactivated by OH-binding reagents. It is concluded that active sites of these two hydrolases differ in such a way that glucanase I preferentially continues to hydrolyse fragments produced by endo-hydrolytic attack on long chains ("multiple attack") while II cleaves internal linkages of the longest chains available ("multi-chain" action pattern).

RÉSUMÉ

Deux endo-1,3-β-D-glucanases (E.C. 3.2.1.6) solubles dans le tampon (buffer-soluble) ont été purifiées jusqu'à homogénéité électrophorétique à partir de tissus de tige étiolée de Pisum sativum. Les glucanases I et II diffèrent quant à leurs mobilités sur des gels dénaturants de SDS-polyacrylamide (Poids moléculaires: 22K et 37K respectivement), et quant à leurs P_I (5.4 et 6.8 respectivement) déterminés par analyse iso-électrique mais montrent des similarités dans leurs pH optima (5.5 - 6.0) et leurs K_m (0.6 - 7.4 mg/ml) pour différents substrats, de même que dans leurs profils d'inactivation thermique. La glucanase I endogène est concentrée dans les tissus juste en dessous de la région croissante (sommet) de la tige tandis que II est principalement localisée dans les vieux tissus à la base de la tige. Cette distribution suit celle des substrats endogènes des deux enzymes que nous avons étudiée avec des techniques biochimiques et histochimiques (fluorescence produite par l'aniline bleue). Ces enzymes et leurs substrats pourraient exister in vivo dans des compartiments cellulaires distincts. L'auxine augmente l'activité spécifique de la glucanase II mais n'a aucun effet sur I tandis que l'éthylène supprime les deux activités.

Les deux glucanases purifiées dégradent la laminarine, les laminaridextrines et leurs dérivés-[^{3}H]-réduits, les 1,3-β-D-glucanes partiellement substituées (O-carboxyméthyle-pachymane), les 1,3-β-D-glucanes cristallins (curdlan), et les β-glucanes à liaisons mixtes. Des cinétiques d'hydrolyse et des caractérisations de produits ont montré une activité endo-hydrolytique de même qu'une faible activité de transglycosylase. Ces

enzymes ne coupent pas les β-glucosides, la laminaribiose, la cellulose et ses dérivés ou les liaisons 1,3 adjacentes aux extrémités réductrices des chaînes. Elles hydrolysent les β-glucanes à liaisons mixtes comme la endo-1,3-β-D-glucanase de Rhizopus, pour former les produits attendus de l'hydrolyse des liaisons adjacentes aux liaisons β-1,3.

La glucanase I produit des groupements réducteurs de tous les substrats plus rapidement que II, et elle hydrolyse les laminaridextrines à chaînes courtes (D.P. petit) aux dernières liaisons non-réductrices tandis que II dégrade de préférence les liaisons internes. La laminarine est hydrolysée jusqu'aux laminaridextrines à chaînes courtes par I plus rapidement que par II, mais I met plus de temps à hydrolyser complètement les chaînes de laminarine. La glucanase I est sensible aux inhibiteurs spécifiques aux résidus de tryptophane et de lysine, pendant que II est inactivée par des réactifs s'attachant aux groupements hydroxyles. Nous pouvons donc conclure que les sites actifs de ces deux hydrolases diffèrent de la façon suivante: la glucanase I continue à hydrolyser préférentiellement les fragments produits par attaque endo-hydrolytique sur des chaînes longues (attaque multiple) tandis que II coupe les liaisons internes des chaînes les plus longues (attaque sur chaînes multiples).

Traduit par Krikor Torossian

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PREFACE

The results of this thesis are organized in accordance with regulations of the Faculty of Graduate Studies and Research into "Chapters", which are written with the expectation that modified versions will be submitted as papers for publication. The first and second of these are biochemical in nature and have been revised and ~~submitted to~~ accepted by the journal Biochim. Biophys. Acta. The third, which is due to be submitted to the journal Plant Physiology, is of more interest to biologists. Further explanations of methods and details not included in these chapters are presented in "Appendices". The thesis is introduced by a more extensive "Literature Review" than is normally acceptable in published papers, and references are assembled in the individual sections in which they are cited. Finally, a section on "Concluding Remarks" contains a Table to summarize the enzymic properties of pea 1,3-β-D-glucanases, as observed in the present study.

ABBREVIATIONS

ABA	Abscisic acid
BA	Benzyladenine
BSA	Bovine serum albumin
CG	3-O-β-cellobiosyl-D-glucose
p-CMB	p-Chloromercuribenzoate
CMC	O-Carboxymethyl-cellulose
CM-pachyman	O-Carboxymethyl-pachyman
2,4-D	2,4-Dichlorophenoxyacetic acid
DANS	5-Dimethylaminonaphthyl sulfonyl chloride
D.P.	Degree of polymerization
D.S.	Degree of substitution
ER	Endoplasmic reticulum
FUdR	5-Fluorodeoxyuridine
G_1	Glucose
G_2	Cellobiose
G_3	Cellotriose
G_4	Cellotetraose
G_5	Cellopentaose
G_6	Cellohexaose
GA	Gibberellic acid
pI	Isoelectric point
IAA	Indole-3-acetic acid
K_m	Michaelis-Menton constant
K_I	Dissociation constant of an enzyme-inhibitor complex

L_2	Laminaribiose
L_3	Laminaritriose
L_4	Laminaritetraose
L_5	Laminaripentaose
L_6	Laminarihexaose
L_7	Lamina riheptaose
L_8	Laminarioctaose
L_{2H}	Laminaribiitol (reduced laminaribiose)
L_{3H}	Laminaritriitol (reduced laminaritriose)
L_{4H}	Laminaritetraitol (reduced laminaritetraose)
L_{5H}	Laminaripentaitol (reduced laminaripentaose)
L_{6H}	Laminarihexaitol (reduced laminarihexaose)
L_{7H}	Laminariheptaitol (reduced laminariheptaose)
LG	4-O-β-Laminaribiosyl-D-glucose
$[M_n]$	Molar rotation
OR	Origin
β-PNPC	p-Nitrophenyl β-D-cellobioside
RSIII	Reduced pneumococcal SIII polysaccharide
SDS	Sodium dodecyl sulfate
UDPG	Uridine-5-diphosphate α-D-glucose
V_{max}	Maximum velocity

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INTRODUCTION

This review is intended to be a more extensive survey of the kinds of 1,3-β-D-glucans and 1,3-β-D-glucanases, and their properties and distribution in the plant kingdom, than is possible in introductions to the chapters of this thesis. The latter have been written in a manner suitable for publication, and they concentrate on specific references to relatively recent studies of such products in higher plants. In fact, 1,3-β-D-glucans and glucanases have been more widely studied in fungi, and this preliminary review therefore includes reference to many of the basic advances that have been made with such systems. No recent comprehensive reviews have been published on these products. For surveys of the older literature, the reader is referred to a pioneer review by Clarke and Stone (1). For briefer more recent surveys of various aspects, including callose (2-6), mixed-linkage β-glucans (7-9) and 1,3-β-D-glucanases (10,11), the references given in specific papers devoted to new results are all that is available.

The present author, however, has been privileged to have access to a draft copy of a monograph written by Prof. B.A. Stone and colleagues (Dept. of Biochemistry, La Trobe Univ., Bundoora, Victoria, Australia) between 1976 and 1978, tentatively entitled: "Biochemistry of 1,3-β-D-Glucans", which is due to be published in book form. When complete, this will be an exhaustive review of the entire subject, and it was an invaluable source of information for the present literature survey. The present review supplements Stone's forthcoming monograph by organizing information concerning these enzymes from a point of view which is more particularly relevant to the studies

reported in this thesis. Table I records the various sources, trivial names and linkages of common 1,3-β-D-glucans, and Table II gives further details of their physical properties. Table III describes a classification based on Stone of the various types of endo-β-glucanases which are known to be capable of hydrolysing substrates containing 1,3-linkages. Table IV indicates the sources from which these various types have been derived and defined. Finally, Table V and VI summarize examples of the many methods that have been employed for purification of 1,3-β-D-glucanases from various sources, and their properties. All of these Tables include relatively recent references, particularly to the higher plant sources from which these enzymes were derived. This review of important papers was concluded at the end of 1978, although some more recent publications that have come to our attention are included.

I. Occurrence, Chemical Structure and Properties of 1,3-β-D-Glucans

Polysaccharides of D-glucopyranose units joined in glucosidic linkages are widely distributed in nature, and those derived solely from glucose are referred to as glucans. They are classified into various types of polymers according to different patterns of glucosidic linkages. For example, cellulose is a 1,4-β-glucan which consists of β-glucopyranose units linked by glucosidic oxygen through the 1 and 4 position. There are eight ways of linking α- and β-glucopyranose units in glucans, and all except the 1,2-α-type have been shown to occur in natural polymers. Apart from the linear homogeneous glucans, other linkages may also be present, either as part of the linear polymer or as branch points or both.

The 1,3-β-D-glucans have been reported in many bacteria, fungi and higher plants (Tables I and II) where they appear to have physiological significance in structural and storage functions. The 1,3-β-D-linkages are probably the exclusive type of bonding in only a limited number of β-glucans, e.g., curdlan, callose, while in most that have been critically examined a 1,3-β-glucan backbone is associated with variable proportions of internal 1,4-β-linkages, e.g., lichenin, or 1,6-β-linkages,e.g., laminarins. Some are water-soluble, e.g., laminarin, while others are partially crystalline, fibrous and alkali-insoluble, e.g., yeast cell-wall β-glucan. Molecular weights are relatively low in most recorded cases (D.P. is seldom greater than a few hundred).

The 1,3-β-D-glucans occur in diverse locations in plants, ranging from intracellular inclusions which may consist of homogeneous 1,3-β-D-glucans, e.g. callose, paramylon granules, etc., to cell-wall components where they may be associated with other carbohydrates, with lipids or proteins (1). On a cellular level, investigations into the spatial relationships between 1,3-β-D-glucans and other macromolecular constituents of cellular structure have been made possible with the techniques of X-ray diffraction (62, 63) and electron microscopy (61, 77), in conjunction with step-wise chemical extractions and histological examination using staining reactions (65, 66). The chemical structure of the glucan can also be studied through X-ray and infra-red analyses (62, 63, 67, 68) and optical rotation measurement (69, 70). The detailed linkage composition and molecular structure may be elucidated by

TABLE I

BASIC STRUCTURE AND SOURCE OF 1,3-β-D-GLUCANS

Source	Distribution	Trivial name	Main linkage(s)	References
BACTERIA				
Alcaligenes faecalis	culture filtrates	curdlan	1,3-	12, 13
Agrobacterium radiobacter	culture filtrates	curdlan	1,3-	12, 13
FUNGI				
Ascomycetes	cell walls	-	1,3-; 1,6-	20, 21
Saccharomycetes	cell walls	-	1,3-; 1,6-	18, 19
Basidiomycetes	cell walls	pachyman	1,3-	14 - 17
Oomycetes	cell walls	-	1,3-; 1,6-	22 - 26
	intracellular	cellulin	1,3-	
	granules	mycolaminarin	1,3-	
Deuteromycetes	cell walls	-	1,3-; 1,6-	28 - 35
ALGAE				
Bacillariophyceae (diatoms)	intracellular constituents	-	1,3-	36 - 37
Chlorophyceae	" "	-	1,3-	38
Phaeophyceae	" "	laminarin	1,3-; 1,6-	39 - 42
Euglenophyceae	intracellular granules	paramylon	1,3-	43 - 45
Chrysophyceae	intracellular constituents	chryso-laminarin	1,3-; 1,6-	46
LICHEN	intracellular constituents	lichenin	1,3-/1,4-	47 - 50

TABLE I

(cont'd)

Source	Distribution	Trivial name	Main linkage(s)	References
HIGHER PLANTS				
Dicotyledonous	cell wall sieve elements	callose	1,3-	51 - 54
Monocotyledonous	cell wall	cereal glucans	1,3-/1,4-	55 - 60

TABLE II

PROPERTIES OF 1,3-β-D-GLUCANS

Source	Linkage composition % 1,3-linkages	others	D.P.	Specific optical rotation $[\alpha]_D^o$	References
BACTERIA					
Alcaligenes faecalis	97	1,6-	455	18^o in 0.1 M NaOH	12, 13
Agrobacterium radiobacter				3^o in $CHCl_3$	12, 13
FUNGI					
Basidiomycetes					
Fomes annosus	25	1,6-; 1,4-	-	10^o in H_2O	14
Poria coccus	90	1,6-	255	32^o in 1 M NaOH	15
Schizophyllum commune	65	1,6-	-	-	16
Polyporus fomentarius	24	1,6-	-	-26^o in H_2O	17
Saccharomycetes (yeast)					
Saccharomyces cerevisiae					
(cell wall, alkali sol.)	94	1,6-	154	-	18, 19
(" ", alkali insol.)	70	1,6-	1500	-10^o DMSO	
(" ", hot H_2O sol.)	5	1,6-	130	-32^o in H_2O	

TABLE II (Cont'd)

Source	Linkage composition % 1,3-linkages	others	D.P.	Specific optical rotation $[\alpha]_D^o$	References
Saccharomyces fragilis	85	1,6-	-	-	
Ascomycetes					
Cyttaria harioti	40	1,6	220	11^o 1 M KOH	20
Claviceps fusiforms	75	1,3-/1,6-	-	9^o 5M NaOH	21
Plectania occidentalis	70	-	1400	-	
Oomycetes					
Pythium acanthicum	63	1,6-; 1,4-	-	-10^o 1 M KOH	22
Phytophthora cinnamonsi	59	1,4-/1,6-	420	-33^o in H_2O	23, 24
Phytophthora pulmivora	90	1,3-/1,6-	4900	-15^o in H_2O	25, 26
Phytophthora megasperma	56	1,3-/1,6-	620	-	27
Deuteromycetes					
Aspergillus niger	85	1,4-	-	30^o 1 M NaOH	28
Piricularia oryzae	83	1,6-; 1,3-/1,6-	238	-	29,30
Aureobasidium pullulans	96	1,6-/1,3-	-	-20^o $CHCl_3$	31
Microsporum quinckeanum	22	1,6-; 1,3-/1,6-	36	-27^o H_2O	32, 33
Trichophyton granulosum	25	1,6-; 1,3-/1,6-	-	-37 H_2O	33

TABLE II (Cont'd)

Source	Linkage composition		D.P.	Specific optical rotation $[\alpha]_D^o$	References
	% 1,3-linkages	others			
ALGAE					
Bacillariophyceae					
Phaeodactylum tricornutum	-	1,3-/1,6-	-	42° H_2O	36, 37
Chlorophyceae					
Caulerpa simphicuiscula	-	1,6-; 1,4-	270	25° H_2O	37, 38
Phaeophyceae					
Laminaria digitata	90	1,6-	16 - 33	-12° H_2O	39, 40
Laminaria japonica	90	1,6-	35	-	41
Fucus vesiculosus	90	1,4-; 1,6-	-	-	42
Euglenophyceae					
Euglena gracilis	-	-	150	28° 1 M NaOH	43, 44
Astasia ocellata	95	1,3-/1,6-	52 - 55	17° 1 M NaOH	45
Chrysophyceae					
Ochromonas malhamensis	90	1,6-	34	10.8° H_2O	46
LICHEN					
Evernia prunastri	75	1,4-	60	12° H_2O	47

TABLE II (Cont'd)

Source	Linkage composition % 1,3-linkages	others	D.P.	Specific optical rotation $[\alpha]_D^o$	References
Cetraria iscelandica	30	1,4-	52 - 410	8^o H_2O; -32^o $CHCl_3$	48 - 50
HIGHER PLANTS					
Dicotyledonous					
Vitis vinifera	90	-	90	37^o $CHCl_3$	51
Lilium longiflorum	66	1,4-	-	12^o 1 M NaOH	52
Larix laricina	81	1,4-	126 - 205	17.6^o 0.25 M NaOH	53
Rosa sp.	95	-	120	-38^o DMSO	54
Monocotyledonous					
Horden vulgare (barley)	32	1,4-	1350	-11.2^o H_2O	55 - 57
Avena sativa (oat)	28	1,4-	160	-8.3^o H_2O	55, 58, 59
	35	1,4-	380	37^o H_2O	
Secale cereale (rye)	36	1,4-	396	-	
Sorghum officinale	58	1,4-	26	9^o H_2O	59
Arundinaria japonica (bamboo leaves)	31	1,4-	56	-6.5^o 0.5 M NaOH	60

methylation analysis (71) and periodate oxidation (72, 73), as well as by the identification of the products of partial acid (74) or enzyme hydrolysis (75, 76). Table II summarizes physical properties, including linkage composition, degree of polymerization (D.P.) and specific optical rotation of 1,3-β-D-glucans from various sources.

a) Bacteria

The best known bacterial 1,3-β-D-glucan is curdlan, a gel-forming neutral glucan extracted from a stable mutant (K) of the soil micro-organism Alcaligenes faecalis var. myxogenes 10C3 (12, 13). Curdlan is water-insoluble but soluble in formic acid, DMSO, and 0.1 M NaOH. It has an optical rotation ($[\alpha]_D^o$) of +3° in $CHCl_3$ (Table II). Structural characterization by methylation, periodate oxidation, infra-red spectroscopy and enzymic hydrolysis show that curdlan is a linear 1,3-β-D-glucan (97% 1,3-linkages) with an average D.P. of 455 (12, 13).

Curdlan molecules self-associate into microfibrils or gels at high temperature (over 60°C). The gel is elastic, resilient and able to resist high pressures. The gel strength increases with temperature or urea concentration (78). Optical rotation, density and viscosity studies show that at low NaOH concentrations the polysaccharide has a regular conformation, whereas at higher alkali concentrations it consists of random coils (79). With respect to the ultrastructure of the polymer, electron microscopy of native and partially degraded curdlan samples (D.P. 400 and 260) revealed microfibrils 10 to 20 nm wide. However,

lower molecular weight insoluble and soluble fractions (D.P. 36 and 13, resp.) did not occur as microfibrils (77).

Due to its gelling properties, curdlan is used as a food additive for adjusting the viscosity of jelly products. The gel of curdlan, which possesses no caloric value to man, displays properties intermediate between the brittleness of agar gel and the elasticity of gelatin, thus acting as a useful ingredient for low-caloric food (80).

b) Fungi

As a result of studies of fungal cell walls, it is clear that in some species cellulose, and in others chitin, constitutes the major part of wall polysaccharides. However, there is also evidence that mannans, 1,3-β-D-glucans and other non-cellulosic glucans occur in a number of filamentous fungi (Table II). In addition to being components of rigid cell walls, some fungi and yeast have intracellular 1,3-β-D-glucans with storage functions (81).

The chemical and physical properties of fungal 1,3-β-D-glucans have been extensively studied using as starting material walls of hyphae, sporangia and conidia and, in some cases, whole fruiting bodies. As most fungal wall 1,3-β-D-glucans are insoluble in water, acid and alkali are generally used as extractants. These treatments, however, may lead to the alteration of the physical properties or to the loss of important structural features of these glucans (82, 83); thus the assessment of homogeneity of fractions during characterization is often difficult.

β-Glucan from the walls of baker's yeast (Saccharomyces cerevisiae) has been the most extensively investigated polysaccharide among different fungi (1, 84, 85). Alkali extraction of whole yeast cells followed by specific enzymic hydrolysis, indicates that the "yeast glucan" consists of linear chains of 1,3-β-linked glucose units with a small number of 1,6-β-linkages cross-linking these chains (84, 85). However, other investigations suggest that this glucan has 1,3-β- side branches attached to a 1,3-/1,6-β-glucan chain (22). About 20% of the total wall polysaccharide consists of this β-glucan.

With respect to the structural organization of 1,3-β-D-glucans in fungal cell-walls, some Oomycete species (e.g., Phytophthora palmivora and Pythium acanthicum, see Table II) consist of a bilayered wall in which the inner-most part is cellulosic and microfibrillar,while the outer-most part contains a mixed 1,6- and 1,3-β-D-glucan (24,25, 28). In contrast to yeast cell walls, the Oomycete hyphal walls often show the presence of relatively high proportions of alkali-insoluble 1,4-β-linkages. However, the chemical evidence obtained to date has not distinguished between polysaccharides with a mixed 1,3-/1,4 -β-linkage within single chains, or a mixture of separate 1,3- and 1,4-β-glucans. In Ascomycete and Basidiomycete species, the organization of hyphal walls is somewhat more complex than in Oomycetes. The Ascomycete Neurospora crassa has multi-layered hyphal walls, and the 1,3-β-D-glucan component, which is mainly in the inner-most layer, appears to be associated with protein (20). Other species of this group appear to have their 1,3-β -D-glucan component covalently attached to a branched galactomannorhamnan

core (86). The hyphal walls of the Basidiomycete Schizophyllum commune (16) contain chitin and mixed 1,3-/1,4-α-linkages (S-glucan) in addition to 1,3-/1,6-β-glucan (R-glucan). The walls are composed of four layers: the outermost S-glucan layer, an underlying R-glucan layer, a discrete protein layer and an inner-most layer of chitin microfibrils.

Intracellular fungal 1,3-β-D-glucans are exclusively found in the mycelia of Oomycetes (81, 26). They are of the laminarin-type 1,3-β-D-glucan and are known as mycolaminarins. Mycolaminarins are water-soluble linear 1,3-β-D-glucans, which are readily metabolized and act as reserves in the cells (storage carbohydrate) to be consumed during growth on glucose-depleted media. This property is unique to the Oomycetes among fungi, and may be indicative of phylogenetic ties between Oomycetes and algae (87). Another Oomycete intracellular inclusion, called cellulin granules, contains 40% β-glucan with 1,3- and 1,6-glucosidic linkages, and the remainder is chitin (88). It has been suggested that this product serves a "plugging" function to block off sectors of the mycelial threads (89).

c) Algae

Algae have long been used in studies of cellulose microfibril deposition (27) but they are also known to contain non-cellulosic polymers in their walls (37, 38). Recent investigation of the chemistry of the cell-wall polysaccharides of some members of this group has shown that the main component present is often 1,3-β-xylan (90,91). Caulerpa simplicuiscula (Chlorophyceae) walls are known to fluoresce brightly with

aniline blue (1), but whether this is due to the 1,3-β-xylan or to some associated 1,3-β-D-glucan, or both, is unanswered. Both 1,4- and 1,3-β-linkages are found in glucans as major constituents of Xanthophyceae cell walls (36, 37), where periodate oxidation showed that the proportion of 1,4- : 1,3- linkages was 85 : 15, a ratio higher than those reported in mixed-linkage glucans of cereals or lichens.

Many intracellular carbohydrate inclusions have been reported in algae which, on analysis, have proved to be either 1,4-/1,6-β-glucans or 1,3-β-D-glucans. The latter occurs either as granular inclusions (paramylon) or as soluble cytoplasmic/vascular constituents (laminarin or leucosin). Paramylon is a linear 1,3-β-D-glucan, typically found in Euglenophyceae, which is insoluble in water but dispersable in dilute alkali (Table II, 43, 44). Laminarin is the most common 1,3-β-D-glucan found especially in the fronds of brown algae (Phaeophyceae). Chemically two distinct laminarins have been recognized, the Laminaria-type and Eisenia-type (39, 40, 41). The Laminaria-type laminarins are water-soluble, linear 1,3-β-D-glucans (D.P. 20 - 25), usually with small amounts of glucose in branches through 1,6-β-linkages. A variable proportion (30 - 90%) of laminarin chains are terminated by mannitol residues (M-chains). Eisenia-type laminarins, however, are linear 1,3-/1,6-β-glucans with 1,3- : 1,6- ratios of approximately 3 : 1. They are readily soluble in cold water and are not terminated with mannitol residues. Higher molecular weight (D.P. 270) soluble laminarin-like 1,3-β-D-glucans have also been isolated from some algal species (e.g., Caulerpa simplicuiscula, 38), although they may occur in association with

1,3-β-xylan in the walls. The term Leucosin (Chrysolaminarin) has been used by phycologists to describe the material of cytoplasmic inclusions of diatoms and Chrysophyceae species. They are highly refractile (1) and water-soluble. A detailed investigation of the structure of Leucosin reported (1) that the polysaccharide contained 99% glucose, and methylation analysis indicated the presence of 1,3- and 1,6- linkages in the ratio of 11 : 1.

d) Higher Plants

Callose, a 1,3-β-D-glucan found in higher plants, appears to bind with stains such as aniline blue and resorcin blue to yield a product which can be localized histologically by its fluorescence under the UV microscope (93, 94). This product is often closely associated with particular regions of the cell wall, e.g., plasmodesmata (4-6), and is insoluble in cold water. Sieve plate callose from celery is the only form which has been isolated, purified and analysed (6). It appears to be a relatively homogeneous 1,3-β-D-glucan containing 2% uronic acid and a D.P. of approximately 120. Because callose, as observed by the fluorescence reaction, appears especially after wounding or injuring the plant (2), it has not generally been regarded as a "normal" vegetative cell-wall component. However, recent reports have been published to demonstrate aniline blue-staining materials in normal cell plates (95), developing cotton hairs (96), walls of cultured Rosa cells (54) and cell walls of Gymnosperms (53). In the cell walls of pollen, pollen tubes and embryo sacs, callose-like β-glucans have long been recognized as

important in their composition and development (97). For example, the tetrads of microspores are surrounded by layers of apparent callose, which undergo dissolution at spore release. Pollen tube walls contain alkali-soluble and -insoluble 1,3-β-D-glucans which appear to form part of the skeletal microfibrils within the structure. Analytical investigations of Lilium longiflorum pollen tubes following successive alkaline extraction suggests that this "callose" is composed of two physically associated crystalline β-glucans, one mainly 1,3-linked, the other mainly 1,4-linked (97). It is not certain whether these entities contain components which are mixed-linkage β-glucans.

With respect to its physiological function, it is suggested that callose plays an important role in newly forming walls (98). In cell plates, it may act as an initial gel separating daughter cells and trapping other components into an increasingly firm wall barrier (95). During development, callose generally disappears as the wall thickens and strengthens as a result of continued deposition of cellulose and other wall components. Callose is the predominant polysaccharide of pollen grain walls and serves to protect the male gametophyte from wounding and infection. In the pollen mother cell-wall, it functions as a molecular filter which permits the entry of nutrients but excludes larger molecules (97). The water-holding capacity of callose may help to prevent the male gametophyte from dehydration under water stress (99). In the endothelial cells of the embryo sac, it also acts as a barrier or possibly water reserve between the zygote and the maternal tissues. In sieve elements, callose is deposited after wounding and may serve to restrict phloem

transport. The removal of the wound stimulus generally results in a slow resorption of the callose deposits (93, 98). As in the case of intracellular 1,3-β-glucans in algae, another role attributed to callose in higher plants is that of a readily utilizable source of glucose and energy for neighbouring tissues following its dissolution. The chemical uniqueness of callose would be expected to render it susceptible to removal by specific endogenous β-glucanases, provided these are secreted into the wall, without causing destruction of other wall components.

Water-soluble mixed-linkage glucans have been clearly identified in seeds of barley (48, 100), oats (44), and rye grass (101). They have been reported also in hypocotyls of dicotyledons (103). Chemical and enzymic analysis shows that at leat 75% of cereal glucan molecules (D.P. 150 - 1500) consist of runs of two or three consecutive 1,4-linked residues separated by single 1,3-linked residues (56, 102). Other reports, however, suggest that runs of up to six 1,3-linked residues may occur although the upper length of the runs of 1,4-linked residues has not been determined (44, 48, 100). The ratio of 1,3- : 1,4- linkages and the D.P. of mixed-linkage β-glucans decreases as the plant tissue matures (104). Since this type of polysaccharide does not appear to consist of a regular repeating unit, molecules are not readily self-aggregated and thus then possess relatively high water-solubility and do not gel. However, by examining the linkage composition of five grass species through enzymic hydrolysis, Nevins et al (7) recently found that Bacillus or Rhizopus 1,3-β-D-glucanases hydrolyse the cell walls of these five grass species to generate very similar products (3-O-β-cellobiosyl-D-glucose 3-O-β-cellotriosyl-D-glucose). The ratios of trisaccharide and tetrasaccharide

are identical in all five species and indicate that 30% of the β -glucosyl bonds in the intact molecule are 1,3-linked (7). This, in contrast to other reports, suggests that cereal β-glucans may consist of rather regular repeating sequences.

e) Animals

Although 1,3-β-D-glucans have been only rarely reported in animal species, the phosphate esters of glucans called onuphic acid is found in the alimentary tubes of a marine polychaete (Hyalinoecia tubicola) Onuphic acid has a molecular weight of approximately 6,000 and is readily hydrolysed by alkali (105). Studies using periodate oxidation suggest that this polymer is a 1,3-linked β-glucan phosphorylated at the 4 and 6 positions.

II. Distribution and Functions of 1,3-β-D-Glucanases

Enzymes capable for hydrolysing β-glucans containing 1,3-β -D-glucosidic linkages may be classified according to specificity in a system suggested by B.A. Stone (private communication), as shown in Table III. Members of the various classes which hydrolyse 1,3-β-linkages are widely distributed in bacteria, algae, higher plants and invertebrates as recorded in Table IV. In the following sections, the classification is discussed in general terms (section a), and the distribution and potential (suggested) functions of these enzymes in various groups of organisms are described in more detail in later sections.

TABLE III

CLASSIFICATION OF ENDO-β-GLUCANASES (after B.A. Stone)

Type	Minimum glucosyl requirement	Linkage hydrolysed	Substrate	Products	References
Type A (EC 3.2.1.39) e.g. *Nicotiana glutinosa*	G3G3G3G↓3G	1,3-	homogeneous 1,3-glucans (e.g., laminarin CM-pachyman curdlan)	G G3G G3G3G	106
Type B (EC 3.2.1.6) e.g., *Rhizopus arrhizus*	G3G3G↓	1,3-	homogeneous 1,3-glucans	G G3G G3G3G	102, 107
	G3G4G↓	1,4-	1,3-/1,4-mixed linked glucans	G3G G4G3G G4G4G3G	
			alternating 1,3-/1,4-mixed glucan (e.g., RSIII)	G3G G3G4G3G	
Type C (EC 3.2.1.73) e.g., *Bacillus subtilis*	G4G3G4G↓	1,4-	1,3-/1,4-mixed glucans	G4G3G G4G4G3G	108, 109
			alternating 1,3-/1,4-mixed glucan (RSIII)	G3G G3G4G3G	
Type D (EC 3.2.1.4) e.g., *Streptomyces* QM 816 *Pisum sativum* (cellulases)	G4G4G4G↓	1,4-	homogeneous 1,4-glucans (e.g., cellulose)	G G4G G4G4G	102, 110
			1,3-/1,4-mixed linkage glucans	G3G4G	

a) Classification of 1,3-β-D-Glucanases

Apart from the β-glucosidases which hydrolyse β-glucosides (56, 108, 125, 234), 1,3-β-D-glucanases may be classified into two major groups, namely the exo-hydrolases and the endo-hydrolases. The exo-hydrolases remove single glucose residues from the non-reducing end of oligo- and polysaccharides, but are inactive on cellobiose and laminaribiose (110, 126). None are known to cleave disaccharide units from chains in a manner analagous to β-amylase acting on amylose. The endo-hydrolases are classified as such if they cleave the internal linkages of β-glucans (102, 108, 125). There are several methods to determine whether an enzyme catalyses exo- or endo-hydrolysis. For example, simultaneous measurement of reducing group production and decrease in chain length of the substrate (e.g., viscosity) is useful for this purpose. Endo-glucanases lower the viscosity of soluble carboxymethyl-polysaccharide rapidly with a relatively slow increase in reducing groups (110), whereas exo-enzymes reduce viscosity at the same rate as they generate reducing groups. Chemically modified 1,3-β-glucan which has been oxidized by periodate at the non-reducing end, and then reduced, is also useful for the detection of exo-glucanase (129). Exo-glucanases will not act on such substrates since their terminal reducing and non-reducing ends are modified, but endo-hydrolases will continue to act, though sometimes at a reduced rate (118).

Table III summarizes a way in which endo-1,3-β-D-glucanases may be further classified. Type A enzymes (e.g., Nicotiana glutinosa

β-glucanase, EC 3.2.1.39) specifically hydrolyse 1,3-β-glucosidic linkages in homogeneous polymers provided that there is a laminaritriosyl residue adjacent to the cleavage site (see diagram in Table III). The specificity requirements are met by 1,3-β-glucans such as laminarin, O-carboxymethylpachyman (CM-pachyman) and curdlan, all of which generate laminaribiose and a series of 1,3-β-oligosaccharides or derivatives. Type B enzymes, represented by the glucanase from Rhizopus arrhizus (EC 3.2.1.6; 102, 107), cleave either 1,3- or 1,4-β-glucosidic linkages, provided the substrate contains a laminaribiosyl unit at the non-reducing side of the hydrolysed linkage. These endo-hydrolases will degrade internal linkages of homo-polymers, such as laminarin or CM-pachyman, and also hetero-polymers such as mixed-linkage β-glucan, e.g., barley β-glucan, lichenin and RSIII polysaccharide (224). The products are lower D.P. oligosaccharides similar to those generated by Type A enzymes, and also, in the case of mixed-linkage β-glucans, the particular trisaccharide 3-O-β-cellobiosyl-D-glucose (102, 107). Type C endo-hydrolases specifically hydrolyse 1,4-β-glucosidic linkages of mixed-linkage glucans provided that there is a 4-O-β-laminaribiosyl-D-glucose residue (e.g., ---G-1,4-G-1,3-G---, see also Table III) adjacent to cleavage sites. This is represented by the glucanases of the Bacillus subtilis type (EC 3.2.1.73; 108, 109), and products may include the same trisaccharide as found with Type B glucanases. It is most readily assayed by use of an alternating 1,4-/1,3-β-linked substrate (RSIII) and is sometimes referred to as a specific 'mixed-linkage' β-glucanase (109). Type D endo-hydrolases (cellulases) also specifically hydrolyse

1,4-β-linkages, but do not require the presence of 1,3-linkages in the substrate. They are discussed in detail in literature on cellulases (127, 128). If these enzymes are presented with mixed-linkage β-glucans, they are not active on alternating 1,4-/1,3-linked substrates (110, 127) but will produce 4-O-β-laminaribiosyl-D-glucose from β-glucans which contain a run of 1,4-linkages adjacent to a single 1,3-linkage (110).

b) Bacteria and Fungi

β-Glucans, including cellulose, are by far the most abundant organic compounds in nature. Recycling of the β-glucan carbon by micro-organisms are mainly attributed to the activities of secreted β-glucanases. Table IV shows that the 1,3-β-glucanases are widely distributed in bacteria and fungi, and many of these are known to be secreted into the medium. The function of these enzyme systems may be in nutrition, i.e., they may hydrolyse endogenous 1,3-β-D-glucan to generate utilizable sugar molecules as energy sources. But organisms that secret these enzymes may also obtain nutrients through digestion of detritis or the hyphae or cell-wall inclusions of other living species (129). Some pathogenic species may function in an offensive way by secreting β-glucanases which allow penetration of the cell wall of the living host (130). Some may degrade 1,3-β-D-glucans elaborated as a result of potential host-parasite interaction as "elicitors", which provoke defense-reactions in the host (152), thereby penetrating the defences of otherwise resistant varieties.

TABLE IV

DISTRIBUTION OF β-GLUCANASES

Source	Type	Substrate assayed	References
BACTERIA			
Arthrobacter luteus	exo-1,3-	laminarin pachyman	111
Arthrobacter Enz. I, II	endo-1,3- Type B	lichenin	115
Bacillus subtilis	endo-1,3- Type C	barley glucan	109
FUNGI			
Basidiomycete QM 806	exo-1,3-	laminarin laminaridextrins	107
Phytophthora palmivora	exo-1,3-	laminarin laminaridextrins	112
Saccharomyces cerevisiae	exo-1,3-	laminarin	113
	exo-1,3-/1,6-	pustulan	113
Hanseniaspora valbyensis	endo-1,3- Type A	laminarin	114
Rhizopus arrhizus QM 1032	endo-1,3- Type B	lichenin	107
Rhizopus chinensis R 69	endo-1,3- Type B	lichenin	117
ALGAE			
Euglena gracilis	exo-1,3-	laminarin laminaridextrins	118
HIGHER PLANTS			
Nicotiana glutinosa (leaves)	endo-1,3- Type A	CM-pachyman	106
Nicotiana tabaccum (culture cells)	endo-1,3- Type A	laminarin	119, 197
Phaseolus vulgaris (leaves)	endo-1,3- Type A	laminarin	120
Germinated barley	endo-1,3- Type C	laminarin	121
ANIMALS			
Strongylocentrotus purpuratus (sea urchin)	exo-1,3-	laminarin	123
Helix pomatia	exo-1,3-	laminarin	124

Whether the primary role of the 1,3-β-glucanases which are secreted by plant pathogens is for nutritive or infection purposes, this is an active field of research at this time. In Basidiomycete QM 806, the synthesis and secretion of an exo-1,3-β-glucanase is greatly enhanced by growing the organism in low concentrations of a carbon source (131); and this enhancement is repressed when glucose is added to the culture. This process is cycloheximide-sensitive and the inactivation is accompanied by an alteration in molecular weight and immunological properties of the enzyme. The implication is that the enzyme is induced under starvation conditions, and that its potential function is nutritive. In other fungi, e.g., Claviceps species, 1,3-β-D-glucanases appear to be involved in the reclamation of extracellular β-glucans previously secreted by the fungus itself (107). Mycolaminarins, intracellular storage carbohydrates of Oomycetes, are unquestionably utilized during cellular differentiation or carbon starvation for synthesis of new cell-wall glucans (81). The particulate 1-3-β-glucanases responsible for degradation of mycolaminarins are present in mycelia and zoo-spores of the fungi. These enzymes may also have functions in the regulation of sclerotial glucans in higher fungi, e.g., Sclerotinia sclerotiorum. A marked decrease in endogenous 1,3-β-D-glucans is accompanied by an increase in 1,3-β-glucanase specific activity during sclerotial germination (132).

Microbial hydrolytic enzymes for 1,3-β-glucans are also widely reported to be involved in changes in cell walls during growth and morphogenesis (133, 134). During the growth of fungal hyphae, a

delicate balance of wall synthesis (by synthetases) and wall lysis (by β-glucanases) appears to be maintained, and the driving force in wall expansion is provided by the turgor pressure of the fungal protoplast (133). This balance can be shifted in favour of lysis by a variety of stimuli, resulting in violent disintegration of the hyphal apex, probably through the hydrolytic action of 1,3-β-D-glucanases (133). In Saprolegnia monoica and Neurospora crassa, the lytic vesicles containing 1,3-β-D-glucanases are responsible for transporting the hydrolases to sites of wall growth at apices, apical conidia and hyphal branches (134). Discharge of the enzymes into the wall results in softening and initiation of growth centres. During germination of conidia of Microsporum gypseum, the lysosomal vesicles containing 1-3-β-D-glucanases also move to the spore wall and discharge enzymes for initiation of germination (135).

The budding process in yeast growth is usually conceived as a modified type of tip growth in hyphal mycelia. In Saccharomyces cerevisiae, intracellular 1,3-β-glucanases are accumulated in vesicles (lysosomes) derived by proliferation of endoplasmic reticulum, and it is from these sources that the enzymes are liberated into the yeast wall during the budding process (136). Since the budding process in yeasts is a highly localized regional one, it is of interest to understand how the hydrolase activity is directed to the correct locus at a suitable time, i.e., how the yeast avoids a general lysis of its whole wall. Various speculations have been proposed that the enzymes are released to the medium or converted to a latent form, although the

possibility of de novo synthesis of hydrolases upon the appropriate signal should not be ruled out (137).

Also, 1,3-β-D-glucanases have been reported to take part in morphogenesis of fungi, e.g., in formation of pilei and dissolution of septa (138, 139). During the differentiation of thick walled hyphae into pilei in Schizophyllum commune, the formation of pilei occur without uptake of nutrient from the medium, and experimental evidence shows that hydrolysis of cell wall glucans is a prerequisite for the process (138). Following fusion of participating cells during conjugation, there is a localized dissolution of the fused wall to allow the passage of nuclei. The 1,3-β-D-glucanases together with chitinases are reported to play a role in this particular dissolution process (140, 141).

c) Algae

There is a seasonal variation in laminarin content of the fronds of Laminaria cloustoni. The alga exhibits highest concentration of the glucan at the end of the summer, with a minimum in winter (1, 142). This seasonal flux has been suggested to be a result of 1,3-β-D-glucanase activities. Similarly, when Euglenophyceae are grown heterotrophically in the dark, paramylon (intracellular 1,3-β-glucan granules) serves as a hexose source for energy generation (143). Similar phenomena have been observed in Fucus species, where the intracellular levels of laminarin decrease during cell-wall formation in zygotes (144).

d) Higher Plants

Since there is no known accumulation of 1,3-β-glucan in higher plant cytoplasm, it is unlikely that plant glucanases have a physiological role in mobilizaton of storage polysaccharide. However, the relative high percentage of evanescent 1,3-β-glucans in endosperm and pollen mother cell-walls as well as the rapid appearance and disappearance of materials that fluoresce in the presence of aniline blue in localized wall areas (2, 5), may indicate that endogenous hydrolytic enzymes are important in regulation and turnover of these glucans.

A number of 1,3-β-glucanases have been detected in leaves, stems and roots, and some have been purified to homogeneity, but their actual roles are far from understood. In tobacco (145) and grape leaves (146), the levels of endo-1,3-β-glucanases increased 14- and 30-fold, respectively, during the course of senescence. Other leaf enzymes (e.g., Phaseolus vulgaris, 147) which are subject to hormonal control will be discussed later in this review (see section III). During senescence of the corolla of morning glory (Ipomea sp.), there is a decrease of cellulose and hemi-cellulose accompanied by an increase in 1,3-β-glucanase and β-glucosidase activities (145). It appears that 1,3-β-glucan hydrolases may have a role in the reclamation of cell wall material for further flower development. In phloem tissue, 1,3-β-D-glucanases have been postulated to remove callose deposited in sieve tubes which may facilitate transport processes otherwise impeded by the deposits (147).

Glucanase activities in higher plants are widely reported to play a role in a defensive mechanism developed by plants in which these enzymes are secreted and attack fungal cell walls of invading organisms without harming host cells (148). Thus, for example, before exposure to bean rust (Uromyces phaseoli), pre-treatment with ethylene gas, which stimulates an increase in 1,3-β-glucanase activities in healthy bean plants has been found to slow the onset of the disease upon attempted infection (148). In tobacco plants (149), there is also an increase in levels of 1,3-β-D-glucanase activity which accompanies the development of tobacco mosaic virus and tomato spotted-wilt virus. This may be related to the general effect of wounding of plant tissues, since temporary callose deposition in sieve elements is often a result of fungal and viral infection. It is possible that the 1,3-β-D-glucanase activities, which concurrently develop, catalyse depolymerization of host-elaborated callose, thus allowing translocation to continue and spreading of the infecting agents.

It has been suggested that the "loosening" or partial hydrolysis of wall structure may be a pre-requisite for synthesis of new cell-wall material, by providing molecular chain ends for accepting newly added units (158, 173), and α-amylases may perform such a function in starch and glycogen metabolism (240), and the possibility has some evidence to support it in recent experiments on effects of purified pea cellulases on pea cellulose synthesis (158). Thus the endogenous hydrolytic enzymes of glucans may play a paradoxical role by stimulating anabolism of work materials through cleavage of intra- and inter-polymer chain

linkages to generate more primer chain-ends (151, 152). The 1,3-β-D-glucanases could also conceivably loosen the wall and invoke cell expansion by weakening the wall matrix if 1,3-β-D-glucan is considered to be an integral and important part of the normal primary wall (173, 175).

Auxin, a plant hormone which leads to an increased extensibility of the cell wall has been reported to increase the level of 1,3-β-D-glucanase activities in Avena coleoptiles (153) and Pisum epicotyls (176). These observations raise the possibility of involvement of 1,3-β-glucanases in extension growth of plant cells. Exogenous fungal and plant preparations containing plant 1,3-β-glucanase activities have been reported to mimic the effect of auxin on elongation when added to tissue segments of oat and barley coleoptiles (155, 156) although similar effects have not been obtained when the same preparations are added to Jerusalem artichoke tuber slices (156). An enzyme preparation extracted from bean hypocotyls, containing both 1,3-β-glucanase and β-glucosidase, modifies the extensibility of cell walls of Avena coleoptiles to an extent comparable to that found with added IAA (Indole acetic acid) (157). The effect is inhibited by nojirimycin (5-amino 5-deoxy-D-glucopyranose), a specific inhibitor of β-glucosidases (157). Though they are highly suggestive, it is difficult to evaluate the reproducibility and meaning of these results because they have not been repeated by other workers, and they employed enzyme preparations from fungi which may have contained enzyme activities or components besides the β-glucanase and β-glucosidase known to be present.

The cell walls of cereal endosperm contain, amongst other components, 1,3-/1,4-mixed-linkage β-glucans (159). During germination of cereal grains, the dissolution of endosperm walls occurs and appears to facilitate the access of various hydrolases, which are generated in aleurone layers, to substrates, e.g., starch grains, in the endosperm (165, 166, 180). In germinating rice (161), rye (162) and wheat (163) there are progressive increases in 1,3-β-glucanase activities. In studies of endosperm wall-dissolution during barley germination, three types of β-glucanases have been isolated; including a strict endo-1,3-β-glucanase (Type A, see Table II), an endo-1,3-/1,4-β-glucanase (Type C) and a cellulase (Type D) (164). Attention is focused on the Type C enzymes, since these are presumably the main components responsible for hydrolysis of 1,3-/1,4-mixed linkage β-glucan in the endosperm wall, though the cellulase may also be capable of limited attack. Callose deposits have been recently reported to be one of the endosperm wall constituents (160), and Type A hydrolases may be involved in the depolymerization of this polysaccharide, presumably to supply additional sources of carbohydrate for the developing embryo.

Finally, during microsporogenesis in Lilium anthers, the four microspores are released after the callose-like special cell wall has been dissolved (167). The dissolution process may be caused by the hydrolytic action of 1,3-β-D-glucanases, which are known to be secreted from the parental tapetal cells, since a dramatic increase in such activity has been demonstrated during spore dispersal (167).

e) Animals

Exo-1,3-β-D-glucanases are found in the digestive tract of various groups of invertebrates, namely Polychaetes (168), Coelenterates (168), Gastropods (169), Echinoderms (171, 172) and Arthropods (170). The function of the enzymes is unknown, and whether the hydrolases are secreted by the animals themselves or are of bacterial or fungal origin remains uncertain in most instances. In many species of Echinoderms (e.g., sea urchins), contact of unfertilized eggs and sperms results in the breakdown of a peripheral ring of cortical granules. An exo-1,3-β-glucanase found in the walls of unfertilized eggs may catalyse the degradation of this ring, which is regarded as a block to fertilization (171). The 1,3-β-D-glucanase activity may also play a role in hatching the embryo of Echinmetia vaubrunti, since the hydrolase activity level increases before, and decreases after, the hatching process (172).

III. Hormonal Regulation of 1,3-β-D-Glucanase Development in Plants

It is generally believed that the integration of plant growth and development is achieved in part through the action of plant growth regulators (hormones), and one of the mechanisms of hormonal action may be the exertion of control over the anabolic side of turnover of various components of the wall structure. In this view, wall rigidity may be reduced by the action of specific wall hydrolases, and this could be a primary regulatory point for designating the direction and rate of growth. The idea was originally enunciated by Heyn (238) in 1931 and has been elaborated by many authors and research studies since

then (76, 239). A number of hypotheses concerning the mechanism by which the hormone auxin may increase extensibility of the cell wall have been proposed, including induction of enzymes potentially responsible for degradation of restrictive cell-wall constituents (151, 153, 157, 173, 174). An important observation is that the cell-wall-bound 1,3-β-D-glucanase activity in Avena coleoptiles increases as a result of the treatment of segments with IAA, and the enhancement is inhibited by cycloheximide (174). Hormonally-evoked activities have also been observed when IAA is applied to etiolated barley coleoptiles (175). 1,3-β-D-Glucanase activity in pea epicotyls is augmented by auxin treatment (176), and there may be changes in the intracellular distribution of the enzyme (176, 210), although the specific activity is apparently not increased under the particular hormonal regimes which are employed (176). Masuda and colleagues (173, 175) reported that exogenous addition of fungal 1,3-β-D-glucanase provoked the elongation of barley coleoptile segments; an observation which if verified would clearly strengthen the notion that such enzymes may have a physiological role in intitiating cell expansion. However, Nevins et al (149) do not observe such an elongation response when Avena sections are treated with highly purified 1,3-β-D-glucanase. Such experiments and their interpretations are, of course, critically dependent on the purity of the enzymes employed.

Enzyme inhibitors have also been employed to assess the role of hydrolases in cell-wall elongation. There is evidence that nojirimycin, an inhibitor of many β-glucosidases and exo-glucanases

(178) suppresses IAA-induced growth of excised Avena coleoptile and pea epicotyl segments (157, 177). What other enzymes this derivative may inhibit has not been documented to the extent required to attribute its effects solely to β-glycosylase action. There is no evidence for competitive effects between nojirimycin and IAA, since increasing the auxin concentration does not reverse the inhibition. With cellobiono-1,4-lactone (179, 201), which also inhibits growth, inhibition occurs only with relatively high added concentrations and there is little difference between coleoptiles grown ± the inhibitor in 2 μM IAA or 2% sucrose medium.

With respect to the potential function of 1,3-β-D-glucanases in seed germination and the degradation of wall barriers in seed reserves, there is little doubt that gibberellic acid (GA) is a major regulatory hormone for the whole process. GA has been shown to induce de novo synthesis and secretion of α-amylase in isolated barley aleurone layers (180, 181), and it also enhances the release of 1,3-β-D-glucanase from aleurone layers, although this hormone apparently has no significant effect on β-glucanase synthesis (166). Other authors (182) have confirmed that there is only a slight increase in the total activity of Type A 1,3-β-D-glucanase in extracts when whole barley grains are germinated in the presence of GA, but there is no question that the hormone regulates its intracellular distribution. When the time-course of 1,3-β-glucanase - release from aleurone layers is compared to that of α-amylase-synthesis and -release, the 1,3-β-D-glucanase is totally secreted by a time when only 20% of the α-amylase is released (166).

For enzymes capable of hydrolysing 1,3-/1,4-mixed linkage β-glucans, GA has also been shown to stimulate the activity in whole grain. This type of enzyme (Type C) appears in barley grain extracts before α-amylase and protease (183), and therefore it may well be important in the physiology of grain cell-wall dissolution, and it merits further study. These observations strongly suggest that the various 1,3-β-glucanases which are produced by aleurone cells are responsible, albeit on different time scales, for part of the observed cell-wall digestion, and that they function in mobilizing reserves for seedling nutrition.

The elementary studies of hormonal regulation of 1,3-β-D-glucanases which have been recorded to date are not comparable to such studies on the cellulases (110, 179, 201). The latter have drawn more attention due to the fact that results are more precise, and to the more obvious potential function in cell-wall expansion of cellulolytic activity (133, 179). The mechanisms whereby hormones may alter levels of 1,3-β-D-glucanase activity have not been clarified, and undue speculation is justly subject to criticism (184). It is premature to draw any conclusion as to which mechanism or steps in the cell-wall turnover process may be mediated by plant hormones via induction, activation or repression of 1,3-β-D-glucanase activities.

With respect to the potential role of 1,3-β-D-glucanses in plant aging, or "wound" responses, and the hormones which might be involved, Abeles and Forrence (147) published evidence that 1,3-β-D-glucanases in excised (aging) petiolar tissue of Phaseolus vulgaris are subject to temporal and hormonal regulation. Excision of intact plant tissue

results in an increase in this hydrolytic activity after a 6-hour lag period (aging) but the increase can be prevented by growth promotors, e.g., IAA, GA or cytokinin. On the other hand, ethylene, an activator of abscission process (213), and a wound-elicited product of metabolism, promotes the synthesis of 1,3-β-D-glucanase in this tissue. Cycloheximide prevents the enhancement of this enzyme activity by hormone, but actinomycin D and chromomycin A_3 have only partial effects. In tobacco (145) and grape (146) leaves, the endo-1-3-β-D-glucanase activities increase markedly during the course of senescence, and the augmentation appears to be due neither to removal of an inhibitor nor to the appearance of an activator. A comparatively high level of activity of 1,3-β-glucanase is present in the leaves of Nicotiana glutinosa (145), and it is therefore an exprimentally interesting system. The relationship between the hydrolase activity in tobacco and the degree of leaf senescence has been investigated by examining the effect of plant regulators on the changes in level of hydrolase, protein and chlorophyll in leaf discs during artificial (excised) senescence. IAA and GA do not change the normal pattern of development, but cytokinin delays the loss of protein and chlorophyll and also delays and reduces the rise in hydrolases activity (145). In contrast, abscisic acid (ABA), which increases the rate of loss of protein and chlorophyll, also causes a decrease in the rate and extent of the rise in hydrolase. These results suggest the possibility of a functional relationship between the rate of rise in hydrolase level and the rate of onset of senescence. Mutually contradictory though some of

these results appear to be, in-so-far as generalizations concerning the roles of 1,3-β-glucanases are justified, it is clear that these enzymes are generally subject to regulation by hormone and antibiotic treatments. The implication is that these enzymes have important functions related to those that hormones control.

IV. Purification of 1,3-β-D-Glucanases

Standard purification techniques, such as ammonium-sulfate fraction, ion-exchange chromatography and gel flitration, have been applied at various times with success in the purfication of 1,3-β-D-glucanases from different sources. Many of the procedures used, and the efficiency of purification, as well as recoveries of enzyme activities, are summarized in Table V. The fact that some of the materials used as adsorbants in purification are potential substrates for the enzymes has created problems. Accordingly, it has been suggested (199, 200) that addition of disaccharides in extraction media in order to inhibit the enzyme activity, and acetylation of fractionation supports (e.g., dialysis membranes), may help to overcome these problems. Thus, procedures which do not involve 1,3- or 1,4-linked glucans, such as Sephadex and Bio-Gel chromatography, are usually adopted in preference for fractionation, although even these media sometimes generate artefactual peaks due to adsorption. This may lead to controversies concerning the multiplicity of enzymes (isozymes) (179).

TABLE V

PURIFICATION OF 1,3-β-D-GLUCANASES

Source	Enzyme type	Purification procedures	Fold	Recovery (%)	References
BACTERIA					
Flavobacterium sp. strain M 64	-	$(NH_4)_2SO_4$, 0-60% DEAE-cellulose Sephadex G-200	90	7	185
Bacillus circulans WL-12	-	Glucan adsorption Hydroxylapatite CM-cellulose	10	17	186
FUNGI					
Rhizopus arrhizus QM 1032	endo-1,3-	Conc. - dialysis $(NH_4)_2SO_4$, 35-75% CM-cellulose Sephadex G-100	304	69	187
Rhizopus niveus	-	Ca - acetate Polyethylene glycol CM-cellulose DEAE-cellulose Sephadex G-150	39	38	188
Mucor hiemalis	endo-1,3-	$(NH_4)_2SO_4$ ppt. Sephadex G-100, KCl CM-cellulose	11	18	189
YEAST					
Candida utilis	exo-1,3-	DEAE-Sephadex A50 Sephadex G-50 Sephadex G-200 Bio Gel P-10 Concanavalin A Sepharose 4B	17	30	190
Kluyveromyces aestuarii	exo-1,3-	DEAE-cellulose DEAE-Sephadex (adsorption) DEAE-Agarose Sephadex G-100	8333	46	191
Oerskovia sp.	1,3-	ECTEOLA-cellulose CM-cellulose Bio Gel p-100	-	-	193

TABLE V (Cont'd)

Source	Enzyme type	Purification procedures	Fold	Recovery (%)	References
Schizosaccharomyces versatilis	exo-1,3-	DEAE-cellulose CM-cellulose ultrafiltration Sephadex G-100	299	44	192
Candida utilis	endo-1,3-	DEAE-Sephadex A50 Sephadex G-50 Sephadex G-200 Bio Gel P-10 Concanavalin A Sepharose 4B	4	0.1	205
PLANTS					
Euglena gracilis	exo-1,3- endo-1,3-	CM-cellulose Bio Gel P-200 Bio Gel P-300	53 6.5	26 8	196
Malted barley	endo-1,3-	$(NH_4)_2SO_4$, 0-50% Bio Gel P-60 Bio Gel P-30	95	-	194
Malted barley	endo-1,3-	$(NH_4)_2S)_4$, 0-80% DEAE-cellulose Amicon conc. CM-cellulose	-	76	121
Rye	endo-1,3-	DEAE-cellulose CM-cellulose Bio Gel P-60	58	-	195
Phaseolus vulgaris	endo-1,3-	DEAE-cellulose $(NH_4)_2SO_4$, 0-60% Hydroxylapatite CM-Sephadex	15	0.8	120
Nicotiana tabaccum (culture)	endo-1,3-	$(NH_4)_2SO_4$, 0-80% DEAE-Sephadex A50 CM-Sephadex C50 Sephadex G75	44	21	197
Nicotiana glutinosa	endo-1,3-	DEAE-cellulose Bio Gel 150 isoelectric focusing	280	40	106

TABLE V (Cont'd)

Source	Enzyme type	Purification procedure	Fold	Recovery (%)	References
ANIMALS					
Helix pomatia (snail)	exo-1,3-	Bio Gel P-60 Sephadex G-100 DEAE-cellulose DEAE-Sephadex	20	5.4	124
Helix pomatia	endo-1,3- exo-1,3-	GP_2B-Sepharose	-	-	198

Manners and Wilson (121) have reported a one-step purification of an endo-1,3-β-glucanase by what appears to be affinity-binding to DEAE-cellulose. In such tests, most of the applied protein, but not the enzyme activity, is eluted from DEAE-cellulose with prolonged washing with salts (106, 191, 192, 196). A pH-gradient applied after the salt-wash elutes the enzyme in a narrow band (179, 201). Carboxymethyl-polysaccharides (negatively-charged) have also been used as supports with success for purification of 1,3-β-D-glucanase activities (121, 186, 188, 192, 193).

a) Solubility and Extraction

No special problems have arisen in the extraction or solubility of most of the bacterial and fungal 1,3-β-D-glucanases:- they are excreted enzymes found in culture filtrates, and are almost entirely water-soluble. For yeast 1,3-β-glucanases, however, the cells have to be mechanically disrupted, e.g., with glass beads in 0.05 M sodium acetate buffer, pH 5.5, in a Braun homogenizer, before enzyme extraction is complete (191, 192, 193). Plant β-glucanases also require grinding or blending of tissues to completely extract the enzymes, suggesting that part of the activity is intracellular, e.g., stored in vesicles or vaculoes (242). While some plant β-glucanases are readily soluble in dilute buffers (106, 196) others require more complex ionic or salt media in order to bring about complete extraction (121, 194, 197). Endo-1,3-β-glucanases from germinated rye and suspension-cultured

tobacco cells are readily soluble, but only in a relatively concentrated buffer (0.1 M sodium acetate and phosphate at pH 5.0; 197, 164). Acetate buffer containing 2% NaCl is required to extract these enzymes from Lavats patonica var. grains (195). Malted barley 1,3-β-D-glucanases also require high ionic strength for complete extraction (194). This suggests that a fraction of these enzymes is membrane-bound or sequestered in organelles or partially crystalline in vivo.

b) Ion-exchange and Affinity Chromatography

β-Glucanases from various sources have been subjected to a great range of chromatographic procedures, including DEAE-cellulose (106, 121, 185, 188), CM-cellulose (187, 189, 193, 195), hydroxylapatite (120, 186) and DEAE-Sephadex (124, 191) fractionation (see also Table V). While the principle on which these supports fractionate proteins may be based on ion-exchange and ion interactions, in the case of β-glucanases, it is possible that such proteins may have some affinity for the 1,4-β- or 1,6-α-linked polysaccharides from which the supports are synthesized.

The principal feature underlying ion-exchange chromatography is the attraction between oppositely charges particles. Proteins have ionizable groups and the fact that they may carry a net positive or negative charge can be utilized in separating mixtures of such compounds. The net charge exhibited by such compounds is dependent on the pH of the solution and the isoelectric point of the compound. The

actual procedure involved in column ion-exchange chromatography may be illustrated by reference to a cation-exchange resin, where the exchange material is at a pH at which it is fully ionized and fully saturated with a cation (e.g., A^+). This would be the situation in the column while a buffer containing A^+ is passing through it. If a solution containing a different cation (e.g., B^+) is added to the column at a pH at which the ion-exchange material remains fully ionized, some A^+ will be displaced by B^+ until an equilibrium is established; and when ionic strength and pH of the buffer are correctly chosen, B^+ may completely bound to the exchanger in a narrow band on the resin material. The adsorbed cation B^+ can be eluted from the column by applying a different buffer of low pH or stronger ionic strength. Various types of ion-exchange chromatography have been adopted in purification of 1,3-β-D-glucanases, and the results are summarized in Table V. Among these, DEAE-cellulose is the most widely used one although in many cases the recoveries of these enzymes are found to be extremely low (120, 185, 205). Marshall (202) pointed out that the β-glucanases are unlikely to be inactivated during chromatography, but they may well be exceptionally strongly bound, possibly as a result of affinity for the polysaccharide matrix rather than by ion-exchange *per se*. It is then suggested that advantage should be taken of the observed differences in affinity of various glucanases for ion-exchangers in order to obtain better separations of them by pH gradient procedures (202).

In addition to ion-exchange chromatography, biospecific affinity chromatography is a method of purification of enzymes based on

biospecific adsorption and subsequent desorption. Basically, the technique consists of covalently attaching the ligand, which may be a substrate or a reversible competitive inhibitor, to a suitable insoluble matrix in such a way that its potential for combination with the enzyme is not impaired. A soluton of crude enzyme to be purified is applied to a column of the ligand-matrix immersed in the correct buffer system and the enzyme is selectively retained. Impurities which are not bound are eluted and the enzyme is subsequently displaced by elution with a buffer of different pH and/or ionic strength. Although not yet as widely used as ion-exchange chromatography, affinity chromatography has been applied to purify a number of β-glucosidases (243) and β-glucanases (244-248) using such polysaccharides as pachyman or crystallized insoluble-laminarin (245), or ligands of small molecular weight covalently attached to Sepharose (244,246, 248) as supporting materials. In some cases, natural ligands are covalently immobilized on Sepharose as an affinity support in purification of some 1,3-β-D-glucanases (244). A glycoprotein extracted from yeast cell wall has been partially characterized as having a branched carbohydrate structure composed of chains of 1,3-linked β-D-glucosyl residues, some of which are attached by 1,6-linkages to the backbone of the molecule. Immobilization of this glycoprotein is achieved by covalent attachment to Sepharose 4B, and the product has been used to purify a number of 1,3-β-D-glucanases from Helix pomatia, malted barley and Basidiomyces fungi (244).

c) Gel Filtration and Ultrafiltration

Gel filtration on Sephadex or Bio Gel has been the most widely-used procedure for purification and characterization of glucanases (see Table V). However, the yields from this procedure are frequently very low. It has been suggested that Sephadex chromatography should not be used as the sole criterion for establishing the multiplicity of β-glucanases, since many of the observed peaks in the chromatograph profile are in fact the artefacts of binding and solubility (179), and elution in buffer of high ionic-strength usually reduces the apparent number of components.

After chromatography on inert gels, the enzyme preparations with high specific activity are generally pooled and concentrated to a smaller volume for further purification steps. Pattersson et al (203) reported that concentration of β-glucanases by either lyophilization or dialysis often leads to dramatic losses in activity. To avoid this, they suggested the use of Sephadex for concentration procedures, which utilizes the capability of the gel matrix to take up water and low-molecular-weight materials rapidly, excluding higher molecular weight components (204). The procedure is efficient, and it has the advantage of keeping the temperature, buffer-strength and pH unchanged, despite the disadvantage of possible adsorption of the enzymes to the gel.

Ultrafiltration using the "Amicon" apparatus (a molecular sieve) has been increasingly used in concentration of enzymes during purification (110, 154, 179). This procedure has all the advantages of

the Sephadex method plus the bonus that loss of the enzyme due to adsorption is eliminated. The basic principle of this method is removing the water, salts and other lower molecular-weight materials under high pressure (nitrogen gas) through a synthetic membrane (anisotropic) impermeable to high molecular weight components.

d) Electrophoresis

Analytical gel-electrophoresis is one of the most powerful methods available for determining the complexity or purity of protein solutions by separating the molecules in aqueous buffers supported within a polymerized gel-matrix (206). Gels have been employed such as starch, polyacrylamide, agarose and agrose-acrylamide,and these supporting media vary in the degree to which they enhance resolution of proteins or nucleic acids (See also Table V). Frequently, however, it becomes necessary to go beyond the analytical stage by attempting to isolate specific porteins after electrophoresis by extraction with buffer or by using commercially available preparative gel-electrophoresis equipment (207). However, extraction of protein from homogenized gel-slices often results in poor recoveries and usually requires long periods for diffusion, during which inactivation or degradation can occur (208).

Some of the matrices used in electrophoresis, e.g., paper or cellulose acetate, may give rise to artefacts with β-glucanases due to affinity to the supports. Polyacrylamide gel-electrophoresis avoids this problem, and has been appplied successfully to both crude and purified 1,3-β-glucanase preparations. In many tests with crude fungal

extracts, for example, several components have been resolved for the first time. Most fungal 1,3-β-D-glucanases are acidic proteins with isoelectric points ranging from 3 to 3.5 (190, 205), and those bacterial enzymes that have been examined are neutral (Table VI). For plant enzymes, however, Ballance and Manners (195) reported a basic pI value (10.3) for an endo-1,3-β-glucanase purified from germinated rye. Endo-1,3-β-glucanases from bean leaves (120), tobacco-leaf cells (197) and barley (209) have also been found to be basic proteins (Table VI). The significance, if any, of these limited number of observations has not become apparent. Curiously, the use of polyacrylamide gel-electrophoresis in the presence of the anionic detergent sodium dodecyl sulfate (SDS), which separataes polypeptides which are not co-valently bound, although extensively applied to purify other enzymes, has not been used for determination of true molecular weights of purified 1,3-β-glucanases (see Table V).

Discontinuous gel-electrophoresis, as a method for assessing the purity of proteins, is frequently carried out at relative extremes of pH, where inactivation of enzymes may occur during the procedure. The standard anionic system is run at pH 8.0 and the cationic system at pH 4.3. These conditions may not be denaturing with fungal enzymes, which are generally found to be heat-stable and tolerable towards pH extremes. Glucanases from higher plants, however, are not known for heat-stability and some of them are extremely unstable at pH values above 8 (179, 210). In comparison to disc gel-electrophoresis, isoelectric focusing is less hazardous. Although high voltages are

generally applied in this technique, resistance is low, and heat generation is minimized. The procedure can be performed in the cold (2-4°C) in the presence of enzymic-stabilizing agents, such as glycerol, even at very high concentrations. Recently, a technique has been developed for the separation of proteins by two-dimensional polyacrylamide gel-electrophoresis (212). Due to its resolution and sensitivity, this technique is a powerful tool for the analysis and detection of proteins from complex biological sources. Proteins are separated according to change by isoelectric focusing in the first dimension, and according to molecular weight by SDS gel-electrophoresis in the second dimension (212).

V. Properties of 1,3-β-D-Glucanases

a) Substrates and Assays

Unlike cellulase, the natural substrate of which is limited to cellulose and certain substituted 1,4-β-glucans, e.g., xyloglucan (240), the endogenous substrates of 1,3-β-D-glucanases have generally not been well defined in higher plants. If they prove to resemble those which have been examined in fungi and algae, they will be found to be variable in crystallinity, chain length, kind and proportions of other linkages in main chains and branches, and the degree to which other sugars are substituted in the molecules. Thus, for example, detailed studies on "yeast glucan", a polysaccharide consisting of β-glucosidic linkages, with variable contents of 1,3-β- and 1,6-β-glucosidic branches, show that this potential substrate is

heterogeneous and varies in composition and percentage of 1,6-linkages, depending on the strain, culture conditions and procedures of preparation (84, 85, 222). Specificity data of β-glucanase action vs. such substrates should be cautiously interpreted, unless the particular polymer employed has been very thoroughly characterized.

A great variety of substrates, including both soluble and insoluble glucan preparations, have been used in numerous assay systems and detection procedures for 1,3-β-D-glucanase. Insoluble β-glucans with relatively high D.P., such as paramylon (43), pachyman (44) and curdlan (12, 13; see also Tables I and II) have been used as substrates for both exo- and endo-hydrolases. These insoluble polysaccharides are usually first dispersed in alkaline solutions and neutralized or carboxymethylated (e.g., CM-pachyman) in order to increase their accessibility and subsceptibility as substrate (44, 118, 163). If pachyman is treated with β-glucanase until the enzyme ceases to release soluble reducing groups from the polymer, a swelling treatment with alkali results in a preparation which has acquired renewed accessibility to β-glucanase. Similar results are observed in the action of cellulase on native cellulose (127). In the case of Arthrobacter 1,3-β-D-glucanase acting on pachyman, it is only necessary to heat the substrate in water at 100°C for 15 min in order to render it fully susceptible to hydrolysis (111, 214). This procedure is not suitable for application to all insoluble 1,3-β-glucans since some, e.g., curdlan tend to form a very firm gel at high temperatures (12, 13).

With respect to soluble polysaccharides, laminarin is the most commonly used substrate for glucanase assays, probably because it is readily available commercially. Such samples possess a comparatively low D.P. (e.g., 20-30), and hence they have a low viscosity in solution and a substantial endogenous reducing value. Many samples are also heterogeneous in terms of linkage composition and branching. Laminaridextrins are not available commercially, but they can be prepared by fractionation of partial acid-hydrolysates of laminarin or pachyman on a charcoal/celite column (127, 241). A homogeneous series of these oligosaccharides would be extremely useful in the exploration of action patterns of the 1,3-β-D-glucanases, e.g., as has been reported in studies of fungal and pea cellulases acting on cellodextrins (110, 127). To date, the only detailed studies that have been published on the action of 1,3-β-glucanases on defined laminaridextrins are those of Barras and Stone (118, 196) who examined the rates and products formed from laminaripentaose and other 1,3-linked oligosaccharides in the presence of Euglena β-glucanase. In addition, Clarke et al (187) recently reported the relative rates of purified Rhizopus 1,3-β-D-glucanase on a series of reduced laminaridextrins (i.e., laminaribiitol to laminariheptaitol), although no similar data using unaltered laminaridextrins as substrates are presented.

Chemically-modified pachyman derivatives, such as CM-pachyman, and the cereal glucans (1,3-/1,4-mixed β-linkages), are also soluble substrates of 1,3-β-D-glucanases which have the advantage of forming

solutions with high viscosity. This property can be exploited in β-glucanases assays. Viscometric assays are much more sensitive than reductometric assays when the enzyme is an endo-glucanase, since it will bring about rapid losses in viscosity that are proportional to the rapid reduction in D.P. Concurrent determination of reducing power production allows a comparison of the initial rate of loss in viscosity (change of D.P.) per hydrolysed linkage (106, 109). Such measurements have often been used to determine the "degree of randomness" of hydrolysis of a substrate by different 1,3-β-glucanase and cellulase preparations (106, 110, 127, 179, 201). CM-lichenin (223) has also been used to examine the action pattern, e.g., of Streptomyces β-glucanase (223), and it could equally well be applied to an investigation of Type B and C endo-1,3-β-glucanases. Most recently, a soluble polysaccharide derived from the cell wall of Pneumococcus consisting of a strictly alternating sequence of 1,3- and 1,4-β-linkages, has been prepared (224) to act as potential substrate of β-glucanases. This substrate allows a more precise definition of the linkage requirements for hydrolysis of mixed-linkage glucans by 1,3-β-glucanases and cellulases (224, 110). The linear 1,3-1/1,6-mixed β-glucan extracted from Eisenia (225) is also a potentially useful substrate, especially for specificity studies.

b) Physical and Enzymic Properties

Only data obtained with highly purified 1,3-β-D-glucanases will be discussed, except for some of the higher plant enzymes for which no

such data are available. Physical and enzymic properties of purified 1,3-β-glucanases from different origins are summarized in Table VI.

Reliable molecular weights from 10 000 to 40 000 have been reported for many bacterial and fungal enzymes. There are two reports of endo-1,3-β-glucanases, one from the bacterium Flavabacterium sp. (strain M 64, 185), and the other from yeast (192), which showed relatively high molecular weights of 180 000 and 97 000, respectively. Horitisu et al (188) reported an extremely low molecular-weight (2 500 daltons) for one of the Rhizopus 1,3-β-glucanases, which may have been artifactual (See discussion by Christou, 226). Higher-plant 1,3-β-glucanases mostly fall within the range of the fungal enzymes, although in some species they display slightly higher molecular weights (106, 197; see also Table VI). 1,3-β-D-glucanases from snail and sea urchin have molecular weights of 82 000 and 60 000, respectively (123, 124).

Amino-acid analyses (120, 187, 190) show that 1,3-β-glucanases purified from fungi, yeasts and plants have several characteristics in common. These includes a low content of basic amino acids and a high content of acidic amino acids, yielding pI values in the range of 3.0 to 5.0. In bean 1,3-β-glucanase (120), however, there is a relatively high content of acidic amino acids (aspartic and glutamic acids constitute 24% of total amino acid residues, w/w) which is combined with a high (basic) pI value of 11.0. This presumably means that much of the dicarboxylic acid is present as amide. Two 1,3-β-D-glucanases from barley and germinated rye also display relatively high pI values (9.2 and 9.8, respectively), but their amino acid contents are not known.

TABLE VI

PROPERTIES OF 1,3-β-D-GLUCANASES

Source	Physical properties					Enzymic properties			References
	Mol. wt.	pI	pH optimum	Temp. optimum	Thermal-stability	Hydrolytic pattern*	β-linkage specificity	K_m values	
	(daltons)			(C°)					
BACTERIA									
Arthrobacter luteus	21 000	-	7.0-7.5	35	70% act. loss 50°C/5 min	exo-1,3-	1,3-; 1,6-	1.09% (laminarin) 0.046% (curdlan) 0.04% (pachyman) 0.019% (yeast glucan)	111, 214
Bacillus circulans									
glucanase I	40 000	-	5.5	-	-	-	1,3-; 1,3-/1,4-	0.105 mg/ml (laminarin)	186
glucanase II	-	-	6.5-7.0	-	-	-	1,3-; 1,3-/1,4-1,6-	-	
Flavobacterium sp.	16 000	-	5.0	-	-	endo-1,3-	1,3-	-	216
Flavobacterium sp. strain M 64	180 000	-	5.8	35	80% act. loss 45° C/10 min	-	1,3-; 1,3-/1,4-1,6-	1.7 mg/ml (succinoglucan) 1.2 mg/ml (desuccinylated succinoglucan)	185
Bacillus subtilis	-	-	6.5	-	-	endo-1,3-/1,4-	1,3-; 1,3-/1,4-	3.4 mg/ml (lichenin)	216
Bacillus pumilus	-	-	6.5	57	100% act. loss 60°C/30 min	endo-1,3- (Type C)	1,3-; 1,3-/1,4-	0.7 mg/ml (barley glucan)	222
FUNGI									
Rhizopus niveus	2 450	-	5.5	60	-	-	1,3-	3.2×10^{-6} M (laminarin)	188
Mucor hiemalis	30 000	-	5.0	50	95% act. loss 60°/10 min	endo-1,3-	1,3-; 1,6-	0.048% (laminarin)	189
Basidiomycete sp. QM 806	51 000	6.5	5.6	60	80% act. loss 70°C/60 min	exo-1,3-	1,3-	2.6×10^{-6} M (laminarin) 1.7×10^{-6} M (L_5)	107, 217, 218
Rhizopus arrhizus QM 1032	28 800	7.5	4.8	-	-	endo-1,3-	1,3-; 1,3-/1,4-; 1,6-	0.314 mg/ml (laminarin)	187
Corticium rolfeii	-	-	4.5	55	77% act. loss 60°C	exo-1,3-	1,3-; 1,6-	-	219
Phytophthora palmivora	-	-	5.5	40	100% act. loss 60°C	exo-1,3-	1,3-; 1,6-; 1,3-/1,4-	3.3 mM (laminarin) 4.9 mM (L_5) 6.1 mM (lichenin	112
Rhizopus arrhizus	10 000	-	3.5-3.7	55	55% act. loss 74°C/3 min	endo-1,3- (type B)	1,3-; 1,3-/1,4-	3.33 mg/ml (lichenin)	221

TABLE VI (Cont'd)

Source	Physical properties					Enzymic properties			References
	Mol. wt.	pI	pH optimum	Temp. optimum	Thermal-stability	Hydrolytic pattern*	β-linkage specificity	K_m values	
	(daltons)			(C°)					
YEAST									
Schizosaccharomyce sp.	97 000	-	5.0-6.0	-	stable at 40°C 90% act. loss 60° /8 min	endo-1,3-	1,3-	0.33 mg/ml (laminarin)	192
Oerskovia sp.									
F - 1	29 000	-	6.5	50-55	100% act. loss	-	1,3-; 1,3-/1,6-	-	193
F - 2	20 000	-	5.5	50-55	60°C/15 min	-	" "	-	
F - L	12 000	-	6.0	45-50		-	" "	-	
Candida utilis	21 000	3.0	5.5	-	-	exo-1,3-	1,3-; 1,3-/1,6-	1.8×10^{-4} M (laminarin)	190
Candida utilis	21 000	3.2	5.0	50	7% act. loss 50°C /10 min	endo-1,3-	1,3-; 1,3-/1,4-	0.21 mM (laminarin) 0.19 mM (oxid. laminarin)	205
Kluyveromyces aestuarii	43 000	-	5.5	30	stable up to 52°C for 20 min	exo-1,3-	1,3-; 1,6-	0.23 mM (laminarin)	191
Fabospora fragilis	22 500	-	5.5	-	-	exo-1,3-	1,3-; 1,3-/1,6-	1.24 mg/ml (laminarin) 1.85 mg/ml (pustulan)	113
Schizosaccharomyces japonicus var.	43 000	-	5.0	-	unstable 50°C	exo-1,3-	1,3-; 1,6-	6.25 mg/ml (laminarin) 166.6 mg/ml (pustulan)	220
PLANTS									
Euglena gracilis	-	-	5.0	-	-	exo-1,3-	1,3-	0.008% (laminarin) 11.1×10^{-5} M (L_5)	196
Nicotiana glutinosa (tobacco leaves)	45 000	4.9	5.0	-	-	endo-1,3-	1,3-	0.13 mM (CM-pachyman)	
Nicotiana tabaccum (Tobacco cell culture)	45 000	4.9	5.0	45	50% act. loss 50°C /10 min	enod-1,3-	1,3-	0.22% (laminarin)	197
Phaseolus vulgaris	12 000	11.0	-	-	-	endo-1,3-	1,3-	-	120
Malted barley	12 800	9.8	5.5	37	unstable 60°C	endo-1,3-	1,3-	0.27 mM (laminarin)	194
Germinated rye	24 000	9.2	5.0	45	stable 30-55°C for 40 min	endo-1,3-	1,3-	0.25 mg/ml (laminarin)	195
ANIMALS									
Strongylocentrotus purpuratus (sea urchin)	60 000	-	4.8-5.6	40	unstable up to 60°C	exo-1,3-	1,3-	-	123
Helix pomatia (snail)	82 000	-	4.5	53	stable up to 50°C	exo-1,3-	1,3-; 1,3-/1,4-	1.22 mg/ml (laminarin) 2.22 mg/ml (lichenin)	124

* See also Table III for classification of endo-1,3-β-D-glucanases.

Unlike fungal cellulases (179), which are generally optimally active between pH 3.5 to 5.0, most fungal 1,3-β-D-glucanases have higher pH optima from 5.0 to 6.5 (see Table VI). Higher plant cellulases and 1,3-β-glucanases show pH optima between 5.0 and 6.5. In general, most cellulases are more heat-stable than 1,3-β-glucanases (127, 179, 201; see also Table VI), but the physiological significance of this generalization, if any, is obscure.

Michaelis-Menton constants (K_m) of 1,3-β-glucanases have been determined using various substrates as shown in Table VI. Most enzymes have K_m values at a concentration of a few mg/ml when using laminarin, lichenin or barley β-glucan as substrates. One yeast hydrolase (from Schizosaccharomyces japonicus var.) shows an unusually high K_m value of 167 mg/ml (w/v) for action on pustulan (220), which is partially crystalline. β-Glucanases purified from Basidiomycete sp. QM 806 (217, 218) and Euglena gracilis (196) display particularly low K_m values towards laminarin (less than 0.04 mg/ml, w/v), which may reflect the particularly crucial importance of these enzymes in the nutrition of this organism.

With respect to inhibitor sensitivities of the 1,3-β-glucanases, only information is available on substrate-binding or the nature of the active site. Mercuric ions strongly inhibit most of these β-glucanases, while copper, zinc, iron, calcium, magnesium, manganese, barium and cobalt ions generally have no or little effect on activities at low concentrations (124, 188, 192, 194, 197). Silver ion has been reported to strongly inhibit tobacco 1,3-β-D-glucanases (197), but no similar effect has been recorded for such enzymes from other sources.

Iodoacetamide and N-bromosuccinimide also inhibit the Nicotiana enzyme, suggesting that lysine, tyrosine and or tryptophan residues are essential for activity (228). Nojirimycin and D-glucono-1,5-lactone are known as powerful competitive inhibitors of β-glucosidases (177), but generally they have little or no effect on exo- or endo-hydrolases (178), and thus they may serve as useful tools to differentiate β-glucosidases and exo-β-glucanases (178). However, Nevins (177, 230) reported that nojirimycin has an inhibitory effect on oat exo-1,3-β-glucanase, and IAA-induced growth of Avena coleoptiles. The specificity of this inhibitor requires further study.

c) Substrate Specificity and Action Pattern

Bacterial 1,3-β-glucanases

The bacterial 1,3-β-glucanase which is excreted from Arthrobacter luteus (231) hydrolyses pachyman and curdlan to liberate predominantly laminaripentaose (L_5). Glucose and other lower oligosaccharides are not detected (111, 231). The enzyme is not active on laminaridextrins (D.P. 6 - 10), but it is active on an enzymatically synthesized, linear 1,3-β-oligoglucan preparation again yielding L_5 as the major product early in the reaction. Periodate oxidation or reduction of the chain ends does not significantly lower the catalytic rate, indicating that intactness of the glucose residues at the chain ends is not necessary for the action of this enzyme.

Two 1,3-β-glucanases (I and II) excreted from Bacillus circulans WL-12 (186) hydrolyse yeast cell-walls to generate a series of

laminaridextrins with D.P. ranging from 2 to 5, as well as gentiobiose (1,6-β-linked). The enzymes are specific for 1,3-β-glucosidic bonds but do not degrade laminaribiose. Synergism between 1,3-β-D-glucanase and 1,6-β-D-glucanase is observed during yeast glucan hydrolysis (186). Bacillus subtilis 1,3-β-glucanase has been found to be of the Type C variety, i.e., specific for 1,3-/1,4-mixed linkage β-glucans such as lichenin and oat glucan (216). This enzyme does not hydrolyse cellulose, laminarin, or their derivatives, but it degrades barley β-glucan to yield 3-O-β-cellobiosyl-D-glucose as the predominant product (109). Such an action pattern reflects a specific cleavage of 1,4-β-glucosidic linkages in the 1,3-/1,4-mixed linkage β-glucans. This hydrolytic pattern differs from that of the cellulases extracted from pea and Streptomyces, which although they readily cleave 1,4-β-glucosidic linkages of mixed-linkage β-glucan, the main product is 4-O-β-laminaribiosyl-D-glucose (see also Table III and 110). When acting on RSIII (alternating 1,3-/1,4-) glucan, endo-enzymes from Bacillus pumilus and Bacillus subtilis release oligosaccharides of D.P. 2, 4, 6, 8 and 10 during early stages of the incubation (224).

Fungal 1,3-β-D-Glucanases

Purified exo-1,3-β-D-glucanase from culture filtrates of Basidiomycetes sp. QM 806 releases single glucose residues from non-reducing ends of 1,3-β-glucans (107). The periodate-oxidized laminarin (at non-reducing end) is degraded at approximately one

hundredth of the rate of the unaltered substrate. Clearly, this is a useful technique for identifying exo-action. In addition, the reaction rate in this system is restored to normal by mild-acid hydrolysis of the modified substrate (129). This enzyme is of the Type A hydrolase (see Table III), since it is highly specific for 1,3-linked β-glucans and does not degrade 1,3-/1,4-mixed-linkage or 1,6-β-glucans (217). The enzyme must have binding sites which recognize substantial parts of its substrate beyond the non-reducing end because it preferentially cleaves long chains of 1,3-β-glucans (231).

The action pattern of Basidiomycete exo-glucanase has been studied using reduced $[^3H]$ laminarin as substrate (232). The enzyme forms a tight complex with individual molecules and continues to generate glucose (or gentiobiose from branch points) until the reduced laminarin molecules reach a critical minimum D.P. of L_{3H} to L_{4H} (laminaritriitol to laminaritetraitol). This action pattern resembles that of certain α-amylases, where it has been referred to as "single-chain" attack (233, 234). Eventually, this glucanase will hydrolyse the lowest laminaridextrins, though much more slowly and apparently at random, i.e., by a "multi-chain" mechanism (233).

Exo-glucanases from other fungi such as Thermomyces ibadanensis (112), Phytopthora palmivora (112), Trichoderma viride (235) and Myriococcum albomyces (112) display similar specificities and action patterns to the Basidiomycete enzyme (231, 232). All of these enzymes show a high specificity for 1,3-β-D-glucosidic linkages and are not active on 1,6- or 1,4-linked β-glucans or mixed-linkage β-glucans.

Enzymes from Rhizopus species are classified as Type B endo-1,3-β-D-glucanases (see Table III). Detailed investigations into their

specificities show that they hydrolyse either 1,3- or 1,4-β-linkages, provided 3-substituted glucosyl residues are present on the non-reducing side of the linkage which is hydrolysed (102). Thus 1,3-linkages in laminarin are hydrolysed to liberate glucose and laminaridextrins, but 1,4-linkages are hydrolysed in 1,3-/1,4-mixed linkage β-glucans to liberate L_2 and 3-O-β-cellobiosyl-D-glucose (103). This groups of enzymes also cleaves the 1,4-linkages of RSIII (224) to produce L_2 and oligosaccharides of D.P. 6 and 8. Rhizopus arrhizus β-glucanase has been employed to hydrolyse part of the products generated by β-glucan synthetase in peas (76) to glucose and laminaribiose. This enzyme is a recognized as a reliable and useful tool for helping to identify the kinds of linkages in such products (76).

Both exo- and endo-1,3-β-glucanases have been isolated and purified from a large number of yeasts (113, 191, 192, 193, 205). These enzymes are capable of hydrolysing 1,3- and mixed 1,3-/1,6-linked β-glucans to release laminaridextrins, glucose and gentiobiose (190, 192, 220). The exo-glucanases also degrade p-nitrophenyl-β-D-glucopyranoside (a substrate for β-glucosidase) and yeast cell-walls, although they usully have no activity on other simple aryl- and alkyl-β-glucosides (190). Exo-1,3-β-D-glucanase from Candida utilis hydrolyses laminarin from the non-reducing end, releasing glucose as the exclusive hydrolysis product (190). Endo-enzyme from the same yeast generates laminaridextrins but no glucose when the activity is tested against laminarin, oxidixed laminarin or yeast cell-wall 1,3-β-glucans (205).

Plant 1,3-β-glucanases

All plant 1,3-β-D-glucanases which have been examined are endo-enzymes (Tables IV and VI). The enzyme from Nicotiana is highly specific for linear 1,3-β-glucan substrates and yields a collection 1,3-β-oligosaccharides of D.P. 2 to 7 and a trace of glucose, as reaction products (106). Laminaribiose (L_2) and -triose (L_3) are not hydrolysed, and L_4 and L_5 are only slowly attacked. The enzyme does not degrade Claviceps β-glucan, and the rate of hydrolysis of CM-pachyman decreases as the degree of substitution (D.S.) increases. The findings suggest a requirement for at least three unsubstituted 1,3-β-glucosyl residues in substrates of this enzyme. Very limited action was observed in the 1,3-/1,4-mixed-linkage β-glucans from cereals and this was probably due to cleavage at the few consecutive 1,3-β-linked glucose units which are present in these substrates. This action pattern is consistent with the basic requirements of a Type A enzyme. A similar group of enzymes has also been found in leaves of Phaseolus vulgaris (120) and suspension-cultured cells of Nicotiana tabaccum var. BY-2 (197). Transglycosylase activity, which has been reported in some cellulases and fungal 1,3-β-glucanases (106, 120, 197), has not been detected with these enzymes.

Type C endo-1,3-β-D-glucanases which act specifically on 1,4-β-glucosidic bonds in 1,3-/1,4-mixed linkage β-glucans have been found in several cereals (121, 236, 237). Some investigators have reported that endo-1,3-β-glucanases of the Type A category also occur in malted barley (194) and germinated rye (195). These β-glucanases appear to

be comparatively specific for homogeneous 1,3-β-glucans and to liberate laminaridextrins, though they do show limited activity on mixed-linkage cereal glucans, and therefore the category into which they should be placed is uncertain.

Animal 1,3-β-glucanases

The so-called "exo-1,3-β-D-glucanases" which have been purified from sea urchins (171, 172) degrade linear 1,3-β-glucan to L_2, two unidentified oligosaccharides and glucose as the main products. Like the Basidiomycete "exo-enzymes", the sea urchin enzymes do not cleave L_2, and their reaction rates increase rapidly with increasingly chain length of the substrate, i.e., they are more properly characterized as endo-hydrolases. A 1,3-β-glucanase (see Table VI), present in the digestive juice of the snail (Helix pomatia), has also been purified to homogeneity. This enzyme degrades 1,3-β-linked oligo- and polysaccharides rapidly to completion, yielding glucose as the predominant product of enzyme action (124). It is capable of acting on 1,3-, 1,4-, and 1,6-linked β-glucans, thus exhibiting the broadest apparent specificity of any of the β-glucanases examined so far. It is unquestionably an exo-hydrolase since it does not show preferential attack for higher-D.P. substrates (124), and this should be regarded as a critical criterion on which to base a β-glucanase classification.

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EXPERIMENTS AND RESULTS

Chapter 1

ISOLATION, PURIFICATION AND PROPERTIES OF 1,3-β-D-GLUCANASES FROM *PISUM SATIVUM* SEEDLINGS

SUMMARY

Two buffer-soluble endo-1,3-β-D-glucanases (E.C. 3.2.1.6) have been purified to within 1% of electrophoretic homogeneity from etiolated Pisum sativum stem tissues. Physically, purified glucanase I and II differ in their electrophoretic mobility in sodium dodecyl sulfate polyacrylamide gels (mol. wt. = 22K and 37K, respectively), as well as in isoelectric focusing (pI = 5.4 and 6.8, respectively). Enzymically, they are similar in pH optimum (5.5-6.0), in K_m values for various substrates (0.6-7.4 mg/ml) and in thermal inactivation profiles. However, they differ markedly in tissue location and 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 (1-8), localized wall digestion (9-11), β-glucan synthesis (8,12), rate of translocation (9,13,14), and substrate mobilization in nutrition (11,15). 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 (7,16), plus two endo-1,4-β -D-glucanases (E.C. 3.2.1.4) assayed against CM-cellulose or cellodextrins (17,18) and an unknown number of 1,3-β-D-glucanases as assayed against CM-pachyman or laminarin (19). 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 (2,16,19,20). Such observations have been taken to mean that they catalyse important events in the sequence of reactions leading to plant growth. To date, the cellulases are the only members of these glycosidases that have been purified to homogeneity (17,18,19) and characterized enzymically (18). One of them has been shown to

be truly hormone-induced (8,21). The other has been localized in the wall in close association with its substrate (22), and perhaps with cellulose synthetase (8,12,20), for which it may provide the primer. Properties of the other glycosidases are largely unknown.

This paper reports on purification, physical properties and distribution of two pea endo-1,3-β-D-glucanases (E.C.3.2.1.6). A subsequent paper provides details of their substrate specificities and modes of action. We have also examined (manuscript in preparation) the biological functions of these glucanases, including distribution of their endogenous substrates in the growing plant.

MATERIALS AND METHODS

Enzyme Sources and Assays. Pea seedlings (Pisum sativum, L., var. Alaska) were grown in darkness for 7 to 8 days and harvested as previously described (16,19). Particular care was taken to ensure that the tissue was free of detectable fungal contamination before extraction. Preliminary tests (23) established that the capacity of buffer-soluble extracts of the tissue to generate reducing 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), which differed in their relative "exo"- and "endo"-hydrolase mode of attack. 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 (<5%) of either activity was detected in buffer-insoluble residues by these assay procedures (see also ref. 19).

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 of rection mixture were withdrawn at intervals for estimation of reducing power (24), and activities were expressed as initial (maximal) rates of production of glucose equivalents during the period when activity was linear. The particular stock of laminarin which was used throughout these tests yielded only glucose upon complete hydrolysis, and reducing power estimation indicated an average D.P. of 20. Partial acid hydrolysis with fuming HCl and chromatography on a charcoal-celite column (18) 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 as described earlier (18), and curdlan (25), a partially crystalline insoluble 1,3-β-D-glucan formed by a mutant strain of Alcaligenes faecalis var. myxogenes supplied by Dr. T. Harada, Osaka University, Japan. The latter was used as a suspension in reductometeric assays.

CM-pachyman with a D.S. of 0.32 was prepared by the method of Stone (26) from water-insoluble pachyman (1,3-β-D-glucan, D.P. 255), obtained from Poria cocos Wolf (27). CM-pachymanase activity was assayed viscometrically

(14,19,27) 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.

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 vol 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 x g for 10 min. Crude glucanase I (apical) and II (basal) extracts were precipitated with ammonium sulfate (Schwartz-Mann, N.Y.)., 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 x 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 x 1.5 cm) of Sephadex G-50

(fine grade, Pharmacia Co., Uppsala, Sweden), equilibrated in acetate buffer. 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 to 250 g fresh weight of tissue.

Gel Electrophoresis. Purified glucanase I and II preparations (approx. 10 μg protein) were subjected to discontinuous electrophoresis (28) on polyacrylamide (7%) gels (50 x 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 dry ice 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.

Sodium dodecyl sulfate (SDS) gel electrophoresis (29) was conducted using purified glucanase I and II (approx. 10 μg protein) 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 glucanase I and II were determined (30) by electrofocusing proteins at room temperature in 4% polyacrylamide gels

(130 x 2.5 mm tubes) containing 2% ampholines (comprised of 1.6% pH range 5 to 7, plus 0.4% pH range 3 to 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 gradual increase in voltage (200 V to 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 then placed in 30% methanol/7% acetic acid (v/v) for 2 to 4 h in order to fix proteins and wash out ampholines. Gels were 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 1 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 in the basal regions is about six-fold that in apical regions. Detailed studies on the distribution of these two activities (data not shown here) indicate that concentrations follow 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 "exo"- and "endo"-glucanase activities in pea seedlings appear to be developed at opposite ends of the plant.

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 glucanases from growing and mature parts of the pea stem appear to attack their substrates.

The existence of two major 1,3-β-glucanases in crude extracts of total pea stem is readily shown by gel chromatography on Sephadex G-100 columns. Peaks of laminarinase and CM-pachymanase appear at different elution volumes, representing molecular weights in the ratio of approx. 1:2 respectively (data not shown).

Laminarinase and CM-pachymanase activities in total pea extracts can also be partially separated by ammonium sulfate precipitation. As shown in Table II, most 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 the fractions eluted from the DE-52 cellulose column with a continuous gradient of NaCl, most protein was removed at a salt concentration below 1.0 M, and glucanase activity eluted between 1.0 and 2.0 M (Fig. 1). When pooled glucanase fractions after ion exchange chromatography were concentrated by ultrafiltration, the specific activity of glucanase I (1.2 - 1.8 M NaCl) increased about sixfold with 46% recovery in total activity, and that of glucanase II (0.9 - 1.2 M NaCl) increased 120-fold with 82% recovery (Table III).

After fractionation on Sephadex G-50 columns, protein eluted mostly in void volumes and glucanase I and II fractionated (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 III). 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. Figure 3 shows the distribution of laminarinase and CM-pachymanase activities and stainable protein following non-denaturing disc-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. 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 (31). Both purified preparations contain single bands of proteins which coincide with their catalytic activities.

Figure 4 shows SDS gel-electrophoresis profiles of enzyme preparations during 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 single 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 may bind to the gel (see also ref. 20). Crude pea extracts also chromatographed on this gel with elution volumes of laminarinase and CM-pachymanase activites 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, glucanase I and II are visible as single proteins, with isoelectric points of 5.4 and 6.8 respectively (Fig. 5). Neither purified glucanase contains significant amounts of carbohydrate (phenol-sulfuric acid test, 32).

Basic 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 and Table V).

Figure 6 shows that 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 (data not shown). 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 IV summarizes V_{max} and K_m values for the purified glucanases, using assays of activity with laminarihexaose, laminarin, CM-pachyman and the insoluble substrate, 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 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/Km and V_{max} values for both enzymes are highest for

laminarin, which combines substantial chain length (D.P. 20) and maximum accessibility (solubility) as a substrate.

With respect to stability of the purified glucanases, both show similar sensitivities to thermal inactivation (Fig. 7). 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 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 to homogeneity, and physical and enzymic properties of the purified enzymes were determined. In most respects, these glucanases resemble those from various higher plant sources (27,33-38), all of which possess relatively low molecular weights and acidic pH optima. K_m values of a few mg/ml have been recorded with both laminarin and 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_{max} values for various substrates (Table IV). 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 V). This does not mean that glucanase I is strictly an exo-glucanase and II an 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 (multiple attack pattern), whereas II preferentially attacks the longest chains at random (multiple-chain pattern, 39). Even though K_m values for CM-pachyman are similar for the two enzymes (Table IV), glucanase I is much less effective than II in reducing the viscosity of a large population of CM-pachyman chains (Table V). However, I is much more effective than II in hydrolysing laminaridextrins (Table IV). Thus the differences between the enzymes can be accounted for by differences in substrate affinity and randomness of attack, rather than any fundamental difference 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., pI, Fig. 5) 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 V). The distribution of glucanase I in the pea stem resembles that of many other

glycosidases and phosphatases (1,16,41), however the near absence of II in the apex and its concentration in mature tissues (Table I) is unique. Thus, the two glucanases may well be regulated by very different controls and, by implication, they may have different functions and/or substrates during plant growth.

The two endo-1,4-β-D-glucanases (EC 3.2.1.4) which have been purified from pea stems (17,20) differ from the 1,3-β-D-glucanases in a number of respects besides substrate specificity (18). Buffer-soluble cellulase is generated by rough endoplasmic reticulum (21,22) and is confined within vesicles in vivo (22), while buffer-insoluble cellulase is secreted and bound firmly to cellulose in the wall (8,22). 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 (41). 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 (18) that they may possess the same active sites. K_m values for standard substrates (D.P. 6 dextrins and CM-polysaccharide) of the four enzymes are all very close (a few mg/ml), but V_{max} values are higher for the cellulases (18), 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 (18) as indicating a binding site for substrates that accomodates 6 glucose units. In contrast, the pea 1,3-β-D-glucanases degrade laminarin about 5 times more effectively than laminarihexaose (Table IV) suggesting, on analogy, that these enzymes possess a relatively larger binding site which distinguishes between chains that are much longer than D.P. 6.

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TABLE I

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

Segments (10 mm) of apical and basal regions of 7-day old pea seedlings were used as sources of crude enzyme preparations which were assayed reductometrically and viscometrically as described in Materials and Methods.

Assay	Units	Apical Region	Basal Region
Laminarinase (reductometric)	µg glc equiv./segment/h	63	19
	µg glc/mg protein	604	182
CM-pachymanase (viscometric)	units/segment	11	67
	units/mg protein	105	650
$\frac{\text{Reducing power}}{\text{Viscosity loss}}$	µg glc equiv./ unit viscosity loss	5.9	0.28

TABLE II

DISTRIBUTION OF 1,3-β-D-GLUCANASE ACTIVITIES IN AMMONIUM SULFATE PRECIPITATES OF TOTAL PEA STEM SOLUBLE PROTEIN.

Ammonium sulfate saturation*	Protein	Laminarinase (reductometric)	CM-pachymanase (viscometric)	Reducing power / Viscosity loss
	(% total)	(% total)	(% total)	
20 to 45%	20	18.2	73.7	0.15
50 to 70%	68	71.6	15.0	1.85

* The fraction between 45-50% saturation was discarded since it contains a mixture of both activities.

TABLE III

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 to 250 g of tissue.

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

* Purified enzymes are stored in the presence of bovine serum albumin (5 mg/ml, w/v) at 2°C, and the stability of the purified preparations under such condition is studied (see Appendix IE).

TABLE IV

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

Substrates (up to 2%, w/v) were incubated (total vol 1.0 ml) with purified glucanase I or II (0.05 μmole) in sodium acetate buffer (pH 5.5) at 35°C and initial velocities were determined as described in Materials and Methods. All assays were reductometric and values were calculated from linear Lineweaver-Burke plots.

Substrate	D.P.	V_{max}		K_m*	
		I	II	I	II
		(μmole glc equiv./ μmole enzyme/min)		(mg/ml)	
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

* Lineweaver-Burke plots of these K_m values are shown in Appendices IA, IB, IC and ID.

TABLE V

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 (pI)	5.4	6.8
Carbohydrate content (%, w/w)	0.5	0.8
pH optimum		
Laminarin	6.0	6.0
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/h)	87	6.4
CM-pachymanase (0.8% CM-pachyman) (units x 10^{-3}/mg protein)	45	680
Laminarinase/CM-pachymanase (ng glc/unit viscosity loss)	1930	9.4
Ratio (reducing power/ viscosity loss)	210	1

*Note: These values can not be compared readily to V_{max} values (Table IV) 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.

Fig. 1

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

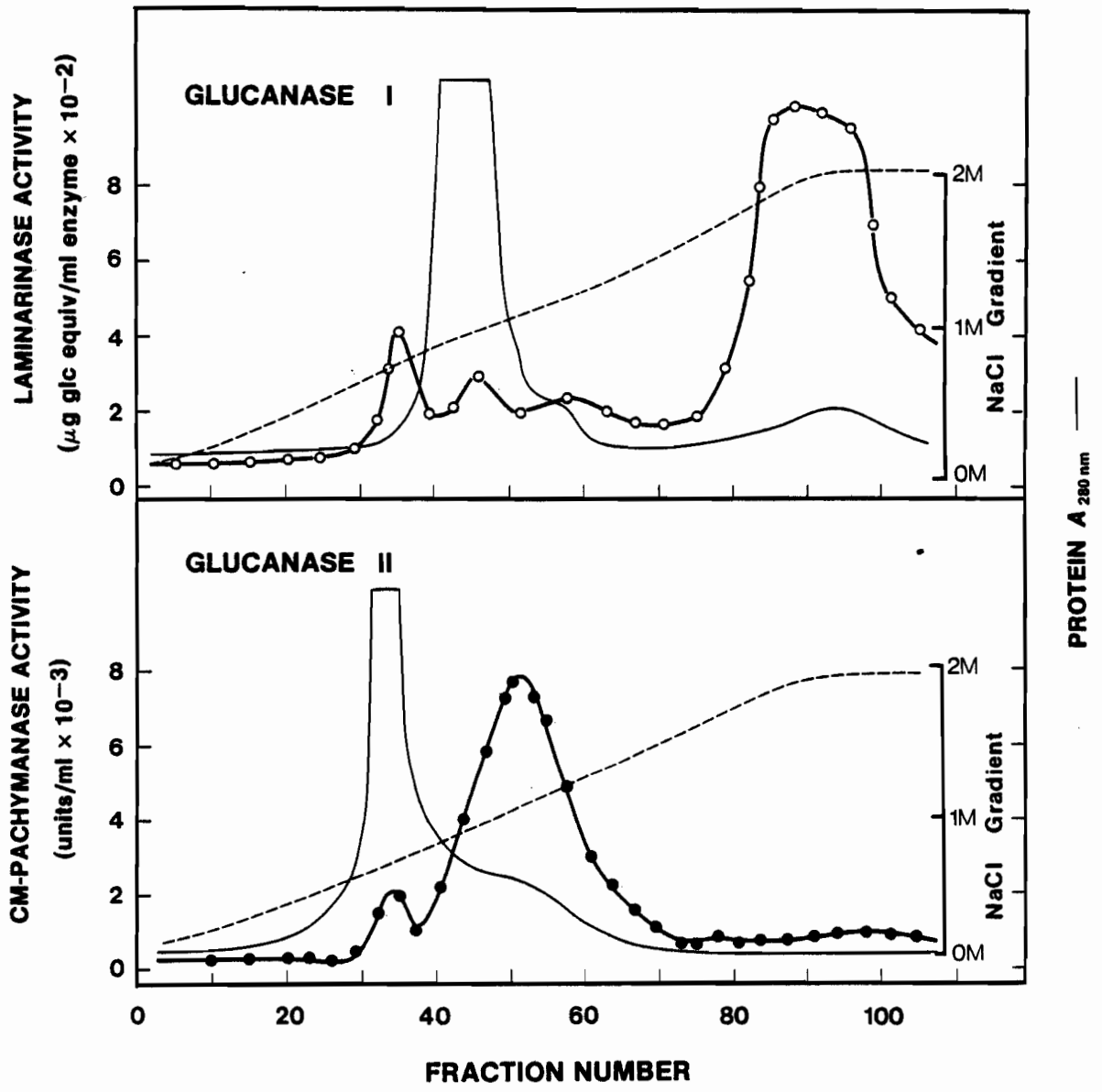

GLUCANASE I
GLUCANASE II
LAMINARINASE ACTIVITY
(μg glc equiv/ml enzyme x 10⁻²)
CM-PACHYMANASE ACTIVITY
(units/ml x 10⁻³)
NaCl Gradient
2M
1M
0M
PROTEIN A 280 nm
FRACTION NUMBER
0
20
40
60
80
100
2
4
6
8

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.

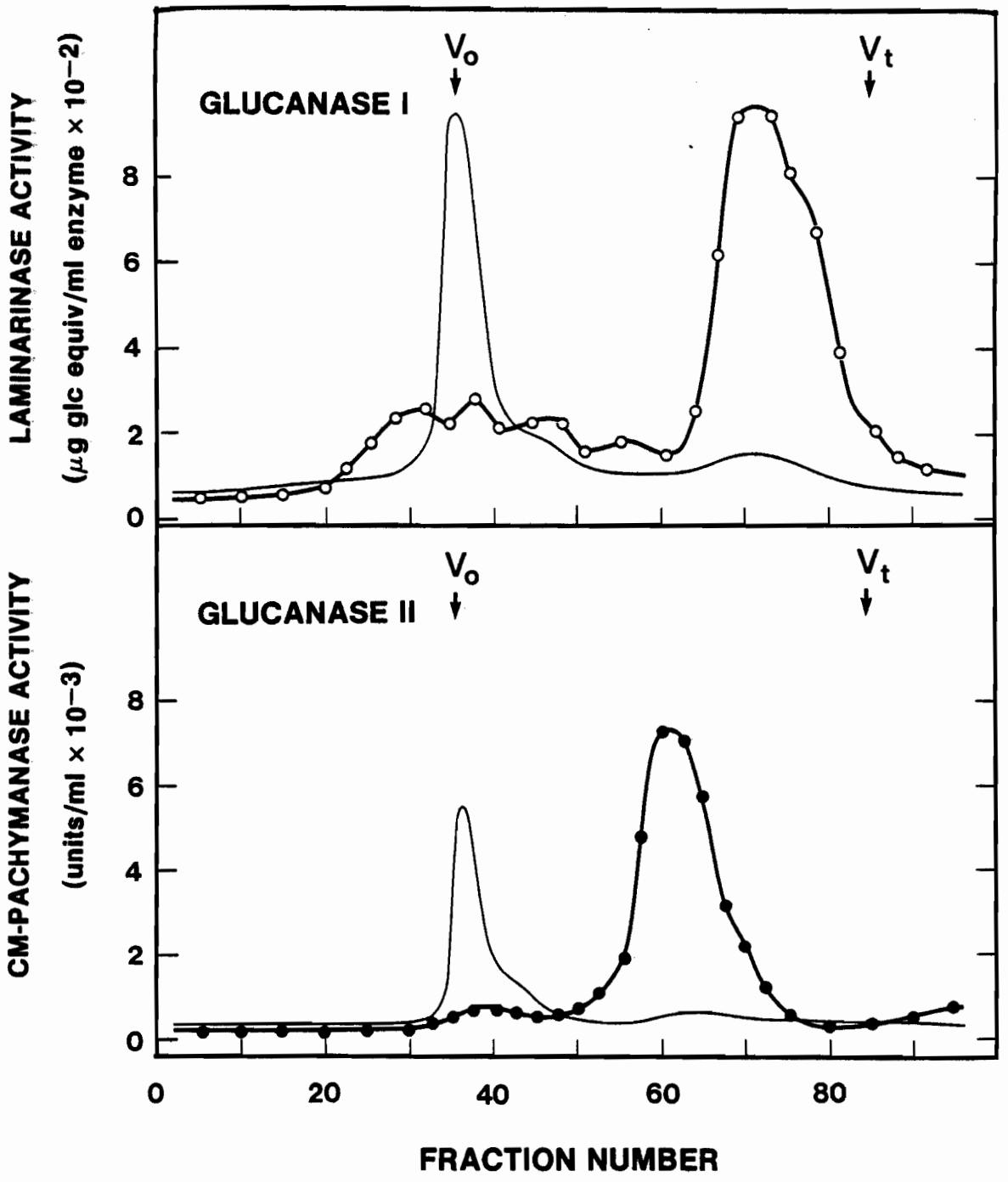

GLUCANASE I
V_o
V_t
LAMINARINASE ACTIVITY
(μg glc equiv/ml enzyme x 10−2)
0
2
4
6
8
GLUCANASE II
V_o
V_t
CM-PACHYMANASE ACTIVITY
(units/ml x 10−3)
0
2
4
6
8
0
20
40
60
80
FRACTION NUMBER

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. Single bands of protein which were present in glucanase I and II preparations coincided with activity as measured reductometrically (o——o) and viscometrically, (•——•), respectively.

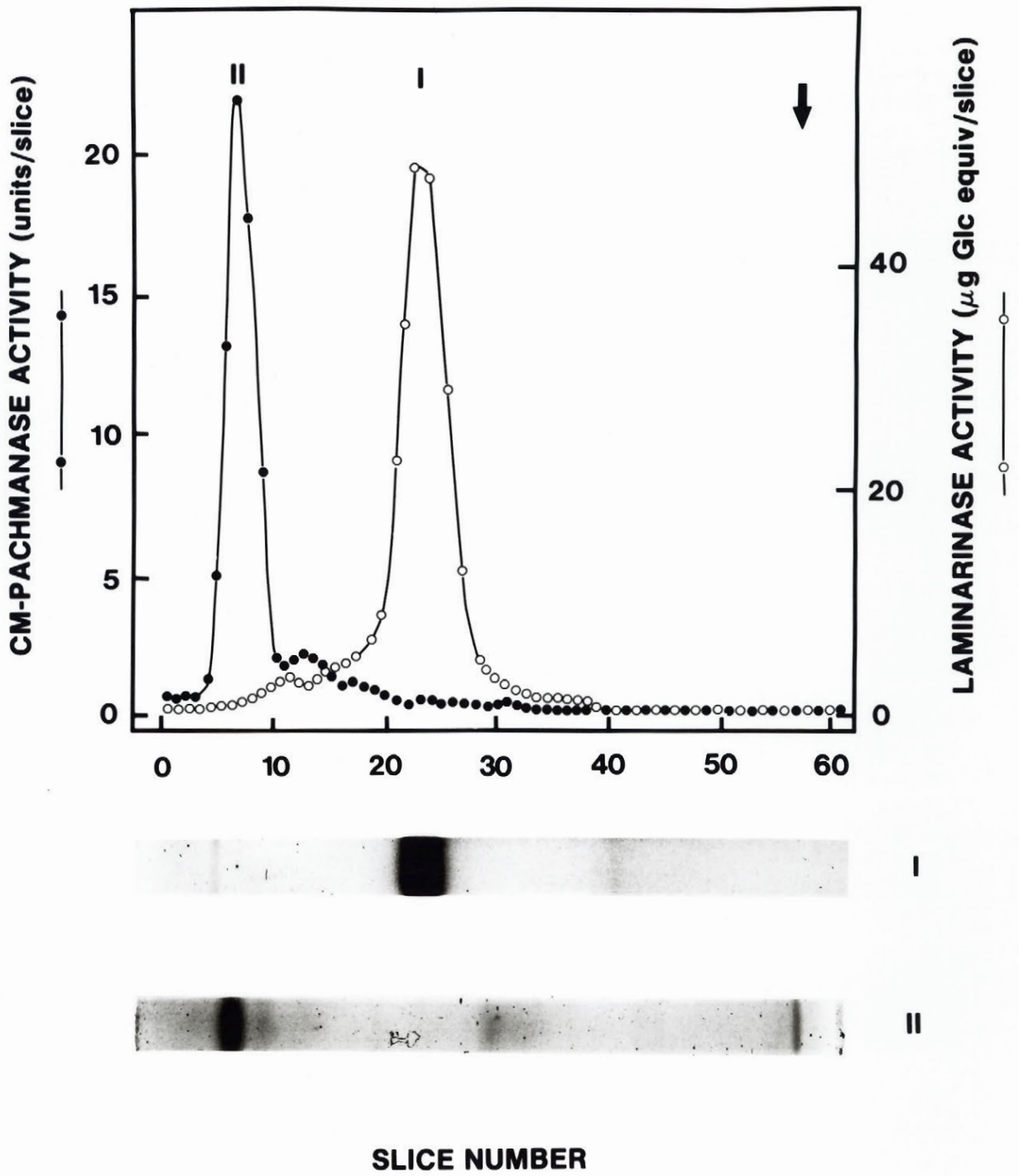

CM-PACHMANASE ACTIVITY (units/slice)
LAMINARINASE ACTIVITY (μg Glc equiv/slice)
II
I
0
5
10
15
20
0
20
40
0
10
20
30
40
50
60
I
II
SLICE NUMBER

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 (68K), ovalbumin (45K), carbonic anhydrase (32K), chymotrypsinogen (24K), β-lactoglobulin (18K) and cytochrome c (13K).

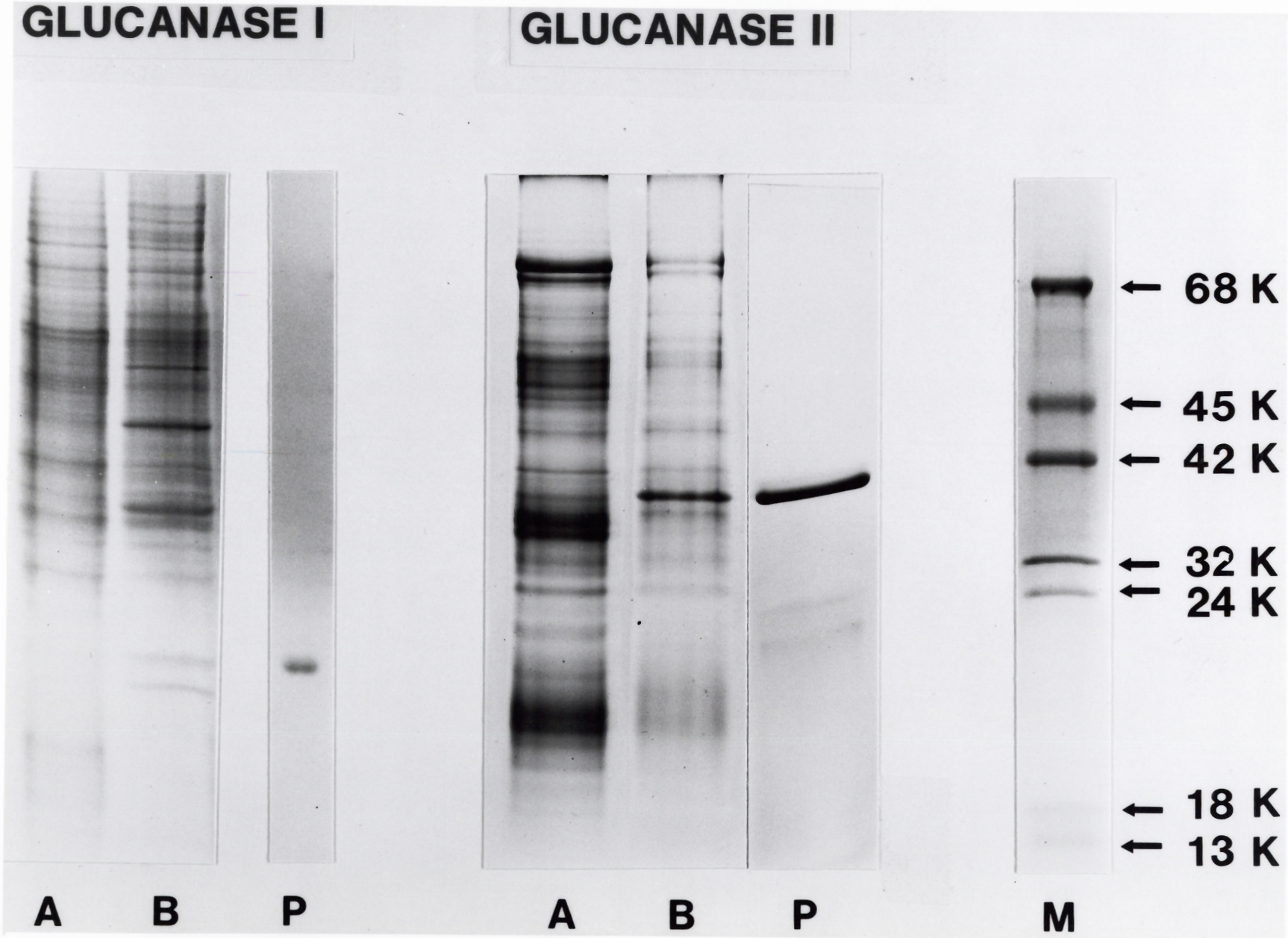

GLUCANASE I
GLUCANASE II
68 K
45 K
42 K
32 K
24 K
18 K
13 K
A
B
P
A
B
P
M

Fig. 5.

Isoelectric focusing of purified 1,3-β-D-glucanases I and II. Details of procedures are given in Materials and Methods.

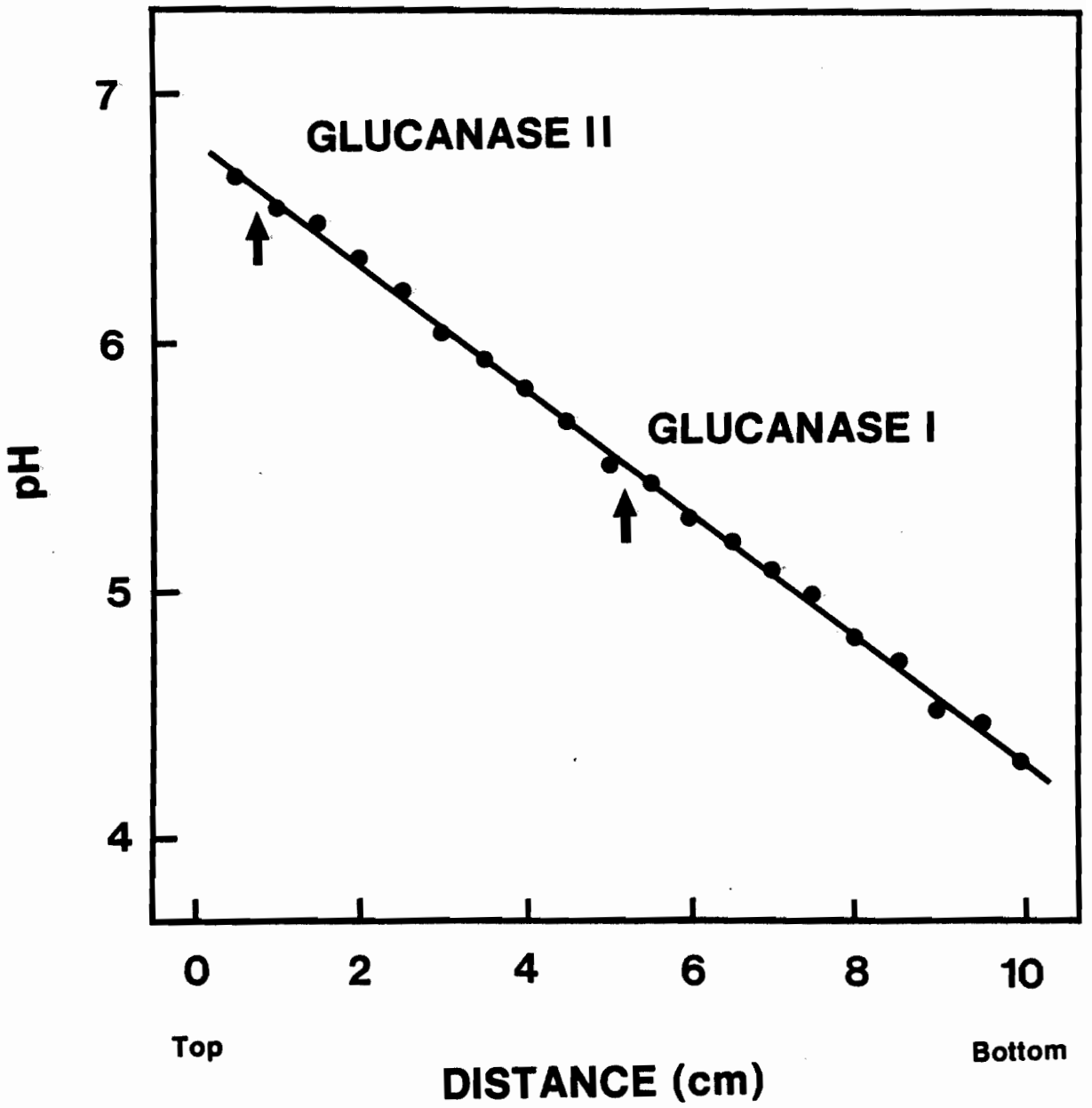

7
6
5
4
pH
GLUCANASE II
GLUCANASE I
0
2
4
6
8
10
Top
Bottom
DISTANCE (cm)

Fig. 6.

pH dependency of 1,3-β-D-glucanases. The activity of purified glucanase I was assayed reductometrically with laminarin (o-----o) and viscometrically with CM-pachyman (o ———— o) as described in Materials and Methods. The pH optima for glucanase II using these two substrates and assays (indicated by arrows) were identical.

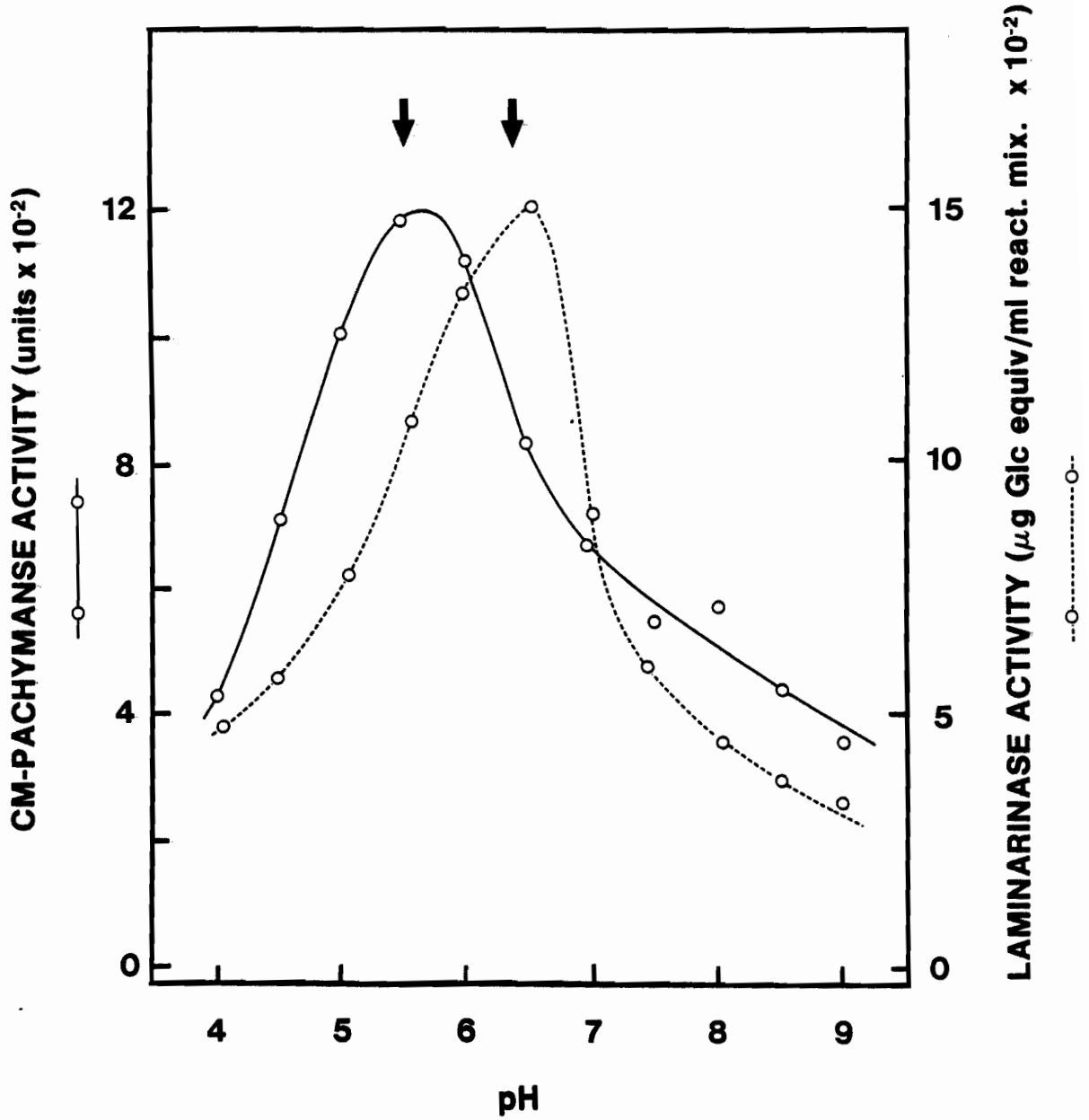
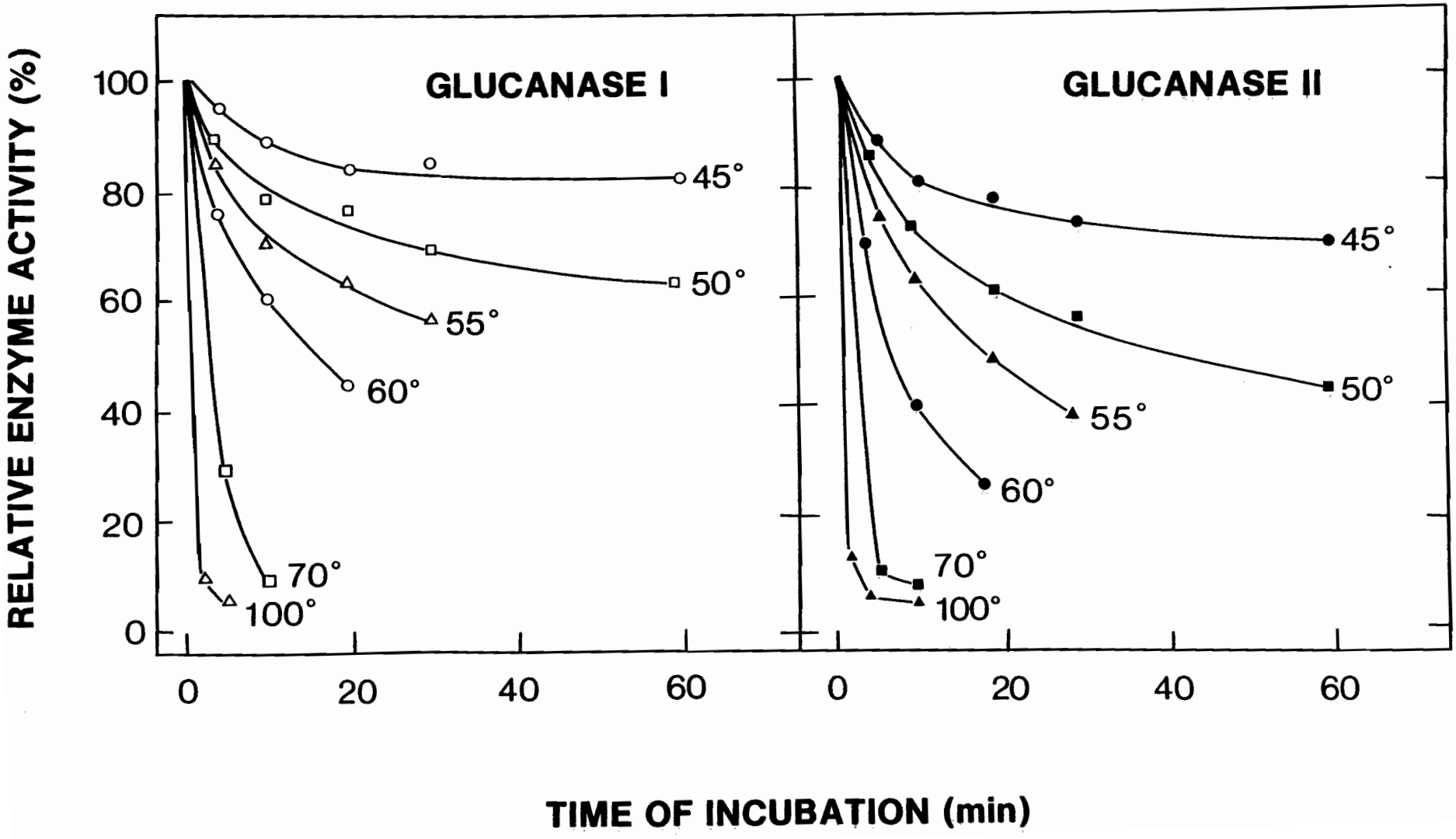

CM-PACHYMANSE ACTIVITY (units x 10⁻²)
LAMINARINASE ACTIVITY (μg Glc equiv/ml react. mix. x 10⁻²)
pH
0
4
8
12
0
5
10
15
4
5
6
7
8
9

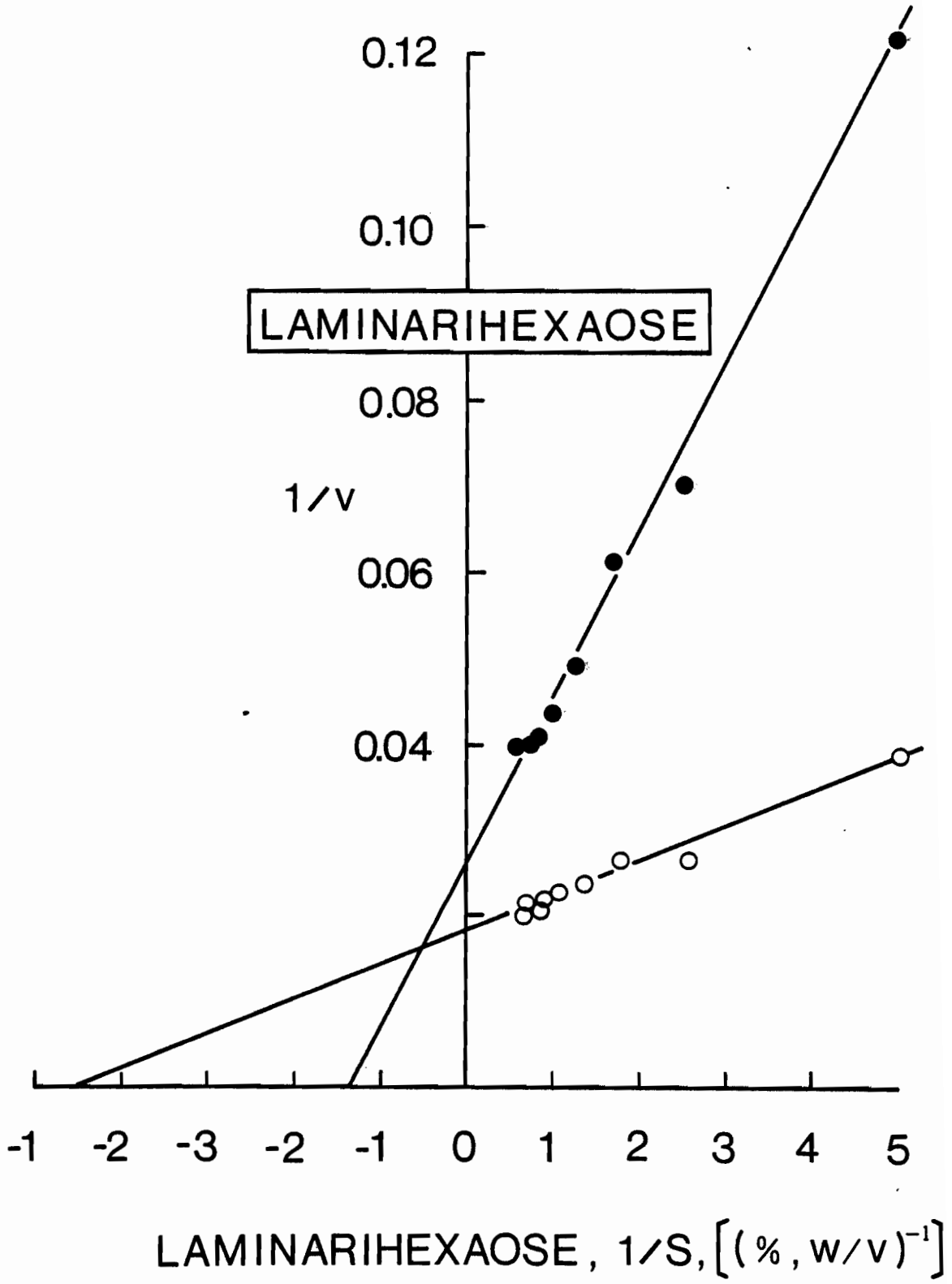
Fig. 7.

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


GLUCANASE I
GLUCANASE II
RELATIVE ENZYME ACTIVITY (%)
TIME OF INCUBATION (min)
100
80
60
40
20
0
0
20
40
60
45°
50°
55°
60°
70°
100°

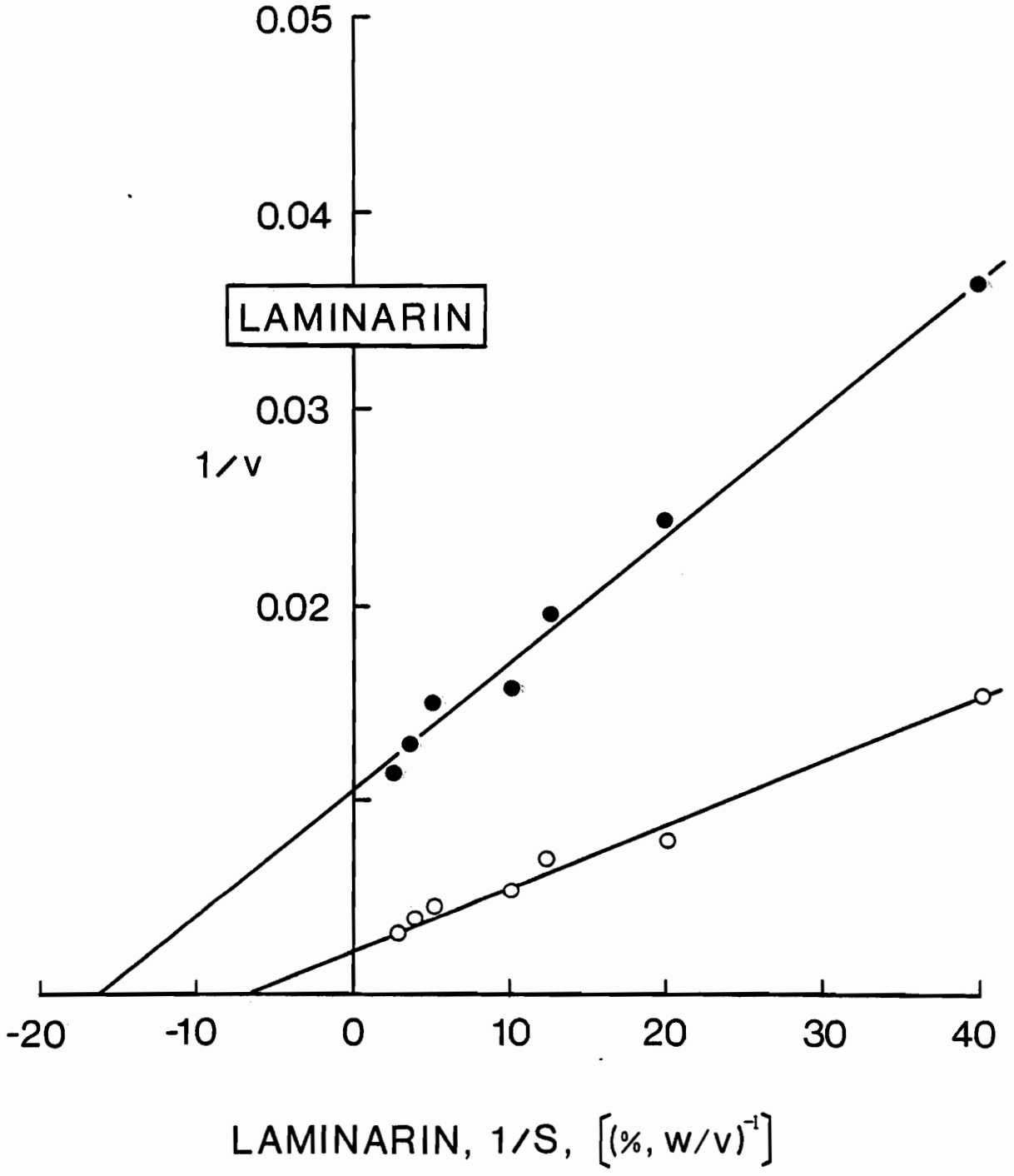
APPENDIX IA

Lineweaver-Burke plots of the action of purified 1,3-β-D-glucanases on laminarihexaose (L_6). The substrate (0.2 - 2.0%, w/v) was assayed reductometrically as described in Materials and Methods, Chapter I. Values for K_m are indicated by the abscissa intercepts and those for V_{max} by ordinate intercepts. These are summarized in Table IV, Chapter I.


0.12
0.10
LAMINARIHEXAOSE
0.08
1/v
0.06
0.04
-1
-2
-3
-2
-1
0
1
2
3
4
5
LAMINARIHEXAOSE, 1/S, [(%, w/v)$^{-1}$]

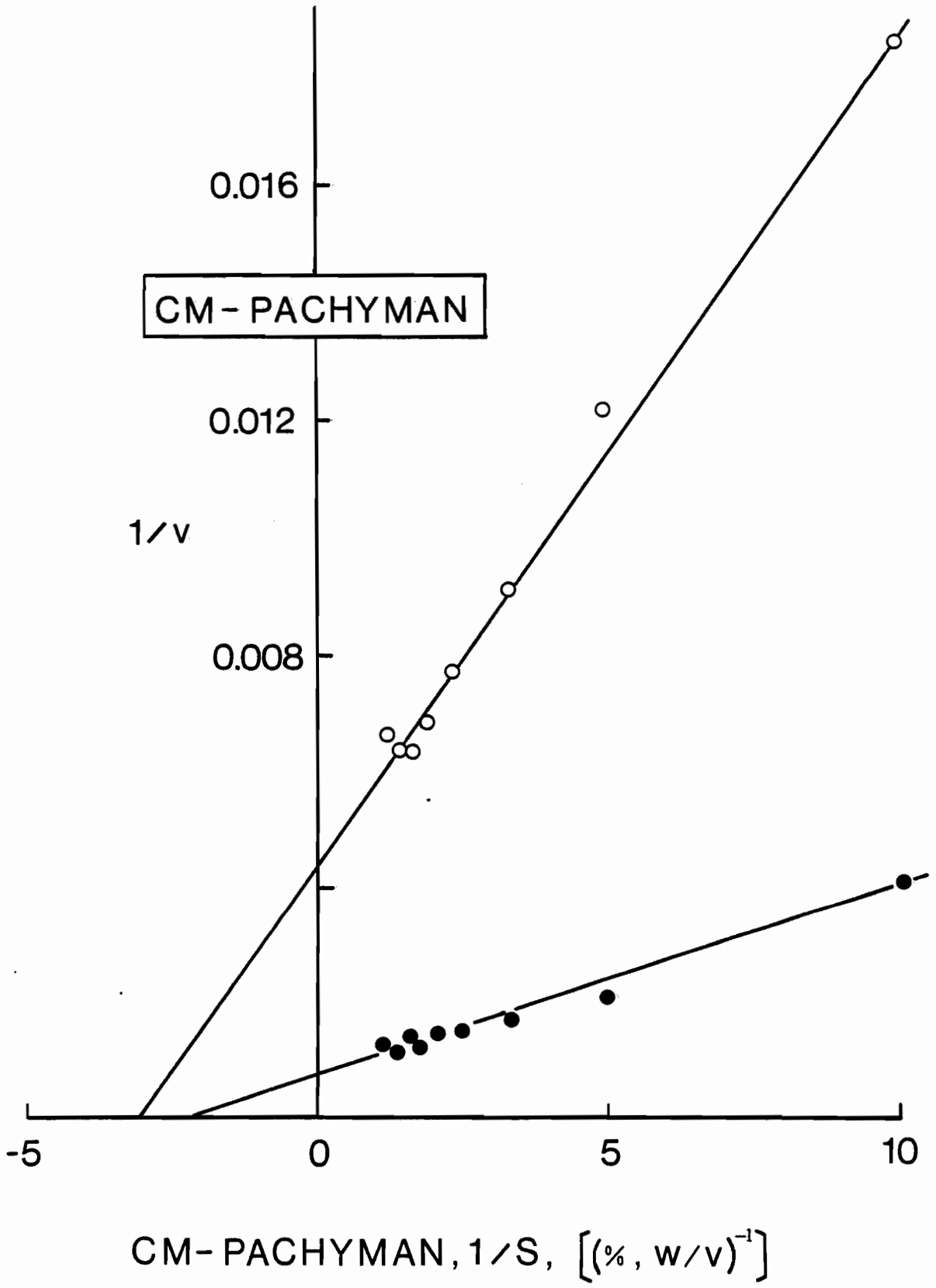
APPENDIX IB

Lineweaver-Burke plots of the action of purified 1,3-β-D-glucanases on laminarin. The substrate (0.025 - 0.4%, w/v) was assayed reductometrically as described in Materials and Methods, Chapter I. Values for K_m are indicated by the abscissa intercepts and those for V_{max} by ordinate intercepts. These are summarized in Table IV, Chapter I.


0.05
0.04
LAMINARIN
0.03
1/v
0.02
-20
-10
0
10
20
30
40
LAMINARIN, 1/S, [(%, w/v)$^{-1}$]

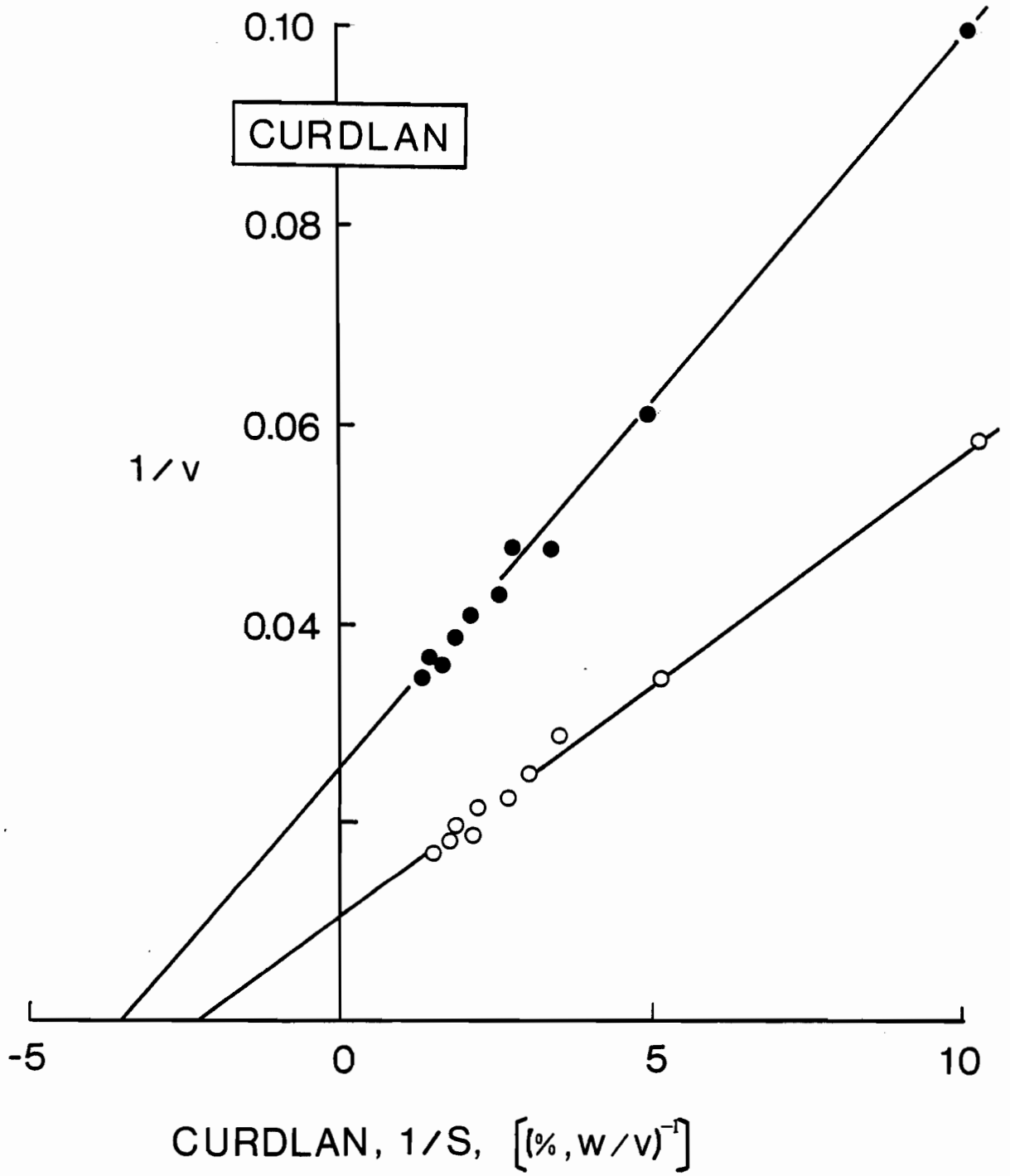
APPENDIX IC

Lineweaver-Burke plots of the action of purified 1,3-β-D-glucanases on CM-pachyman. The substrate (0.1 - 1.0%, w/v) was assayed viscometrically as described in Materials and Methods, Chapter I. Values for K_m are indicated by the abscissa intercepts and those for V_{max} by ordinate intercepts. These are summarized in Table IV, Chapter I.


0.016
CM-PACHYMAN
0.012
1/v
0.008
-5
0
5
10
CM-PACHYMAN, 1/S, [(%, w/v)⁻¹]

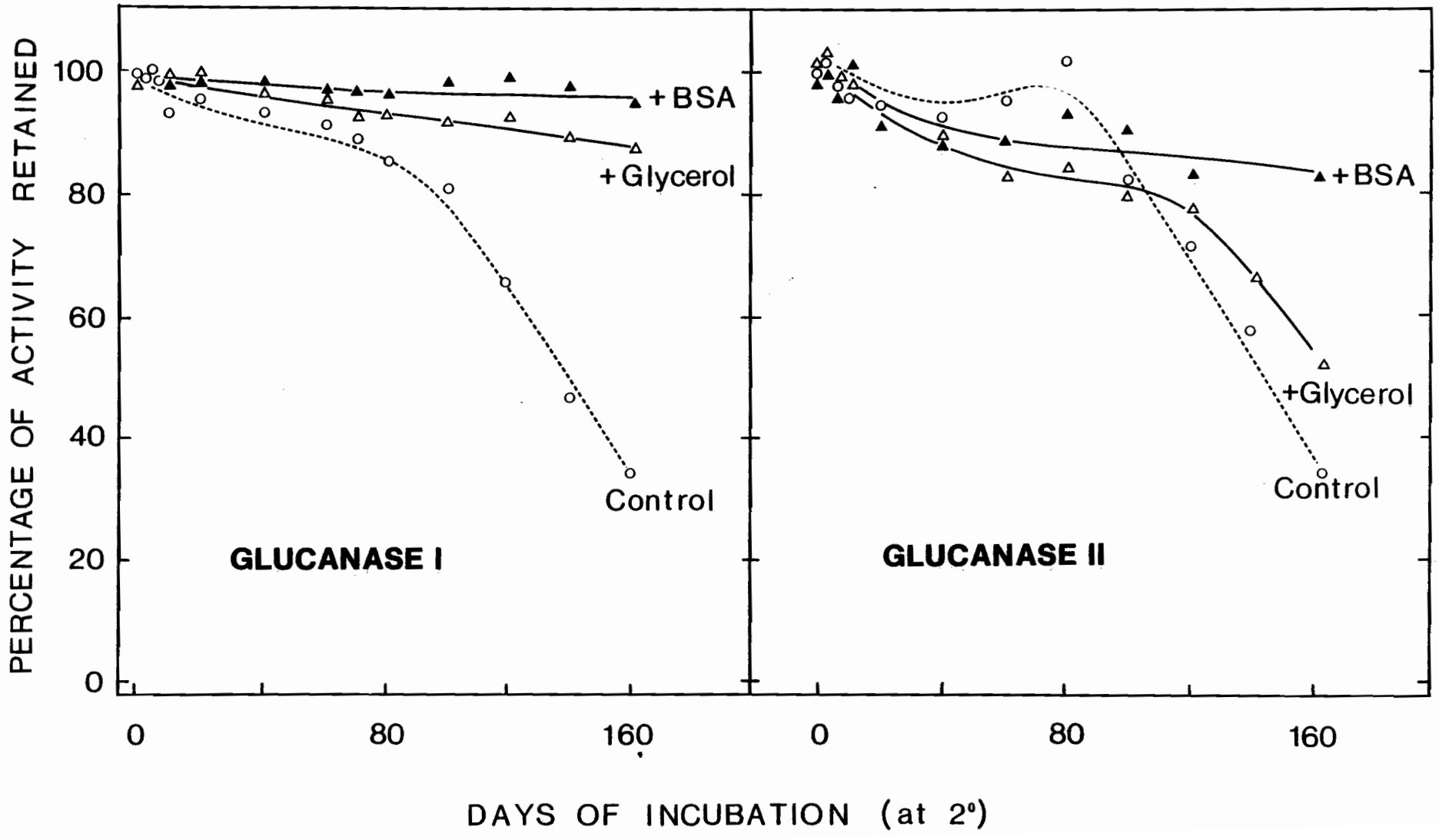
APPENDIX ID

Lineweaver-Burke plots of the action of purified 1,3-β-D-glucanases on curdlan. The substrate (0.1 - 1.0%, w/v) was assayed reductometrically as described in Materials and Methods, Chapter I. Values for K_m are indicated by the abscissa intercepts and those for V_{max} by ordinate intercepts. These are summarized in Table IV, Chapter I.


0.10
CURDLAN
0.08
0.06
1/v
0.04
-5
0
5
10
CURDLAN, 1/S, [(%, w/v)⁻¹]

APPENDIX IE

Stablization of 1,3-β-D-glucanase activities. Purified glucanase I and II (1 ml, 0.3 μmol) were stored with or without bovine serum albumin (5 mg/ml, w/v) or glycerol (2.5%, v/v) at 2°C. Aliquots (50μl) of enzyme preparations were assayed reductometrically (glucanase I) and viscometrically (glucanase II) as described in Materials and Methods, Chapter I.


PERCENTAGE OF ACTIVITY RETAINED
100
80
60
40
20
0
+BSA
+Glycerol
Control
GLUCANASE I
GLUCANASE II
0
80
160
DAYS OF INCUBATION (at 2°)

Chapter II

SUBSTRATE SPECIFICITIES AND ENZYMIC ACTION PATTERNS OF 1,3-β-D-GLUCANASES FROM *PISUM SATIVUM* SEEDLINGS

SUMMARY

Two purified pea 1,3-β-D-glucanases (E.C. 3.2.1.6) hydrolyse laminarin (D.P. 20), laminaridextrins (D.P. 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 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) I is most sensitive to inhibitors that interact with tryptophane and lysine residues, whereas II is inactivated by OH-binding reagents (both are sensitive to SH-reagents and heavy metals). It is concluded that active sites of these β-glucanases differ in such a way that I preferentially continues to degrade fragments produced by endo-hydrolytic attack on long 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 have been purified to homogeneity, and shown to possess markedly different physical and basic enzymic properties (1). Glucanase I, mol. wt. 22 000, is concentrated in apical growing regions and is most effectively assayed reductometrically (as laminarinase), whereas glucanase II, mol. wt. 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 (E.C. 3.2.1.6) which act specifically on poly- or oligo-saccharides containing 1,3-β-D-glucosidic linkages. However glucanase I appears to act via "multiple attack" and II via "multi-chain attack", the 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 purified to homogeneity from extracts of apical and basal stem tissue, respectively, as previously described (1). They were stored at -20°C in 20 mM sodium acetate buffer (pH 5.5) containing 0.25% (w/v) bovine serum albumin to prevent loss of activity. 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 Lab., Mass (3). 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 (4). 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 (5,6),while the Streptomyces glucanase only cleaves 1,4-linkages (3,6).

1,3-β-D-Glucanase activities were routinely assayed reductometrically as laminarinase with the Nelson-Somogyi reagent (7), or viscometrically as CM-pachymanase (1). One viscometric unit is defined as equivalent to the amount of enzyme required to reduce the viscosity of 1.0 ml 0.8% CM-pachyman (D.S. 0.32, pH 5.5, 35°C) by 1% in 2 h during linear stages of the reaction.

Substrates. A number of potential substrates were gifts from the sources indicated: insoluble pachyman (8), prepared from Poria cocus Wolf, Dr. B.A. Stone, La Trobe University, Melbourne; highly crystalline curdlan (9), Dr. T. Harada, Inst. Scientific and Indust. Res., 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., Mass. Commercial sources included: laminarin, chitin and wheat straw xylan from Sigma Chem. Co., St. Louis, Mo; lichenan from Koch-Light Lab., Colnbrook, Bucks. 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 (10,12) by fractionation on a charcoal/celite column of partial hydrolysates of laminarin (Appendices IIA to D). The products co-chromatographed with laminaridextrins prepared elsewhere (gift of B.A. Stone), and yielded R_g values consistent with a homologous series (13). Measurements of optical rotation density (22°C, 589 nm) showed that the oligosaccharides were optically pure and linearly related in a Freudenburg plot (13). Reduced [^{3}H]laminaridextrins were prepared by treating unlabelled laminaridextrins with NaB[^{3}H]$_4$ (New England Nuclear Corp., Boston, Mass.) as described by Cole and King (14). Specific activities of the products were approximately 1.85 μCi/μmole of dextrin (see Appendix IIE and IIF).

Reduced forms of laminariheptaose (L_7), laminarin and curdlan were prepared by treating aqueous solutions with sodium borohydride overnight at room temperature (14). 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 oligosaccharide or polysaccharides (15). 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 (16) and lyophilized. Carboxymethylation of pachyman was carried out as described (17) to form a soluble product, D.S. 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 $AgNO_3$ (18) or potassium periodocuprate (19). Spots were delineated and eluted for estimation of reducing power (7).

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 x 0.7 cm). Laminarin (D.P. 20) 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 (20).

Inhibitors. Reagents used in inhibition studies were commercial analytical grade and were obtained as follows: *p*-chloromercuribenzoate (*p*-CMB), *n*-ethylmaleimide, iodoacetamide, succinic anhydride, 5-dimethylaminonaphthyl sulfonyl chloride (DANS) and *n*-acetylimidazole (Sigma Chem. Co., St. Louis, Mo.); *n*-bromosuccinimide (Aldrich Chem. Co., Milwaukee, Wisc).

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 contiguous 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 D.P. (at least up to L_7 and 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 were required in various substrates to determine susceptibility 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 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 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 Figure 1. Lower laminaridexrins are formed relatively rapidly (within min) in the presence of glucanase I, but only eventually (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_o) remains the predominant component of the reaction mixture, suggesting that after initial endohydrolysis, glucanase I preferentially continues to hydrolyse fragments to lower D.P. 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 minutes to products which distribute broadly over a relatively high D.P. range. In a few hours, essentially no laminarin remains in the reaction mixture. It is clear that both enzymes are endo-hydrolases, 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, though glucanase I has a greater capacity than II to do so. This interpretation is confirmed more directly by examining the products generated from reduced (tritiated) laminarin (Fig. 2). During a brief incubation (30 min), neither glucanase attacks linkages up to the fourth glycosidic bond from the reduced terminal, i.e., no radioactive oligosaccharides are produced with D.P. lower than 5. Glucanase I forms relatively more of radioactive

oligosaccharides with higher D.P. and barely any of D.P. 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 Figure 3. Both pea glucanases generate glucose, laminaribiose and laminaritriose in progressively lower yields. The latter components presumably derive from increasingly uncommon runs of 3 or 4 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 hydrolysis of linkages adjacent to 1,3- bonds, even if these are 1,4-β-linkages (6). These four products are also formed in similar relative amounts by action of Rhizopus endo-1,3-β-D-glucanase (see also refs. 5,6). Cellobiose and 4-O-β-D-laminaribiosyl-D-glucose are formed by hydrolysis of linkages adjacent to 1,4- bonds by pea cellulases (10) 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 D.P. 3 or less, implying that I hydrolysis 1,4-linkages in this substrate more readily than II.

Products formed from laminaridextrins. Figure 4 shows progress curves for production of labelled products from 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 D.P. products, whereas II generates L_{2H} and L_{3H} first and hardly forms any L_{4H}. The pattern shown by glucanase II with these substrates is essentially identical to that shown by pea endo-cellulases acting on [^{3}H]cellodextrins (10), 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 laminaridextrins 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 (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 glucanase 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 D.P. 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 D.P., it requires more laminaridextrin to achieve a given percentage inhibition of CM-pachymanase activity using glucanase I than II, as indicated by the relative K_I values (see insert, Fig. 6).

Glucono-δ-lactone and nojirimycin (5-amino-5-deoxy-D-glucopyranose), which are known to act as competitive inhibitors of some glucosidases and exo-glucanases but not endo-glucanases (21), did not interfere with the CM-pachymanase activities of pea glucanase I or II at concentrations up to 0.1 M.

With respect to non-competitive inhibitors, a brief survey (Table III) indicates that glucanase I and II show differential susceptibilities to different classes. While both glucanases are inhibited by sulfhydryl agents and heavy metal ions, albeit to different degrees, glucanase I is more sensitive than II to reagents that interact with tryptophane and lysine residues, while the reverse is true of hydroxyl-binding reagents. Thus, selective use of these classes of inhibitors could be employed to assay one glucanase in the presence of the other.

DISCUSSION

Pea 1,3-β-D-glucanases hydrolyse poly- or oligo-saccharides and their derivatives containing 1,3-β-linkages between anhydroglucose residues. The highest D.P. 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 Figure 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 (3,6) 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 Figure 7; the controlling feature being contiguity of a 1,3-β-linkage at the non-reducing side (6). The fact that glucanase I completely hydrolyses barley β-glucan to lower laminaridextrins (D.P. < 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 of significant laminaribiase activity (Table I). Moreover, glucose and laminaribiose do not appear amongst the reaction products in the early stages of attack on higher-D.P. substrates (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 (5,6).

The degradation profiles and relative reaction rates observed with several substrates in this study confirm the conclusion (1) 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 hydrolyse substrates with high D.P. 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 six glucose units from the reducing or reduced terminal, i.e., the main products formed in brief incubations are clustered around D.P. 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 Figure 7. Glucanase I is shown as hydrolysing laminarin and [^{3}H]laminarin (D.P. 20) at points between about six glucosyl units from the reducing end and two glucosyl units from the non-reducing end of a given chain. This accounts for the rapid apperance of [^{3}H]dextrins with D.P. 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 low-D.P. dextrins (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 high-D.P. products (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 D.P. 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 attack on long chains. However, glucanase II appears to act via "multi-chain attack", wherein it hydrolyses the longest available chains internally before attacking low-D.P. fragments. Glucanase I, therefore, resembles the α-amylase activity found in many sources, while glucanase II acts more like the random hydrolysis observed with acids (2). To our knowledge, this is the first occasion where 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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TABLE 1

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

Purified glucanase I and II (0.3 μmole 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 β-glucosides, cellulose, cellodextrins (G_2 to G_6), starch, wheat straw xylan, ivory nut mannan, chitin, arabinogalactan or pustulan. β-Glucan synthetase products from peas have also been used as substrates for the hydrolases (see Appendix IIG).

Substrate		Relative Rate of Hydrolysis	
		Glucanase I	Glucanase II
		(μmole glc equiv/μmole enzyme/min)	
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-linkage 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 as described in Materials and Methods. These products (5 mg/ml) were incubated with purified glucanases and initial rates of hydrolysis were assayed reductometrically as in Figure 1.

Substrate	Rate of Hydrolysis	
	Glucanase I	Glucanase II
	(μmoles glc equiv/μmole enzyme/min)	
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

TABLE III

NON-COMPETITIVE INHIBITORS OF PEA 1,3-β-D GLUCANASES

Additives were combined with purified glucanases (0.25 μmole), made to volume (1 ml) with CM-pachyman (0.8%) in buffer (20 mM sodium acetate, pH 5.5), and assayed for initial rates of viscosity loss as described in Materials and Methods. Most additives were dissolved in assay buffer, but some were combined as ethanolic solutions or solids. Many additives had no effects (up to 20 mM), including sodium azide, cyanide, $MnCl_2$, and EDTA.

Inhibitors	Conc.	Glucanase I	Glucanase II	Reactive residues
	(mM)	(% inhibition of CM-pachymanase)		
Metal ions				
$CaCl_2$	5	19	25	
$MgCl_2$	5	12	15	
$CuCl_2$	5	30	86	
$HgCl_2$	5	94	85	
Potential sulfhydryl reagents				
p-CMB	2	32	77	cys
Iodoacetamide	10	87	87	cys, tyr
n-Ethylmaleimide	2	0	29	cys
KI	2	18	0	cys
Hydroxyl binding reagents				
DANS	2	79	100	ser, thr
Succinic anhydride	10	0	77	lys, ser, thr
Diethyl pyrocarbonate	0.5% (v/v)	0	100	ser, thr
Others				
n-Bromosuccinimide	5	75	0	trp, lys
n-Acetylimidazole	10	55	0	lys, trp.

Fig. 1

Gel chromatography of hydrolysates of laminarin. Purified glucanase I and II (2.0 μmoles/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 prepared as described in Materials and Methods. Fractions (0.15 ml) were assayed for total carbohydrate by the phenol-H_2SO_4 method (O.D. 490 nm). V_o was determined with blue dextran, and the elution volumes of authentic laminaridextrins (G_1 to L_6) are indicated by numbered arrows.

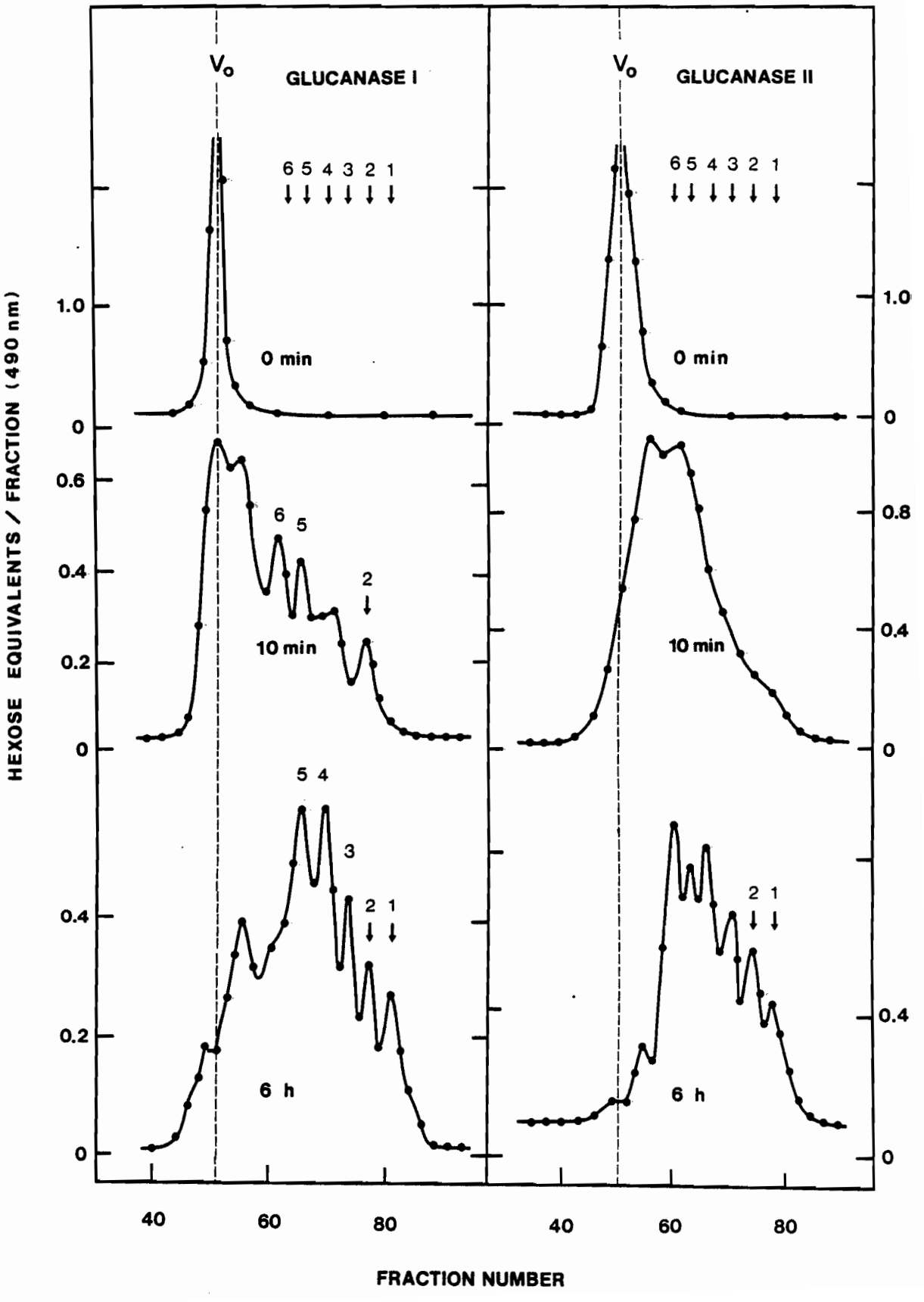

Vo
GLUCANASE I
Vo
GLUCANASE II
6 5 4 3 2 1
0 min
10 min
6 h
HEXOSE EQUIVALENTS / FRACTION (490 nm)
FRACTION NUMBER
40
60
80
1.0
0.8
0.6
0.4
0.2
0

Fig. 2

Paper chromatography of hydrolysates of reduced [^{3}H]laminarin. Purified glucanases I and II (1.0 μmole/ml) were incubated with [^{3}H]laminarin (2 mg/ml, 1.5 x 10^{6} cpm/ml) as described in Figure 1. Aliquots (0.1 ml) were chromatographed as described in Materials and Methods, along with purified reduced [^{3}H]laminaridextrin standards,(L_{2_H} to L_{7_H}).

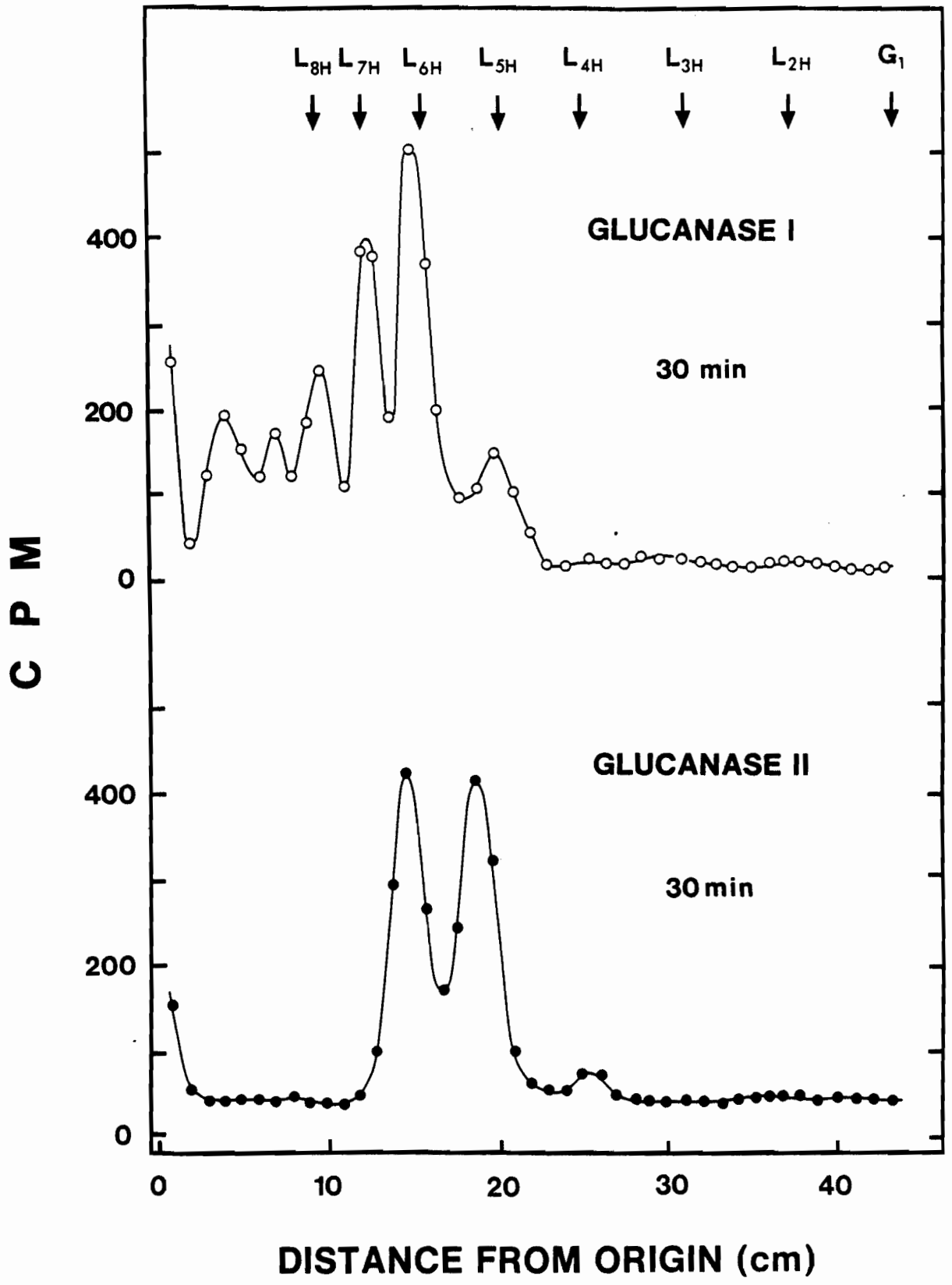

L_{8H} L_{7H} L_{6H} L_{5H} L_{4H} L_{3H} L_{2H} G_1
GLUCANASE I
30 min
GLUCANASE II
30 min
400
200
0
400
200
0
C P M
0
10
20
30
40
DISTANCE FROM ORIGIN (cm)

Fig. 3

Products formed from barley β-glucan by pea glucanase I and II and fungal glucanases. Reaction mixtures (1 ml) contained barley β-glucan (10 mg) plus 0.4 μmole 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).

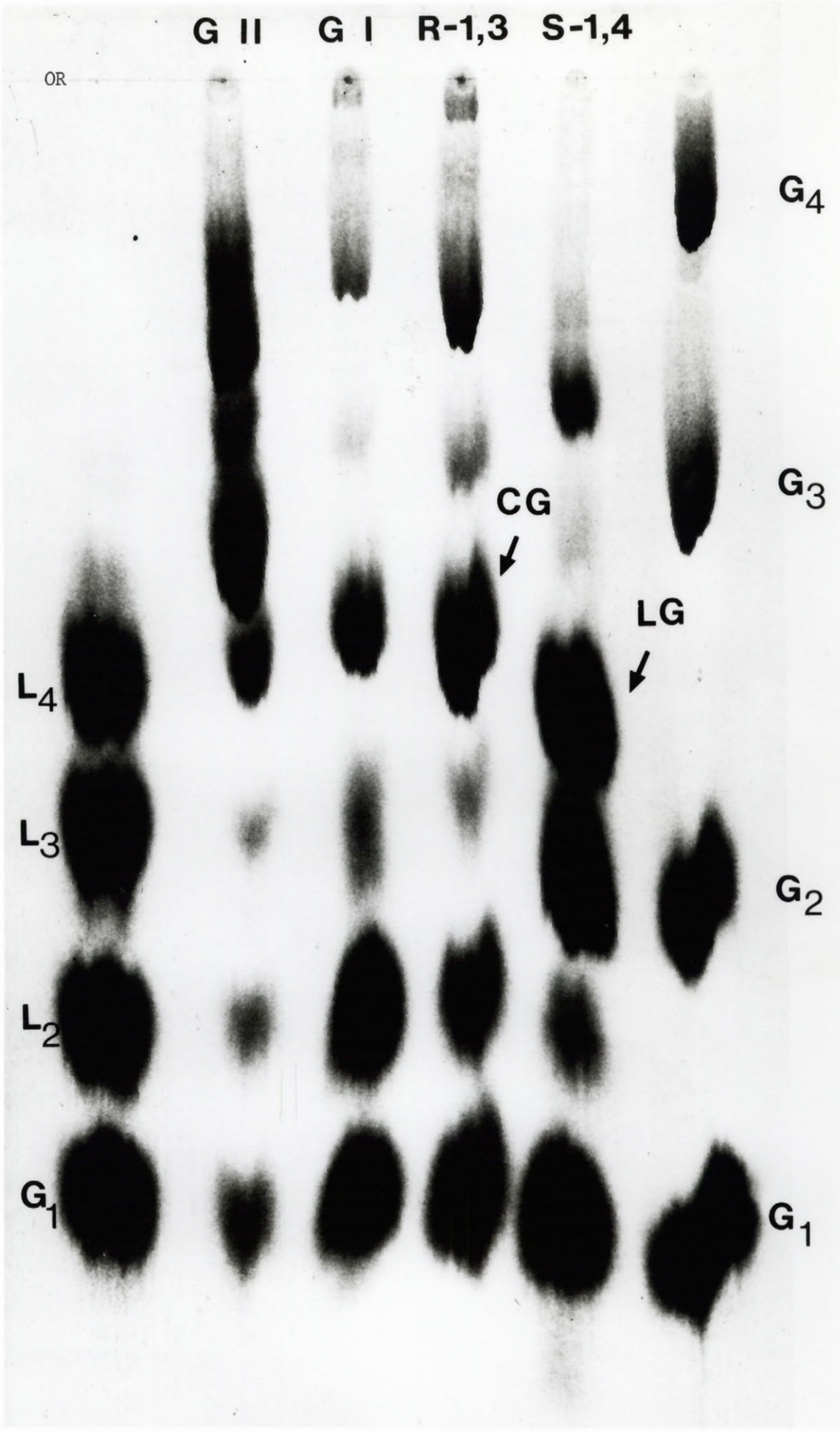

G II
G I
R-1,3
S-1,4
OR
G4
G3
CG
LG
L4
L3
L2
G1
G2
G1

Fig. 4

Products formed from reduced [^{3}H]laminaridextrins by glucanase 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 Figure 2.

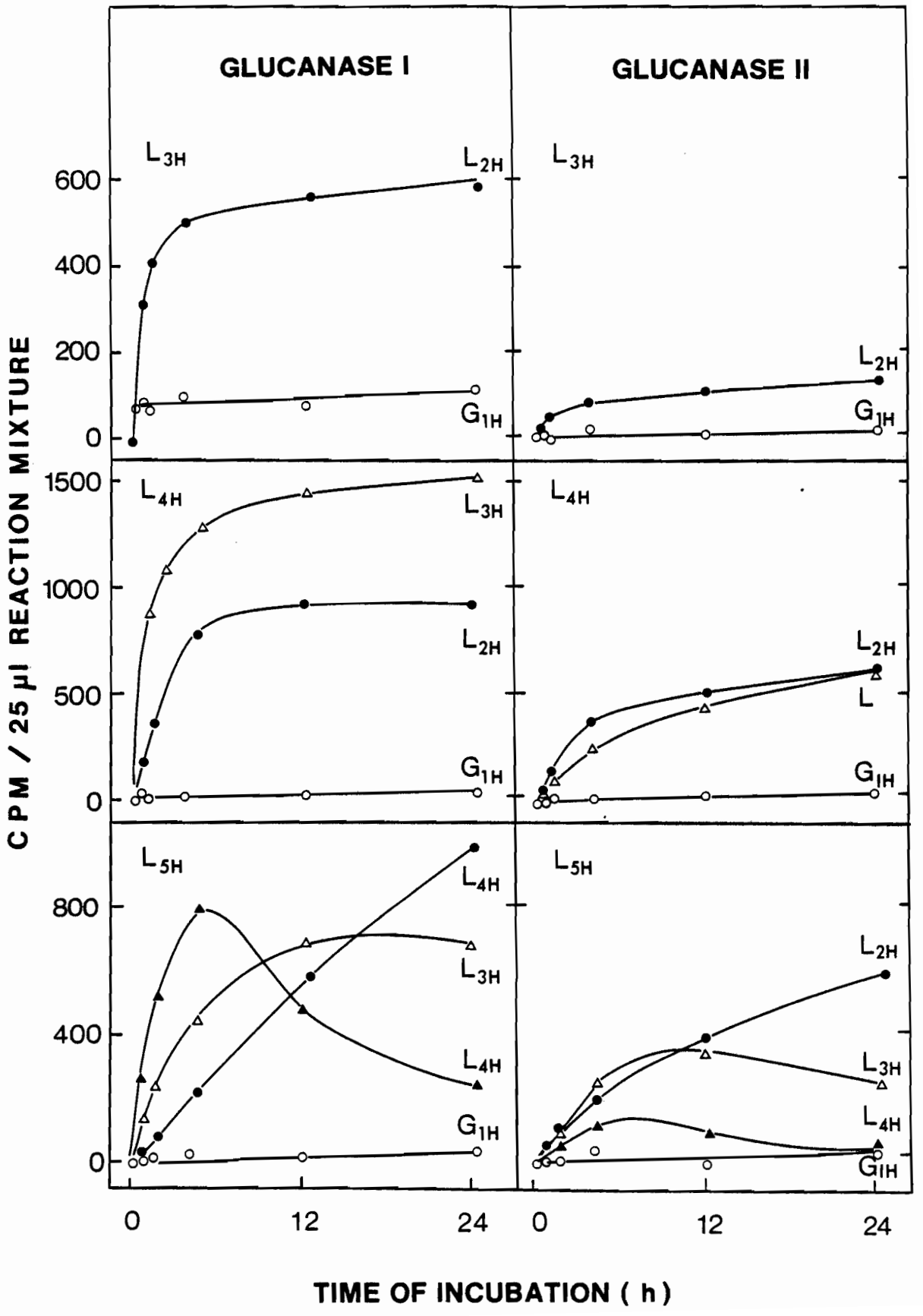

GLUCANASE I
GLUCANASE II
CPM / 25 µl REACTION MIXTURE
TIME OF INCUBATION (h)
0
12
24
600
400
200
1500
1000
500
800
L3H
L2H
G1H
L4H
L5H
L

Fig. 5

Transglucosylation by purified pea 1,3-β-D-glucanases. Glucanase I and II (0.1 ml, 0.5 μmole) were incubated with a mixture of 10 mM D-[^{14}C]glucose (0.75 ml, spec. act. 313 mCi/mole) plus 10 mM unlabelled laminaritetraose (0.15 ml) at 35°C. Aliquots (50 μl) of reaction mixtures were chromatographed on paper as described in Materials and Methods. Control, zero time (0 ----- 0); after 4 h incubation (● ----- ●).

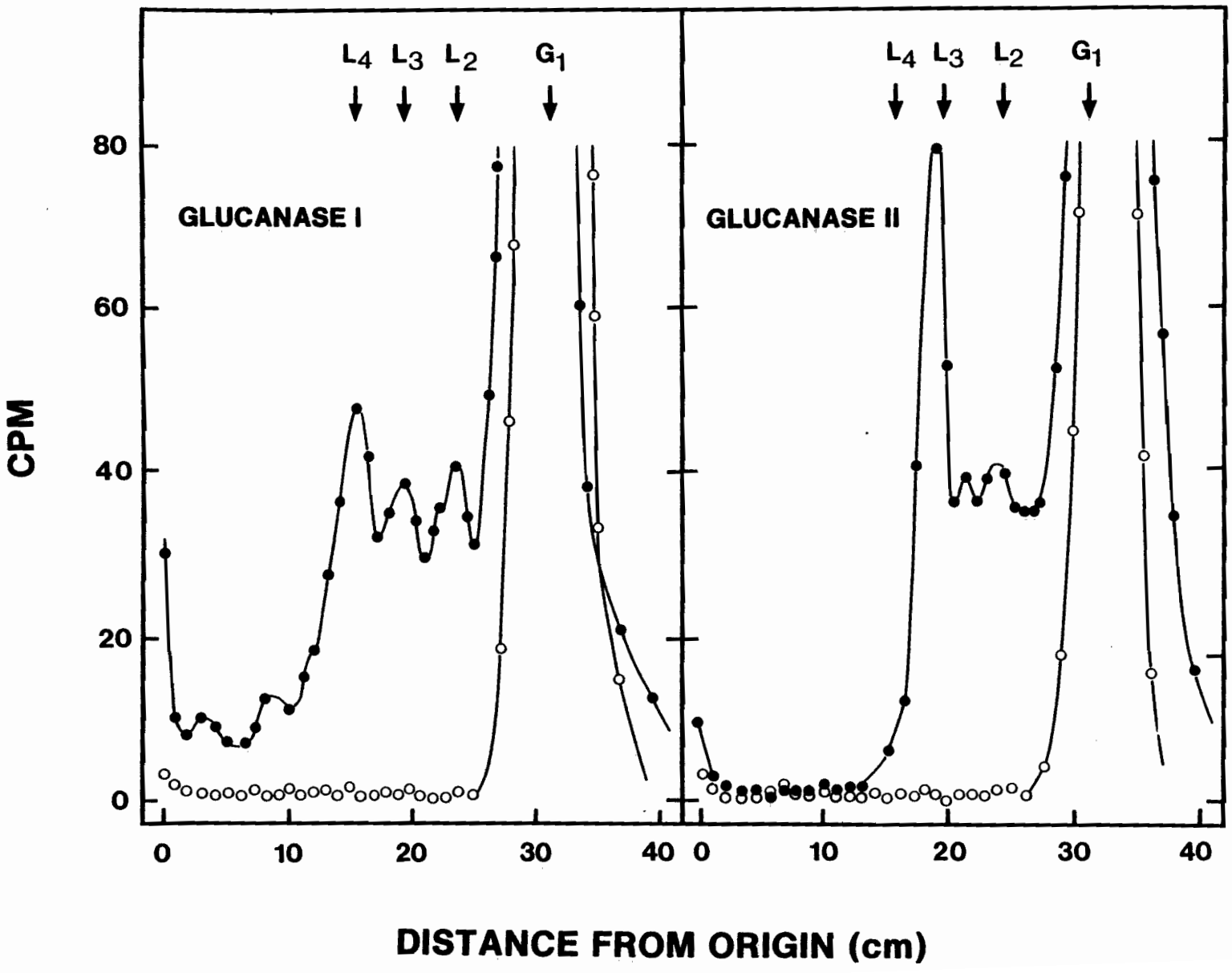

L4 L3 L2 G1
GLUCANASE I
GLUCANASE II
CPM
0
20
40
60
80
0 10 20 30 40
DISTANCE FROM ORIGIN (cm)

Fig. 6

Inhibition of CM-pachymanase by laminaridextrins. Purified glucanase I and II (0.05 ml, 0.1 μmole) 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_I 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.

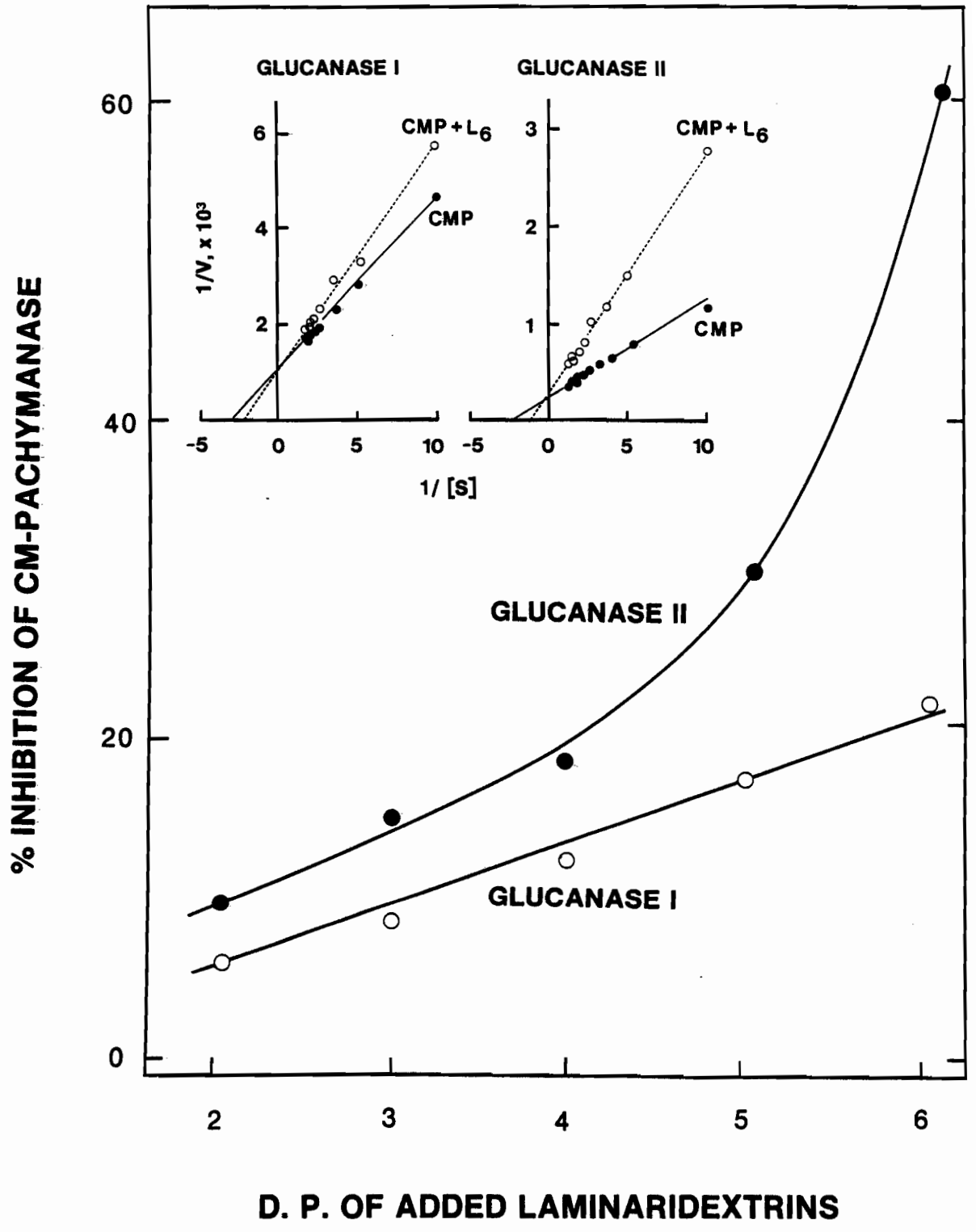
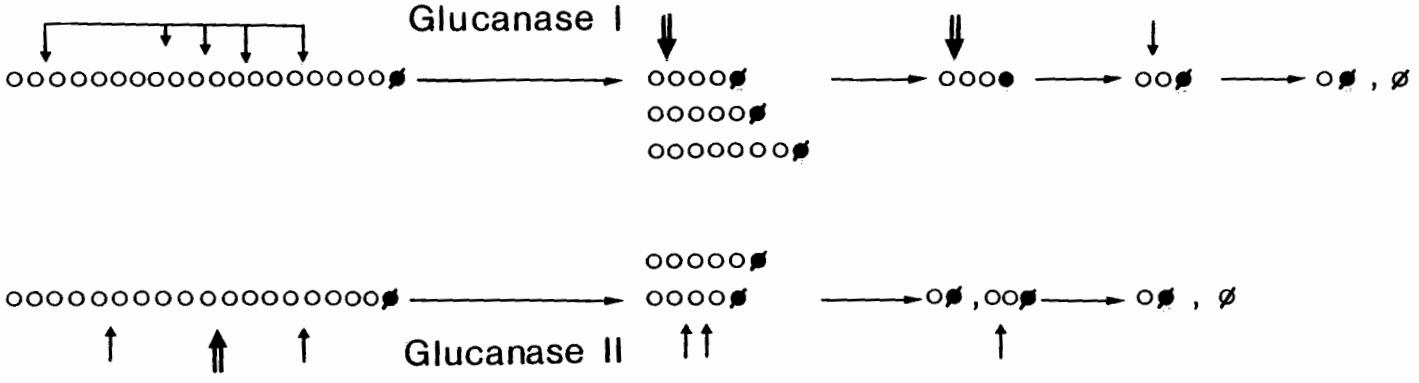

GLUCANASE I
GLUCANASE II
CMP + L6
CMP
1/V, x 10³
1/ [S]
% INHIBITION OF CM-PACHYMANASE
D. P. OF ADDED LAMINARIDEXTRINS
GLUCANASE II
GLUCANASE I
0
20
40
60
2
3
4
5
6

Fig. 7

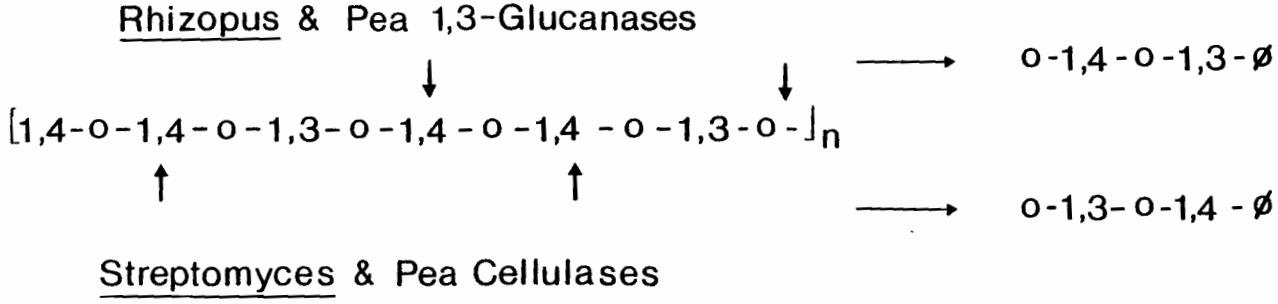
Action patterns of β-glucanases on various substrates. Symbols used are: o - glucosyl unit; ⌀ - reducing glucosyl unit; ⌀ - reduced glucosyl unit; - ⇓ preferential hydrolytic attack; ↓ - slower or later hydrolytic attack.

SUBSTRATES **PRODUCTS**

LAMINARIN & [^{3}H] LAMINARIN **[^{3}H] LAMINARIDEXTRINS**

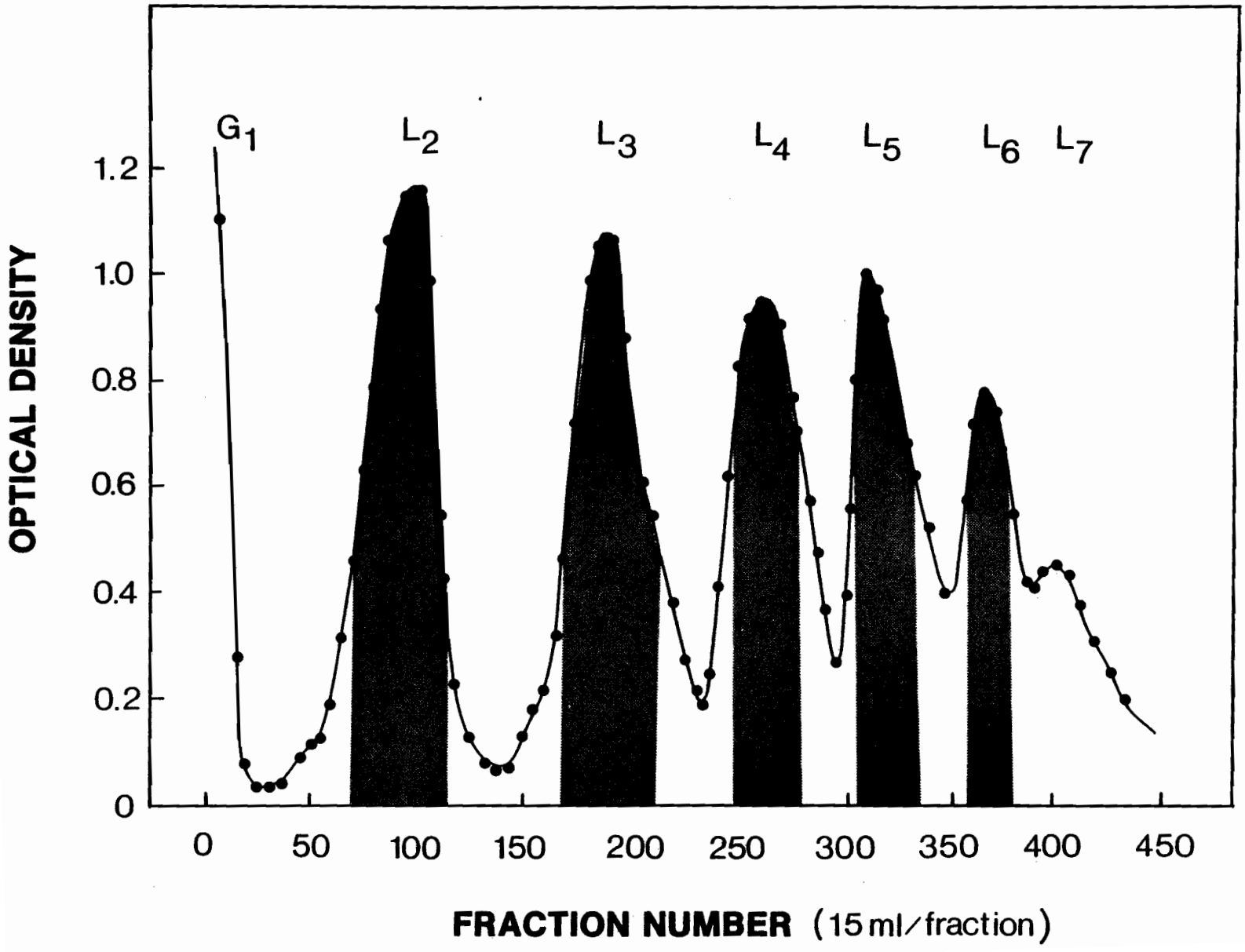


BARLEY β-GLUCAN (1,4- : 1,3- ≡ 2:1) **TRISACCHARIDE**



APPENDIX IIA

Chromatographic profile on a charcoal/celite column of laminaridextrins from partially hydrolysed laminarin. Laminarin (6g) was dispersed in 50 ml of ice-fuming HCl (S.C. 1.21) for 15 min at room temperature. The heavy, brownish liquid was poured into ice-cold distilled water (350 ml) followed by addition of sodium bicarbonate (approx. 115 g) to neutralize the hydrolysate. After leaving at 4°C overnight, the acid hydrolysate was centrifuged and the supernatant concentrated to 200 ml with a rotary evaporator at vacuo. Crystallized salt was removed by filtration and the filtrate was applied to a charcoal/celite column (65 x 4 cm I.D.) which was prepared as described earlier (11, 12). The column was eluted with a linear ethanol gradient (0 - 60%, v/v) at a flow rate of 2 ml/min. Fractions (15 ml) were collected and carbohydrate was detected by the phenol-H_2SO_4 method (20). Major components were combined, as indicated by shaded areas. Note that curves are remarkably smooth with little indication of shoulders, which would occur if there were any significant contamination by oligosaccharides with linkages differing from the backbone 1,3-glucan.


G1
L2
L3
L4
L5
L6
L7
OPTICAL DENSITY
1.2
1.0
0.8
0.6
0.4
0.2
0
0
50
100
150
200
250
300
350
400
450
FRACTION NUMBER (15 ml/fraction)

APPENDIX IIB

YIELDS OF LAMINARIDEXTRINS FROM A HYDROLYSATE CHROMATOGRAPHED ON A CHARCOAL/CELITE COLUMN*

Fraction	D.P.	Weight	Yield
		(mg)	(%, w/w)
Laminaribiose	2	240	4.00
Laminaritriose	3	255	4.25
Laminaritetraose	4	180	3.00
Laminaripentaose	5	205	3.41
Laminarihexaose	6	140	2.33
Total laminaridextrins		1020	17.00

*See Appendix IIA for fractionation profile of laminaridextrins.

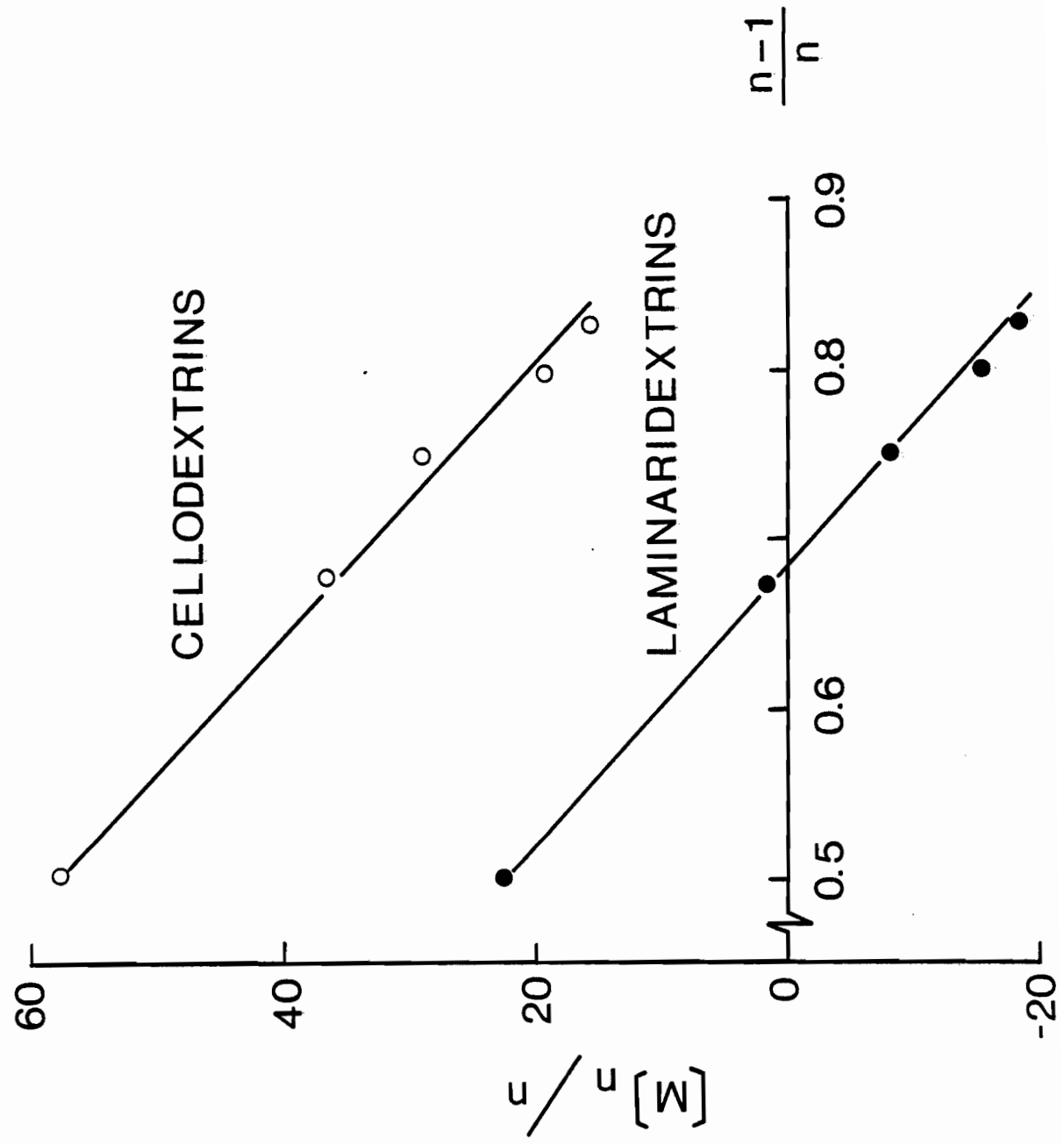
APPENDIX IIC

Paper chromatography of laminaridextrins. Laminaridextrins derived from the charcoal/celite column (Appendix IIA) and authentic standard samples kindly provided by Prof. B. Stone were chromatographed in n-propanol-ethyl acetate-water (6:1:3, v/v/v) for 26 hr on Whatman No. 1 filter paper (20 x 45 cm), and reducing sugars were detected with alkali-$AgNO_3$. Note the absence of any detectable component with R_f value different from authentic laminaridextrins. OR, origin.


G1 to L5
G1 to L6
L2
L3
L4
L5
L6
Authentic
Presently prepared from laminarin

APPENDIX IID

Freudenberg plots of prepared laminaridextrins (Appendix IIA) and cellodextrins (12). Dextrins were dissolved in distilled water (10 mg/ml) and equilibrated for 24 hr at room temperature to ensure that mutarotation was complete. Solutions were clarified by filtration (pore size 0.2 μm, Millipore) and optical rotation was measured at 25°C in a polarimeter (Perkin-Elmer model 141). Calculations for molar rotation ($[M]_n$) were described earlier (12), and n is the D.P. of the dextrins. The Freudenberg relationship (12) states that a homologous series of oligosaccharides should give a straight-line relationship between $[M]_n/n$ and $(n-1)/n$. Values for cellodextrins obtained from previous (12) studies are graphed for comparison. Note that the slopes for the two series are identical but absolute values for relative rotation differ. Also, points are very close to the straight line, indicating very little contamination from components with linkages different from the backbone 1,3-β-glucan.


CELLODEXTRINS
LAMINARIDEXTRINS
[M]n / n
60
40
20
0
-20
0.5
0.6
0.8
0.9
n-1/n

APPENDIX IIE

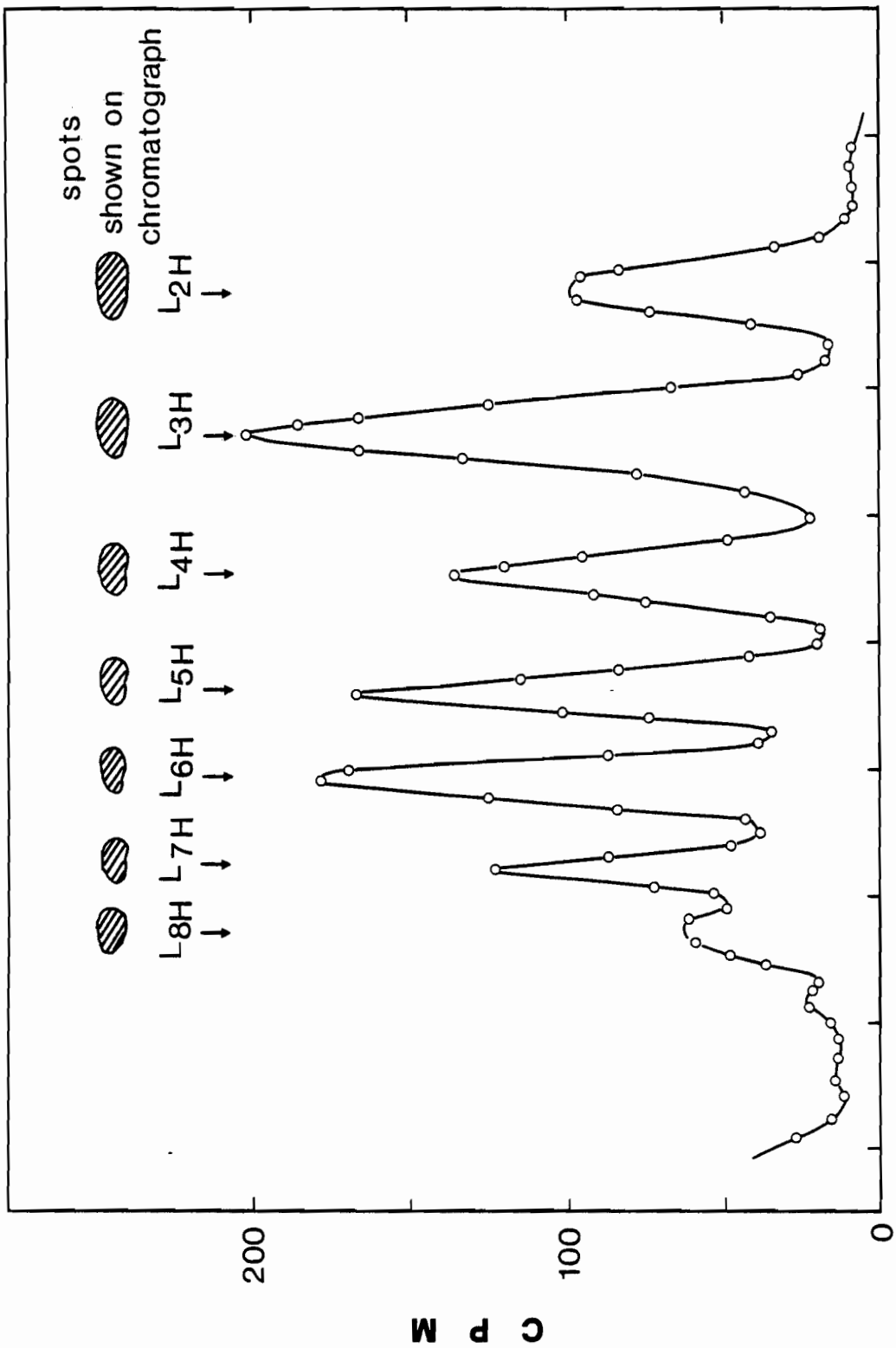
REDUCTION OF LAMINARIN AND LAMINARIDEXTRINS

Laminarin (D.P. 20) and each of the laminaridextrins (D.P. 2 - 6) prepared as in Appendix IIA (1 ml, 10 mg/ml) were added to 0.2 ml of $[^3H]NaBH_4$ (0.7 M, 0.338 mCi/mmol) and heated at 100°C for 15-20 min in sealed tubes. After cooling, glacial acetic acid ~~were~~ was added dropwise until no further hydrogen gas bubbles were released. The solutions were desalted with a bicarbonate form of mixed-bed resin (16) followed by filtration with millipores (0.2 μm). The filtrates were finally lyophilized and resuspended in known amounts of buffer to determine the specific radioactivities of the oligosaccharides.

Samples	Volume	Weight	Conc.	Radioactivity		Specific activity			
	ml	mg	μmol	cpm $x10^{-6}$	dpm $x10^{-6}$	μCi	μCi/ml	μCi/mg	μCi/μmol
L_{2H}	3.5	11.0	32.3	66.2	73.6	33.2	9.5	3.02	1.03
L_{3H}	1.5	10.0	19.9	29.2	33.2	15.0	10.0	1.50	0.76
L_{4H}	1.5	11.3	16.9	26.8	29.7	13.4	8.9	1.19	0.79
L_{5H}	1.5	11.1	13.4	31.0	34.4	15.5	10.3	1.40	1.16
L_{6H}	1.5	10.0	10.0	25.0	27.8	12.5	8.3	1.25	1.25
Reduced Laminarin	1.5	10.8	3.1	11.4	12.7	5.7	3.8	0.53	1.84

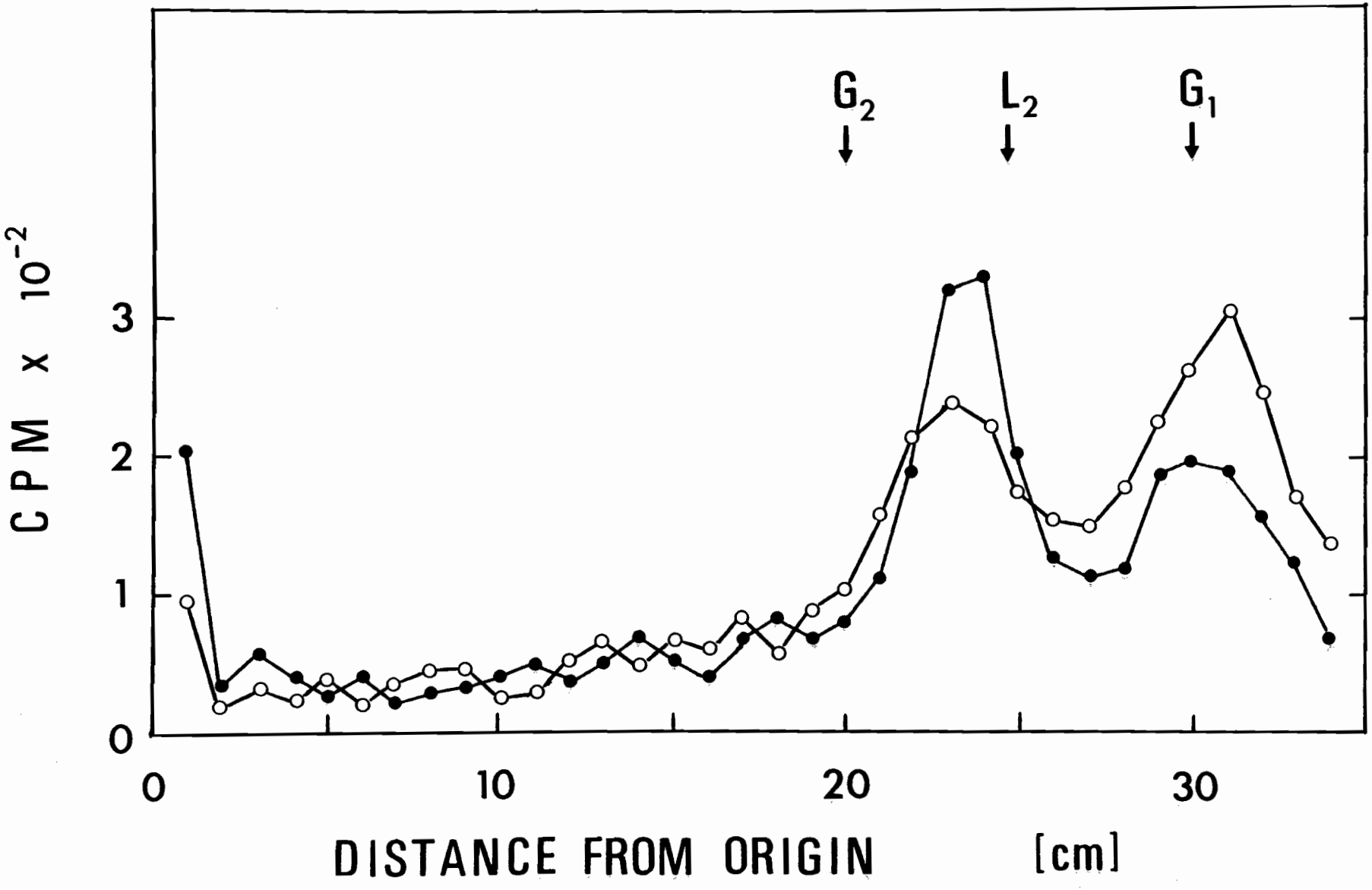
APPENDIX IIF

Paper chromatography of reduced [^{3}H] laminaridextrins (Laminaribiitol to laminariheptaitol). Aliquots (5 µl) of reduced [^{3}H] laminaridextrins prepared as described in Appendix IIE were spotted on Whatman No. 1 paper and developed in n-propanol-ethyl acetate-water (6:1:3, v/v/v) for 26 hr. The chromatograph was sprayed with periodocuprate (19) to locate the reduced oligosaccharides (spots shown on the top of the figure) and then sectioned (0.5 cm/section) for estimation of [^{3}H] in Aquasol.


spots
shown on
chromatograph
L2H
L3H
L4H
L5H
L6H
L7H
L8H
C P M
0
100
200
0
20
40
60
80
DISTANCE FROM ORIGIN (cm)

APPENDIX IIG

Action of purified 1,3-β-D-glucanases on pea β-glucan synthetase products. Pea epicotyl tissue slices were supplied with [^{14}C] UDP-glucose (5 mM) as substrate and alkali-insoluble material was isolated from reaction mixtures described by Raymond (4). Methylation analysis of these products show that they are β-glucans containing mostly 1,3-β-linkages (4). Purified glucanases I or II (0.1 ml, 0.5 μmol) were incubated with these products (approx. 17 000 cpm) at 35°C for 24 hr. Approximately 35% of radioactivity was solubilized, and the supernatant were then concentrated and applied to paper chromatographs. The chromatographs were developed, sectioned and counted as described in Appendix IIF. o——o, glucanase I; •——•, glucanase II.


G2
L2
G1
CPM x 10-2
3
2
1
0
0
10
20
30
DISTANCE FROM ORIGIN
[cm]

Chapter III

DEVELOPMENT OF 1,3-β-D-GLUCANASES AND DISTRIBUTION OF THE ENDOGENOUS SUBSTRATES IN *PISUM SATIVUM* SEEDLINGS

ABSTRACT

Two endo-1,3-β-D-glucanases (I and II, E.C. 3.2.1.6) are present in peas at opposite ends of the stem. Glucanase I from subapical regions degrades substrates to a series of low-molecular-weight dextrins, and is readily assayed reductometrically (e.g., as laminarinase). Glucanase II from basal regions preferentially hydrolyses internal linkages of long chains, and is therefore most sensitively assayed viscometrically (e.g. as carboxymethyl-pachymanase). Glucanase II but not I increases greatly in activity near the apex in response to treatment of the tissue with auxin. Ethylene gas suppresses endogenous activities and the auxin response. Natural substrates for both enzymes occur primarily in tissue fractions soluble in hot water. Substrates for glucanase I are concentrated in apical regions and those for glucanase II in basal regions, implying that enzymes and substrates are normally in separate compartments. The substrates are present exclusively in cell walls, since sections stained with aniline blue show enhanced fluorescence in walls only, and most of this can be removed either by hot water or the appropriate purified β-glucanase. The β-glucanase activities are recovered from homogenates mainly in supernatants, and presumably derive from intracellular compartments, e.g. vacuoles. The small fraction which is particulate (mainly glucanase II) is associated with the endomembrane system. It is suggested that glucanase II is the more likely candidate for an enzyme which is regulated and functions in modifying cell walls during and after growth, but both glucanases may play roles in helping to facilitate translocation.

INTRODUCTION

Two endo-1,3-β-D-glucanases (I and II, EC 3.2.1.6) with physically and enzymically distinct properties have been purified and characterized from pea stems (1-3). Glucanase I, mol wt 23 000, appears to be concentrated in subapical regions of the stem and is most readily assayed as laminarinase, while glucanase II, mol wt 37 000, is found in basal mature tissues and is particularly effective in hydrolysing O-carboxymethylpachyman (CM-pachyman). Both enzymes degrade a variety of 1,3-β-D-glucans and their derivatives, as well as mixed-linkage β-glucans e.g., barley β-glucan. No major distinctions were detected between them in substrate specificity, though they are susceptible to different non-competitive inhibitors, and glucanase I is more active per mole than II. Their action patterns differ in several ways (2,4): glucanase I appears to act via "multiple attack" in which long chains are repeatedly degraded to a collection of dextrins with low D.P., even while other long chains predominate in the reaction mixture, but glucanase II acts via "multi-chain attack" to preferentially cleave long chains before hydrolysing fragments to lower dextrins. The main question addressed by the studies reported here is what functions these two 1,3-β-D-glucanases may have in pea seedlings?

The documentation collected to date (2) on substrate specificity, action patterns, and inhibition do not show such major differences between the two pea 1,3-β-D-glucanases as to suggest distinctive functions for them. The most striking difference is the preliminary observation that they appear to be concentrated at opposite ends of the pea stem. With this as a starting point, the question of function is approached here by examining a) details of the distribution of activities between different stem tissues and subcellular

fractions, b) effects of hormone treatments on β-glucanase levels, c) susceptibilities of endogenous polysaccharide fractions to degradation by the two purified enzymes, and d) distribution of the susceptible polysaccharides in the stem.

Little is known with assurance about the functions of other 1,3-β-D-glucanases in plants, and only a few have been purified to the degree of homogeneity needed to establish modes of action and substrate affinities (5-11). Their tissue and subcellular distribution is also uncertain. They are usually assumed to be secreted enzymes, since putative substrates, e.g., callose, are thought to be deposited extracellularly (12-13). It is also possible that such enzymes are sequestered in vacuoles (14) and mobilized by unknown mechanisms in response to a need to combat invasive organisms (15-17) or to degrade wall or storage glucans during development (18). Potential roles in wall-softening and growth-regulation have been postulated (18-21). 1,3-β-D-Glucanases have also been proposed as agents which increase translocation by dissolving blockage glucans (22), promote nutrition by hydrolysing reserve glucans (23), protect against fungal attack by destroying parasite glucans (elicitors) (17,24) and help in recovery from wound responses by dissolving deposits of wound glucan (22). In different tissues, the levels of 1,3-β-D-glucanases have been reported to increase in total, if not specific, activity in response to auxin (20,25,26), ethylene (27) or gibberellin (28). The spectrum of speculative functions, substrates and regulation of these enzymes is uncommonly wide, but nonetheless potentially important for plant physiology. Clearly, there is a need for more precise investigation into the molecular and cell biology of 1,3-β-D-glucanases in plants.

MATERIALS & METHODS

Plant Materials, Enzymes and Substrates. Seeds of Pisum sativum (L., var. Alaska), were grown in darkness at room temperature as described previously (29). When third internodes were about 3 cm long (7 - 8 days old), segments (10 mm) from sub-apical (third internode) and basal (first internode) tissues were harvested and extracted to yield crude preparations of glucanase I and II, respectively. These were purified, characterized and stored as described (1,30,31). Enzyme preparations were assayed reductometrically and viscometrically with laminarin and CM-pachyman, respectively (1,2). One unit of CM-pachymanase activity is defined as the amount of enzyme required to cause 1% loss in viscosity in 2 hr at 35°C.

Substrates used in this study include soluble laminarin (D.P. 20, Nutritional Biochemicals Corp.), and CM-pachyman which was prepared with a suitable degree of substitution (0.32) by Prof. B.A. Stone (32) and also in this laboratory using Stone's methods. [^{14}C]-labelled 1,3-β-D-glucan, extracted from Fucus embryos (33), was a gift of Dr. R.S. Quatrano (Dept. of Botany and Plant Pathol., Oregon State Univ., Corvallis). The sample was further purified to obtain a 10-fold increase in specific radioactivity (33). The final product (15 500 cpm/mg) was incubated at a conc. of 3.0 mg/ml with purified pea glucanases in assays conducted as for laminarinase.

Regulator Treatments and Enzyme Distribution. Intact six-day old seedlings were sprayed with 0.1% 2,4-dichlorophenoxyacetic acid (2,4-D, Nutritional Biochem. Corp.), 0.1% Tween 80, 0.1 M NaCl (pH adjusted to 7.0). Approximately 10 ml was used per 500 seedlings, after which they were grown in darkness for up to 5 days. 2,4-D prevented growth in length, but led to

markedly swollen sub-apical tissue (29,34). The 10 mm segment below the hooks was excised and used as source of crude 1,3-β-D-glucanase activities as described below.

In order to study the distribution of 1,3-β-D-glucanase activities in pea stems, intact pea seedlings ± 2,4-D-treatment were sectioned into 1 cm segments according to the diagram in Figure 3. The segments were surface-sterilized for 10 min in 0.25% sodium hypochlorite followed by thorough washing with distilled water. One hundred segments of each category were homogenized in 2 vol of 20 mM sodium acetate buffer (pH 5.5) containing 5% glycerol and 0.03% sodium azide. The homogenate was passed through nylon and the filtrate was centrifuged at 130 000 x g for 10 min. The supernatant was assayed reductometrically and viscometrically in order to obtain a rough estimate of glucanase I and II activities.

Ethylene gas also causes swelling in the same sub-apical pea tissue that responds to 2,4-D (29,34), although the mechanism of the effect is probably different because ethylene actually inhibits the increases in protein levels and cellulase activity evoked by auxin (34). Effects of ethylene (100 ppm) ± 2,4-D treatments on development of 1,3-β-D-glucanase activity were studied using seedlings exposed to these regulators for 6 to 8 days in darkness, as described earlier (29,34). Ethylene gas (Lectur Sphere, high purity grade) was obtained from Fisher Co., and seedlings were exposed to it in 6-litre chambers in a constant stream of air-ethylene.

Subcellular Distribution of 1,3-β-D-Glucanase Activities. Pea segments (10 mm) from apical and basal tissues were ground in 2 vol of ice-cold homogenizing buffer containing 5 mM Mg acetate, 20 mM sodium acetate, 20 mM KCl, 5 mM mercaptoethanol and 0.4 M sucrose, pH 5.5. The homogenate was

filtered through nylon cloth and centrifuged at 1000 x g for 10 min to remove wall debris. The supernatant was layered over a discontinuous sucrose gradient composed of 2 ml 70% (w/v) and 3 ml of 15% (w/v) sucrose and centrifuged at 200 000 x g for 4 hr (IEC SB, rotor No. 283). This resulted in a clear supernatant overlying the 15% sucrose layer, a membrane fraction at the 15/70% sucrose interface, and a translucent pellet (polysomal fraction). The supernatant was recovered and used as a source of soluble enzyme. The material collected at the interface was aspirated, washed once and centrifuged at 145,000 x g for 1 hr (IEC A160 rotor) to yield a fraction which is referred to as membrane-bound enzyme. The polysomal pellet contained little glucanase activity and it was included with membrane-bound enzyme.

Methods for membrane fractionation on linear sucrose gradients as described earlier (35,36) were slightly modified. Pea segments (10 mm) from apical and basal tissues were chopped with an array of razor blades (35) in one vol of ice-cold homogenizing buffer containing 0.1 M Tris-HCl, 0.1 M $MgCl_2$, 5 mM dithioerythritol (DTT) and 0.4 M sucrose, pH 7.25. The homogenate was filtered and centrifuged on a discontinuous sucrose gradient as described above. The interfacial material was applied on 15 ml of a linear sucrose gradient (25-60%, w/v) in homogenizing buffer and centrifuged at 140,000 x g for 2 hr (IEC A169). The fractions (0.75 ml) were collected (Gilson microfractionator, FC 80K) and activities were assayed reductometrically (1) and viscometrically (1). Densities were determined by refractometry and the protein level was estimated by the Lowry method (37).

Extraction and Hydrolysis of Endogenous Substrates. Potential endogenous substrates of 1,3-β-D-glucanase activities were prepared from apical and basal tissue segments by standard procedures of lipid, hot-water and alkali

extraction. Tissue segments (200) were homogenized (polytron) in 5 ml of 20 mM sodium acetate buffer (pH 5.5) for 5 min and the homogenate was washed twice with 20 ml of 80% ethanol (80°C). The preparations were centrifuged at 13,000 x g for 10 min and the alcoholic extract was discarded. The sediment was extracted twice with 20 ml distilled water at 85°C. The extract was boiled (15 min), centrifuged and filtered (millipore, 0.2 μ) to obtain high molecular-weight material in the water-soluble fraction. For some tests, the water-insoluble fraction was extracted twice with 20 ml of 1 M NaOH at 85°C. The alkali-soluble fraction retained relatively little carbohydrate (<10% of total insoluble) as assayed with the phenol-sulfuric acid method (38), and was discarded. The water-soluble, total insoluble and alkali-insoluble fractions were all extensively dialysed and lyophilized before tests of their susceptibility to enzymic degradation. These were conducted using approximately 2.0 mg total hexose equiv./ml from apical or basal segments suspended in buffer and incubated with purified glucanases under toluene at 35°C for 24 hr. Aliquots of reaction mixtures were withdrawn for estimation of reducing power (39) and total hexose equivalents (38) in order to determine the extent of hydrolysis. Fractionation was conducted on columns of BioGel P_2 (Bio Rad Corp.) by standard techniques.

Fluorescence Microscopy

Tissue segments were fixed on ice in 3% (w/v) glutaraldehyde in 25 mM sodium phosphate buffer, pH 6.8. Fixed segments were quick-frozen (Cryokwik, Damon/IEC, Needham Hts., Mass.) and sectioned with a razor blade (approx. 50μ). The sections were mounted on concave glass slides in 0.05% (w/v) water-soluble aniline blue (C.I. 42755, BDH Chem., Poole, England) in 0.15 M K_2HPO_4, pH 8.5, and directly examined under a microspectro-fluorescence

microscope (MPV-I, Leitz). Autofluorescence was assessed in sections mounted in this buffer at the same pH (40,41). Exciter filters BG-12, and barrier filters KP 500, TK510 and K515, were used in combination. Illumination was provided by a mercury vapour lamp (HBO-100 W2) connected to a direct-current transformer.

RESULTS

Properties of Purified 1,3-β-D-Glucanases. In earlier tests (1,2), no comparison was made between the relative rates with which pea glucanases generate reducing power and viscosity loss from a given substrate, e.g. CM-pachyman, though this is a classical method for estimating the degree to which a hydrolase cleaves linkages at random (30,42). The methods which were used to examine products generated from β-glucans depended on assays of carbohydrate or tritiated terminal groups, but did not make use of [^{14}C]-labelled substrate, which is probably the most sensitive technique (30). In the following results, data are recorded for the action of the two purified pea 1,3-β-D-glucanases on CM-pachyman and on uniformly [^{14}C]-labelled laminarin purified from Fucus after photosynthesis in $^{14}CO_2$ (33).

Figure 1 shows that glucanase I and II both lower the viscosity of solutions of CM-pachyman rapidly to values that approach the fluidity of buffer, but they generate reducing power from this substrate at a relatively slow and linear rate, properties which are characteristic of endo-hydrolytic attack. Free reducing groups continue to be generated after viscosity losses are complete, and hydrolysis ceases only after very long periods (days) of incubation under these conditions. In plots of the fluidity of CM-pachyman

solutions vs. the percentage of hydrolysis, glucanase I shows a ratio of fluidity increase per reducing group generated (slope) which is only about 1/20 of that of glucanase II. Evidently, glucanase II is some 20-fold more "random" than I in the manner in which it hydrolyses internal linkages of this substrate (42,43).

With Fucus [^{14}C]-1,3-β-D-glucan as substrate (Fig. 2), when reactions are interrupted before completion, i.e., after little of the original substrate remains in the reaction mixtures, an unbroken series of laminaridextrins are still present, with no gross assymetry apparent in the accumulation of any one product. However, glucanase I generates relatively more of the dextrins with low D.P. (<5), and glucanase II shows the reverse profile. Clearly, both enzymes are endo-hydrolases, but I cleaves products to form lower dextrins more rapidly than II.

Distribution of Enzymes. Segments were excised from different regions of pea stems and crude extracts were assayed for protein, fresh weight, laminarinase (reductometric) and CM-pachymanase (viscometric) activities. As shown in Figure 3A, the endogenous laminarinase activity is highest on a per segment (or fr. wt. or protein) basis in apical regions just below the region of most active elongation. This activity declines gradually during further maturation in lower internodes. CM-pachymanase activity is distributed in a reverse pattern, with little or none present near the apex, and a maximum close to the cotyledons. Such a localization has not been observed for any other pea hydrolase (25,44). Levels of both enzymes are relatively low in the plumule and hook (regions of potential growth and cell division), and in the root.

Since most of the reductometric and viscometric activities as assayed here are due to glucanase I and II, respectively (1,2), the true levels of each enzyme resemble the profiles for crude extracts, but with an even more pronounced assymetric distribution. These can be readily calculated from values given earlier (1,2) for reducing power generated per unit viscosity loss for the two purified enzymes. It is also possible to assay for glucanase I in a mixture of I and II by adding high concentrations of a specific non-competitive inhibitor of II, e.g., succinic anhydride, and glucanase II can be assayed in a crude extract in the presence of N-bromosuccinimide, which specifically inhibits glucanase I (2). When such an experiment is performed, the results (Table I) confirm that there is little glucanase I in basal extracts or glucanase II in apical extracts, i.e., the two enzymes are developed separately.

Table II shows the subcellular distribution of 1,3-β-D-glucanase activities between the wall, membrane-bound particulate fraction and supernatant fractions obtained after differential centrifugation. CM-pachymanase activity in basal segments is almost entirely (95%) soluble, and most (70%) of laminarinase in apical segents is also soluble. That part which is associated with membranes separates upon centrifugation in a linear sucrose density gradient and concentrates mainly at a density of 1.11 g/cc, with smaller amounts at 1.12 and 1.15 g/cc (Fig. 4). These loci correspond to those of endoplasmic reticulum (35,36), plasma membrane (45) and Golgi (35,46), respectively. As components of the endomembrane system involved in secretion and sequestration (47), these organelles may be transporting 1,3-β-glucanase to sites of action (e.g., wall) or storage (e.g., vacuole). The small part of activity found at very heavy densities (1.2 g/cc) may be associated with residual plasma membrane: wall material.

Development of 1,3-β-D-Glucanase Activities. Intact pea seedlings were sprayed with 0.1% 2,4-D (29,34) and extracts of the apical growing region, which swells in response to auxin (34,35), were assayed for β-glucanase activity at daily intervals up to 5 days. As shown in Figure 5, after a 2-day lag period a dramatic increase occurs in CM-pachymanase (glucanase II) specific activity as a result of 2,4-D treatment. In contrast, there was no significant increase in laminarinase (glucanase I) specific activity at any time. CM-pachymanase activity was enhanced by 2,4-D treatment in apical parts of the seedling only, while laminarinase activity did not increase significantly over controls in any part of the stem (Fig. 3B). It appears that glucanase II is susceptible to regulation by auxin in growing regions, while glucanase I levels do not change even during massive growth responses.

Ethylene gas also evokes swelling of the growing region in intact peas but, as shown in Table III, the response is not as great as that which follows 2,4-D treatment. Ethylene treatment brings about a decline in both 1,3-β-D-glucanase activities, and it interferes in the 2,4-D-evoked development of CM-pachymanase. Although 2,4-D treatment may result in endogenous ethylene formation in peas (48), ethylene can not be the mediator through which 2,4-D enhances CM-pachymanase activity. Other hormones which cause swelling (e.g., cytokinin) or elongation (gibberellin) in pea tissue were not found to affect the levels of either glucanase. Nor was any change detected in glucanase activity when tissue was increasingly "wounded" (sliced to various degrees, for various periods) before extraction and estimation (data not shown here).

Distribution and Properties of Endogenous Substrates. Water-soluble, water-insoluble and alkali-insoluble fractions of apical and basal stem sections were prepared, dialysed and incubated with the purified 1,3-β-D-glucanases. As shown in Table IV, glucanase I degrades water soluble fractions from apical tissue more rapidly than similar fractions from basal tissue, and glucanase II shows the reverse pattern. Quantitatively, the water-soluble material contains most of the readily degraded endogenous substrate. The distribution along the stem of water-soluble substrates which are degraded by the two β-glucanases (Fig. 6) is parallel to the distribution of the endogenous β-glucanase activities themselves (cf. Fig. 2). Presumably the enzymes and their substrates coexist in vivo in separate cellular compartments.

When purified β-glucanase I and II are incubated with water-soluble fractions extracted from apical and basal tissues, respectively, and the hydrolysates are fractionated on Bio-Gel P-2 columns, both glucanases generate oligosaccharides with K_{av} values corresponding to a series of standard dextrins (Fig. 7). These are formed at the expense of high-molecular-weight polysaccharide (in the void volume), indicating a typical endo-hydrolytic pattern of enzyme action. As with the profiles generated from Fucus 1,3-β-D-glucan (Fig. 3) and laminarin (2), those from endogenous glucan show relatively more of the lower laminaridextrins in the presence of glucanase I than II. Even after prolonged incubation, there is always some carbohydrate remaining in the void volume, which probably represents residual polysaccharide which is not β-glucan.

In an effort to localize the substrate for these β-glucanases more precisely, tests were conducted on the ability of the purified enzymes to remove the components in pea tissue sections which fluoresce in the presence

of aniline blue. These components have been reported to be concentrated in cell walls of sieve-tube plates, plasmodesmata and new cell walls, and they are believed to indicate the presence of β-glucan containing 1,3-linkages (12,22). In the pea stem, endogenous fluorescence can be detected particularly in vascular tissue, but the intensity is weak and the color (yellowish green) is different from the strong green evoked by aniline blue under present conditions. Figure 8A shows that dense regions of fluorescence in the presence of soluble aniline blue are concentrated in the stele in apical parts of the stem. Fluorescence is particularly intense in cell walls and, within them, in localized corners (arrows), i.e., as might be expected from plasmodesmata or intercellular cements. However, when sections of apical tissue are pre-incubated with purified glucanase I (0.5 ml, 0.4 μmol) at 35°C, pH 5.5, for 12 hr before aniline blue treatment, most of the fluorescent material is removed (Fig. 8B). With sections of basal tissue, the prominent aniline-blue-induced fluorescence is observed in cell walls of cortical parenchyma cells. It is particularly marked opposite intercellular spaces (cell corners) on the outer sides of the walls of adjoining cells (arrows, Fig. 8C), and at localized regions of cell-wall junctures (arrows, Fig. 8D). All of these intensely fluorescent regions are eliminated when fresh sections are pre-treated with purified glucanase II before aniline blue staining, though a residual fluorescence remains visible in middle lamellae of the walls (Fig. 8E).

Extraction of apical or basal pea tissues with boiling water before staining also eliminates most of the aniline-blue-specific fluorescence from the stele (apical) or parenchyma cell walls (basal). Extracted sections look very much like those treated with β-glucanases (Fig. 8). Thus, the aniline blue-fluorescent material in these walls is probably equivalent to the

water-soluble substrates of β-glucanase observed biochemically (Table IV). The results indicate that the aniline-blue fluorescence technique is legitimate as a method for visualizing substrates of1,3-β-D-glucanases in these tissues.

DISCUSSION

The question with which this study began can not be answered conclusively as yet, but the results indicate that some of the functions that have been suggested for 1,3-β-D-glucanases are probably not applicable to the pea enzymes, and they help to clarify directions which further research on their roles might profitably pursue. It is most unlikely that the pea glucanases play any role in nutrition, because the proportion of total polysaccharide in pea stem which is susceptible to hydrolysis by them is very small (< 5%, Table IV). Glucanase I may be concerned with maintaining a vertical flow of translocates, in apical tissues, since its substrate is concentrated in vascular regions (Figs. 6 and 8) where it may block intracellular canals (22). This may not be true of glucanase II in basal tissues, however, since most of its substrate is concentrated in cortical parenchymal walls (Fig. 8). In view of evidence here and elsewhere (12,13) for a "callose-like" material in pea plasmodesmata, it may be that both β-glucanases play a role in maintaining potential routes for lateral transport. Clearly, this would require that the enzymes be secreted at selected sites into the wall, and that their intracellular distribution be subject to close regulation.

Most of the pea β-glucanase activity is soluble (Table II) and probably derives from a pool sequestered in vacuoles (14,46) or another compartment

which separates it from the wall. Whether pea cells have mechanisms for relocating such stored glucanase into the wall is unknown. However there is some indirect evidence in the present study to suggest that at least glucanase II may be subject to turnover and secretion. The fraction of glucanase II activity which is associated with particulate material appears to be present mainly in organelles of the endomembrane system which are known to function in enzyme transport (Fig. 4). Glucanase II activity is greatly increased in apical regions in response to auxin treatment (Figs. 3 and 5). In contrast, glucanase I, which is already concentrated in apical regions (Fig. 3), is almost entirely soluble and shows no great fluctuations in level in response to treatments which greatly alter cell dimensions and protein levels (e.g. Table III). Thus, if either of these enzymes is to be further investigated with respect to a possible function in growth and development, paradoxically, it is not likely to be the apparently constitutive and unresponsive glucanase I, despite its suggestive location. Glucanase II is the more likely candidate for an enzyme which is generated and mobilized to perform some essential role in expanding cells, especially since specific substrate for this enzyme appears to be present in the walls of such cells.

It should also be noted that the lowest concentrations of both β-glucanases are found in the hook region of apical tissue (Fig. 3), which may indicate that β-glucan substrates in that region are particularly useful and conserved until tissues develop further. Cell division, in the pea stem occurs in the hook region, and it has been proposed on the basis of histological observations that 1,3-β-D-glucans are deposited in cell plates as an initial foundation for the orderly development of new walls (12). Later, during growth, when primary walls are well established, this callose-like material often disappears, due presumably to activity of

newly-developed or re-located 1,3-β-D-glucanases.

The differences observed to date between the two pea β-glucanases in physical (1) and enzymic (2) properties do not offer any obvious clue as to what differences may exist in their specific functions. Both are endo-glucanases (EC 3.2.1.6) and no qualitative differences were found (2) between their substrate specificities. As observed here (Figs. 1 and 2), glucanase II shows a greater degree of randomness in its action pattern and glucanase I shows a greater capacity for degrading substrates to low-D.P. dextrins. In time, both enzymes generate essentially the same spectrum of hydrolysis products from a variety of substrates, including laminarins and mixed-linkage β-glucan (2). Nevertheless, it is clear that natural substrates exist in the pea stem which have different distributions and are differentially susceptible to degradation by the two β-glucanases. Studies are in progress to define the structures of these substrates, and to compare them to products generated by pea β-glucan synthetase activity which are known to acquire 1,3-β-linkages under certain conditions (49). The aim of these studies is not only to clarify the functions of 1,3-β-glucans in young tissues and, by interpolation, those of their respective hydrolases, but also to relate these to the manner in which the deposition of β-glucans is controlled.

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Table I. Effects of Specific Inhibitors of Glucanase I and II on Laminarinase and CM-Pachymanase Activities of Crude Extracts

Reaction mixtures contained soluble extracts (0.1 ml) and laminarin (0.8 ml, 0.5%) ± N-bromosuccinimide (5 mM) or CM-pachyman (0.8 ml, 0.8%) ± succinic anhydride (10 mM). Assays were conducted as described in Materials and Methods and values are averages of quadruplicate estimates ± S.E.

Assay	Apical	Basal
Laminarinase (μg glc equiv/mg protein/2 hr)	720 ± 45	210 ± 40
+ N-bromosuccinimide	230 ± 34	193 ± 18
CM-Pachymanase (units/mg protein)	460 ± 25	4350 ± 550
+ succinic anhydride	478 ± 65	870 ± 93

Table II. Subcellular Distribution of 1,3-β-D-Glucanase Activities.

Extracts of tissue segments were fractionated by standard methods (35) and fractions were assayed for protein (37) and laminarinase (apex) or CM-pachymanase (base) activities as described in Materials and Methods.

Fraction	Apical		Basal	
	Protein	Laminarinase	Protein	CM-pachymanase
	(μg/seg)	(μg glc/seg/2 hr)	(μg/seg)	(units/seg)
Total homogenate	134	312	60	365
		Distribution (%)		
Supernatant	63	69	74	95
Particulate	25	18	5	1
Wall	12	13	21	4

Table III. Auxin and Ethylene Effects on Development of 1,3-β-D-Glucanase Activities

Intact plants were sprayed once with 0.1% 2,4-D and grown in air or in 100 ppm ethylene for 3 days. Measurements were on the apical 1 cm of stem below the hook.

Treatment	Fr Wt / Length	Protein	Laminarinase	CM-Pachymanase
	(mg/mm)	(μg/seg)	(μg Glc/mg prot)	(units/mg prot)
Zero, Control	2.2	65	890	62
3 Days, Control	2.7	74	700	77
, + Ethylene	5.9	69	120	33
, + 2,4-D	11.5	131	540	2210
, + Both	13.5	117	440	1010

Table IV. Distribution of Endogenous Substrates of 1,3-β-D-Glucanase

Purified glucanase I and II (0.05 ml, 0.5 μmol) were incubated at 35°C with various pea fractions (200 μg carbohydrate suspended in 0.2 ml buffer, pH 5.5), extracted from apical or basal tissue as described in Materials and Methods. The amount of reducing power generated in 12 hr was determined with the Somogyi reagent (39), and percentage of hydrolysis was obtained by determining total initial carbohydrate (38). Values are averages for quadruplicate determinations (average S.E. = 4-10% of values given).

Fraction	Polysaccharide content	Glucanase I	Glucanase II
	(mg glc equiv/ 50 seg)	(% hydrolysis to reducing equivalents)	
Water-soluble			
Apical	2.25	30.4	15.2
Basal	2.82	12.2	44.8
Total water-insoluble			
Apical	12.42	1.4	1.8
Basal	18.75	0.8	1.2
Alkali-insoluble			
Apical	9.76	0.2	0.1
Basal	13.42	0.1	0.3

Fig. 1. CM-Pachymanase activity of pea 1,3-β-D-glucanases. Purified glucanase I or II (0.1 ml, 0.2 μmol) was added to CM-pachyman (0.9 ml, 0.8%, w/v) and incubated at 35°C, pH 5.5, in Cannon-Manning semimicroviscometers. Initial η_{sp} = 4.65. Aliquots were removed at intervals for measurements of reducing power (1,2) and viscosity loss (1,2). Glucanase I, o———o; Glucanase II, •———•.

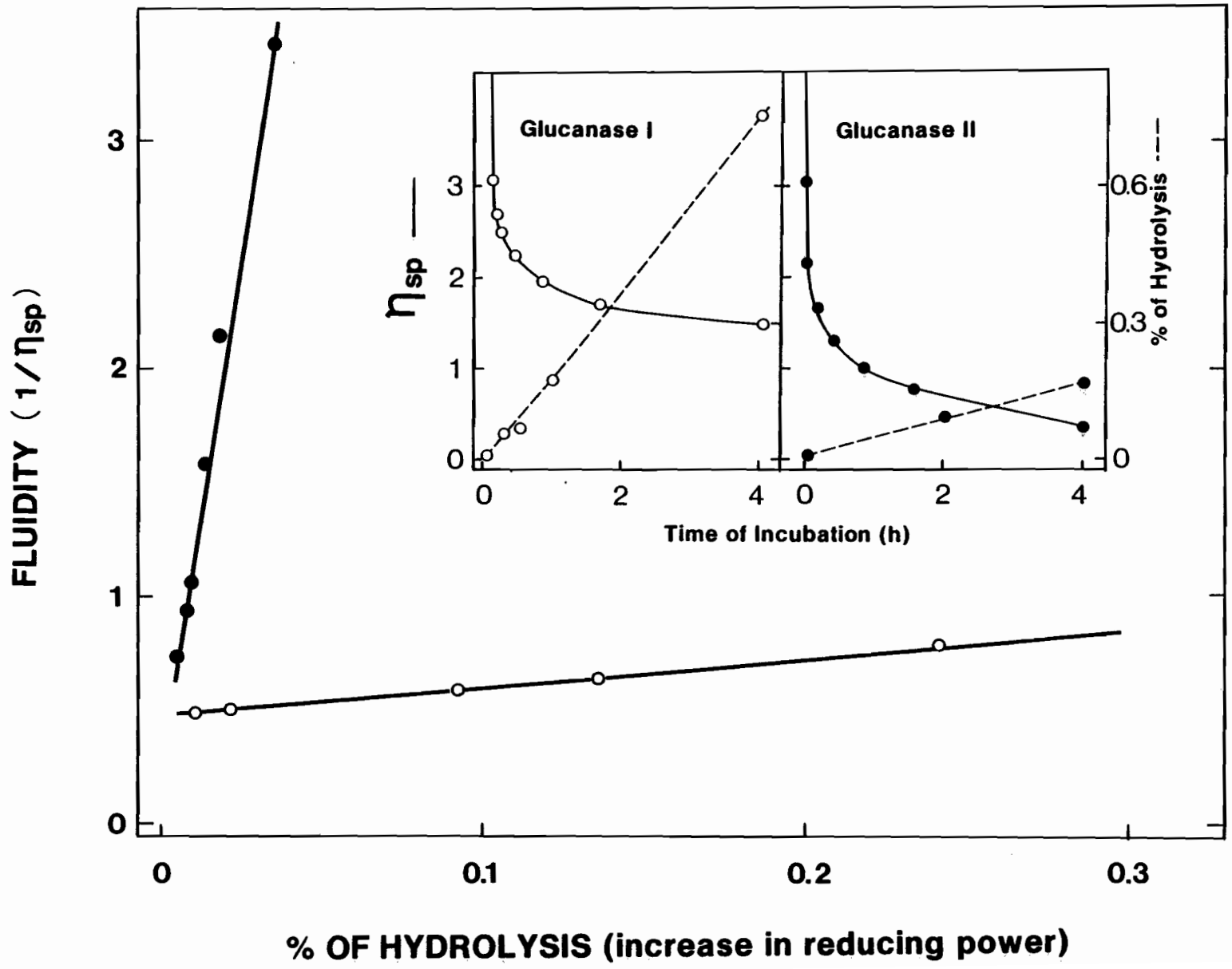

FLUIDITY (1/ηsp)
% OF HYDROLYSIS (increase in reducing power)
Glucanase I
Glucanase II
ηsp
% of Hydrolysis
Time of Incubation (h)

Fig. 2. Degradation profiles of products generated from Fucus [^{14}C]-1,3-β-D-glucan. Purified glucanase I and II (0.05 ml, 0.5 μmol) was incubated with purified [^{14}C]glucan (0.45 ml, 3 mg/ml, w/v, spec. act. = 0.03 μCi/μmol) at 35°C, pH 5.5, for 4 hr. The hydrolysates were lyophilized, chromatographed on paper (Whatman No. 1), and sections of paper (1 cm) were assayed for radioactivity in Aquasol (30,31). Co-chromatographed laminaridextrin standards (L_2 - L_6) and glucose were located with a $AgNO_3$-alkali reagent. Glucanase I, o——o; Glucanase II, •——•.

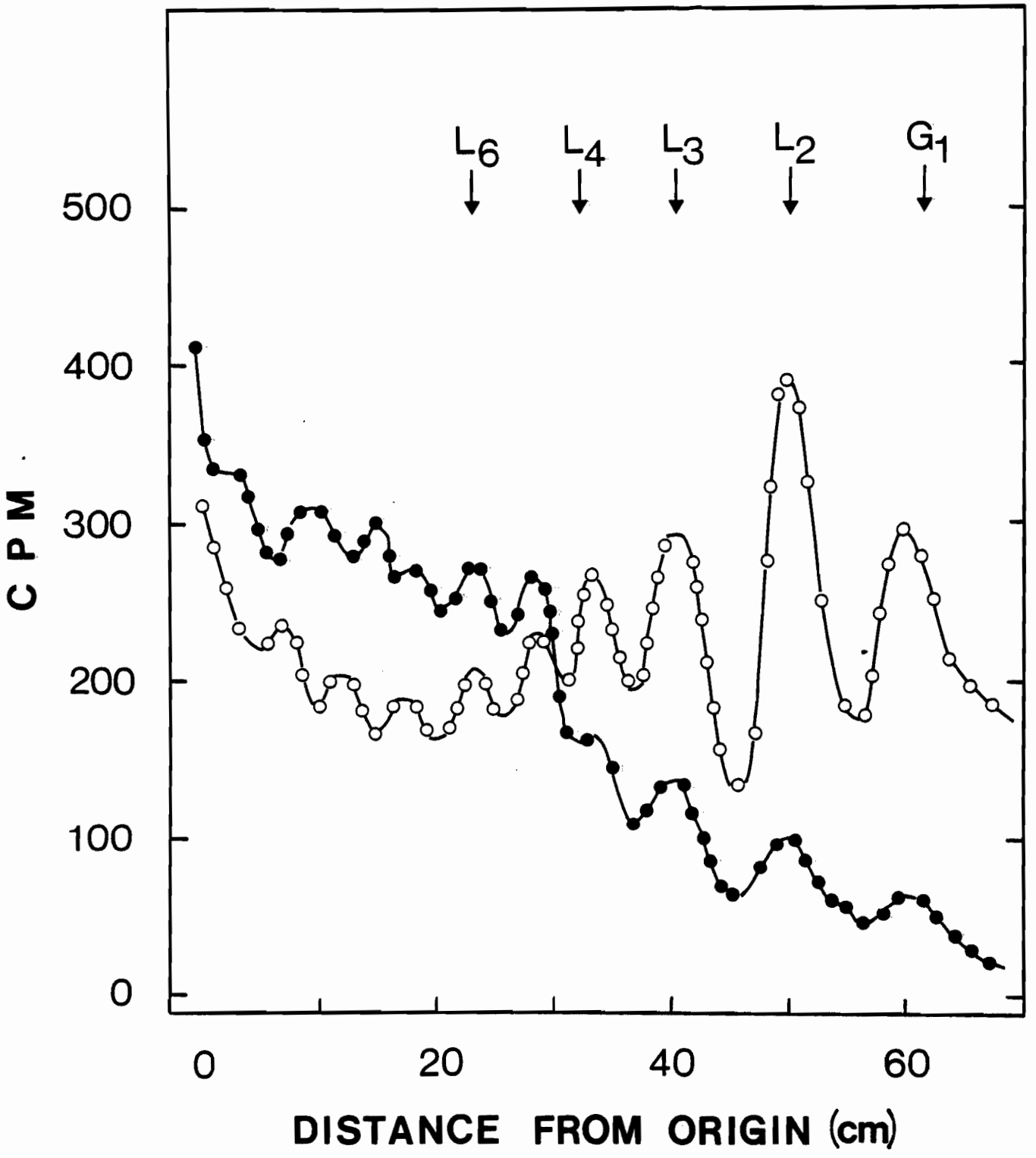

L6
L4
L3
L2
G1
500
400
300
200
100
0
CPM
0
20
40
60
DISTANCE FROM ORIGIN (cm)

Fig. 3. Distribution in etiolated pea stems of laminarinase and CM-pachymanase activities. Segments (1 cm) were extracted and assayed reductometrically (o———o) and viscometrically (●———●) as described in Materials and Methods. The same assays were repeated three days later after spraying with 2,4-D (□———□ and ■———■, resp.). Soluble protein levels decreased from the plumule and hook (235 μg/seg) to the elongating region (segment No. 1, 62 μg/seg) and successively in more basal non-growing regions, including the nodes (e.g., N_1 = 32 μg/seg) and internodal regions (e.g., I_1 = 27 μg/seg).

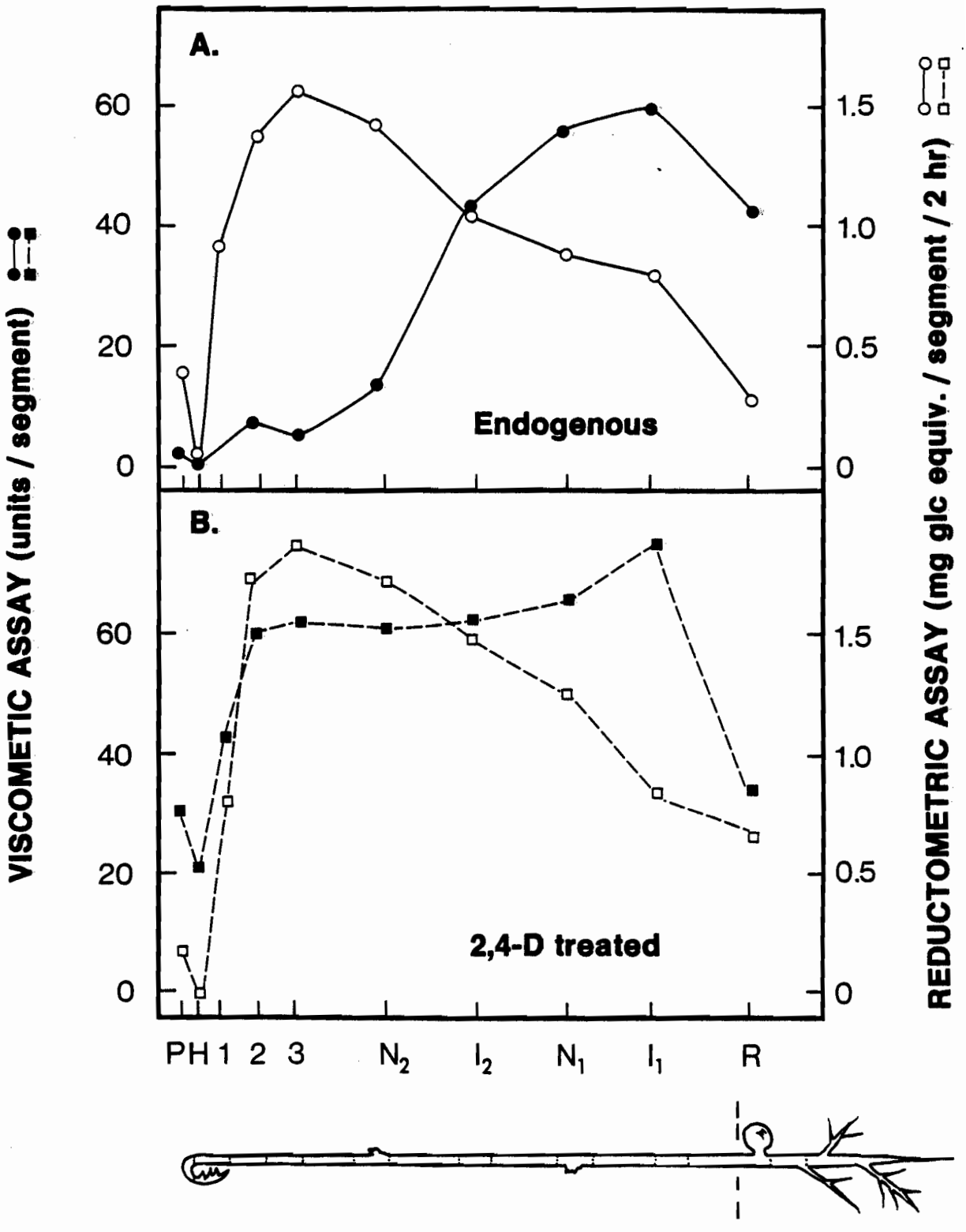

A.
B.
Endogenous
2,4-D treated
VISCOMETIC ASSAY (units / segment)
REDUCTOMETRIC ASSAY (mg glc equiv. / segment / 2 hr)
0
20
40
60
0
0.5
1.0
1.5
PH 1 2 3 N2 I2 N1 I1 R

Fig. 4. Distribution of 1,3-β-D-glucanase activities in linear sucrose gradients after isopycnic centrifugtion of particulate preparations. Membranes prepared by velocity centrifugation (200 000 x g) from chopped pea apical and basal tissue were separated on linear sucrose gradients (25-60%, w/v) for 2 hr at 140 000 x g. Each gradient was divided into 20 fractions which were assayed reductometrically for laminarinase (o———o) and viscometrically for CM-pachymanase (•———•). Protein (-----).

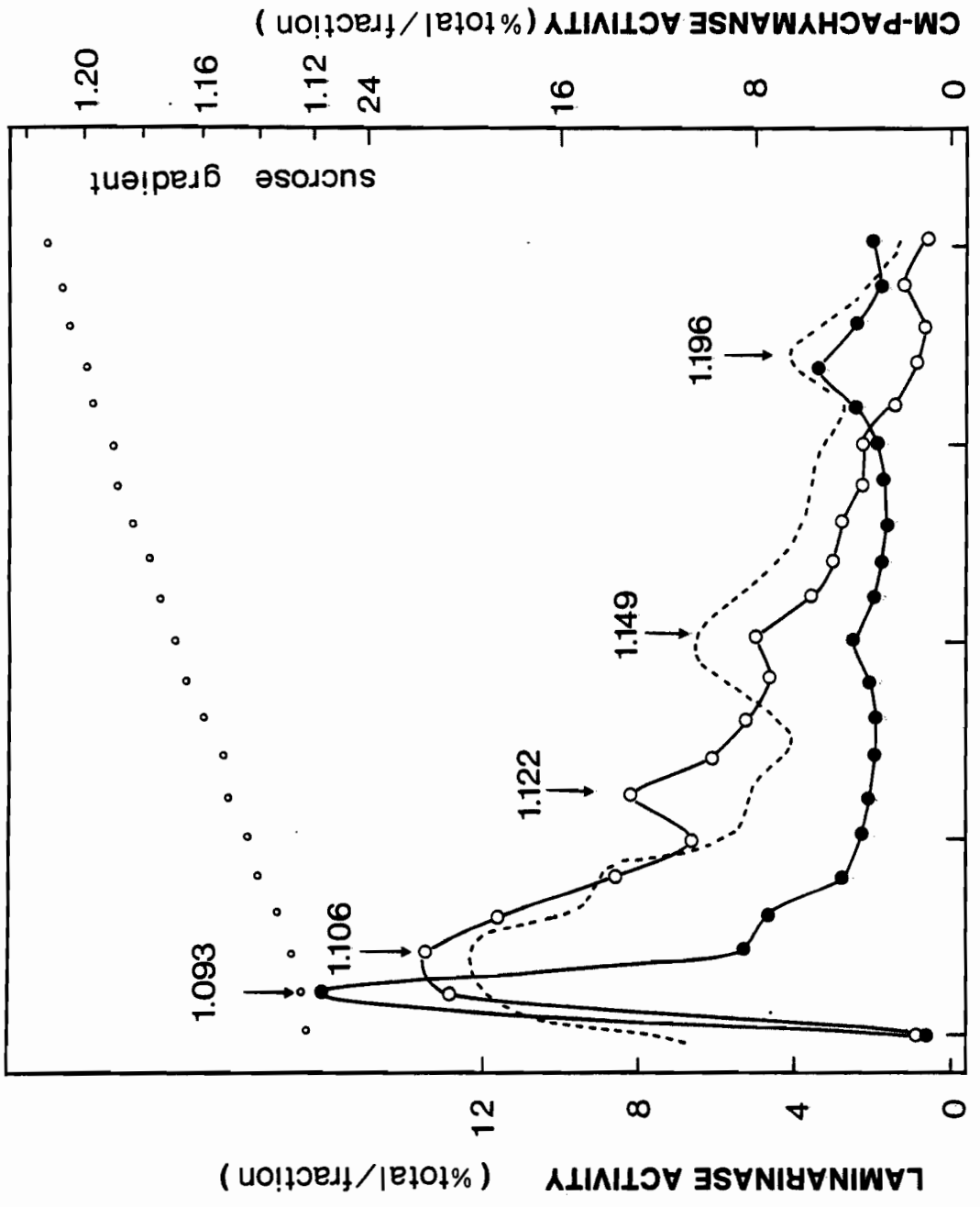

LAMINARINASE ACTIVITY (%total/fraction)
CM-PACHYMANSE ACTIVITY (%total/fraction)
FRACTION NUMBER
sucrose gradient
1.093
1.106
1.122
1.149
1.196
0
4
8
12
16
20
24
1.12
1.16
1.20
0
5
10
15
20

Fig. 5. Development of soluble β-glucanase specific activities in apical tissue following treatment of intact pea seedlings wih 0.1% 2,4-D. Seedlings were sprayed at 7 days and the expanding region (subapical 10 mm) was excised at daily intervals, extracted, and assayed for protein, laminarinase and CM-pachymanase activities as described in Materials and Methods. Over the 5-day period of this test, protein levels increased from 50 to 60 μg/segment as a result of 2,4-D treatment (controls did not change).

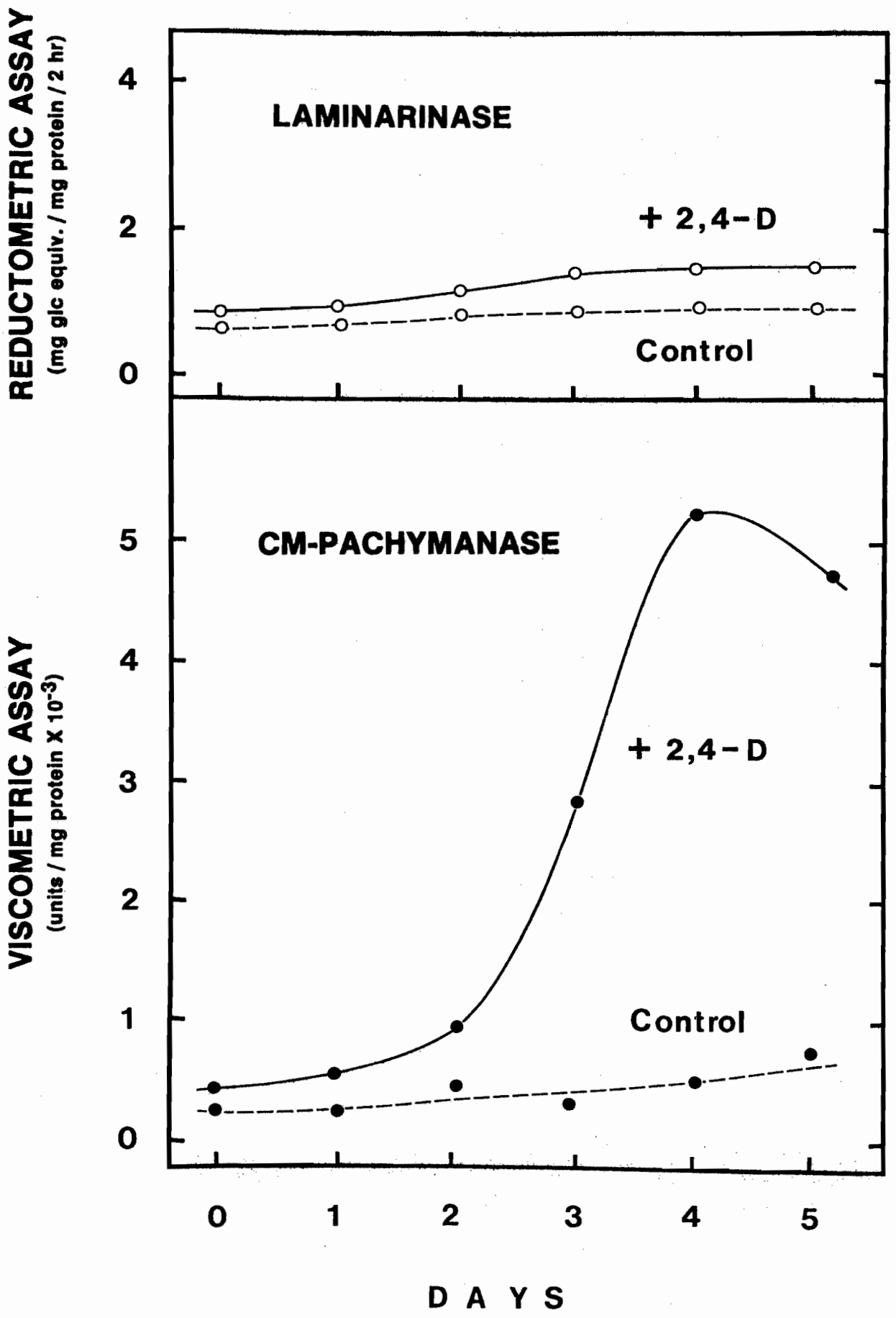

REDUCTOMETRIC ASSAY
(mg glc equiv. / mg protein / 2 hr)
LAMINARINASE
+ 2,4-D
Control
VISCOMETRIC ASSAY
(units / mg protein X 10-3)
CM-PACHYMANASE
+ 2,4-D
Control
DAYS

Fig. 6. Distribution in pea stems of endogenous water-soluble substrates of glucanase I and II. The water-soluble, non-dialysable, fraction was extracted from various tissues (see Fig. 1) as described in Materials and Methods. These preparations were incubated with purified glucanase I and II (0.05 ml, 0.4 μmol) at 35°C for 12 hr and reducing power generated was measured (39) as an estimate of the amount of susceptible substrate.

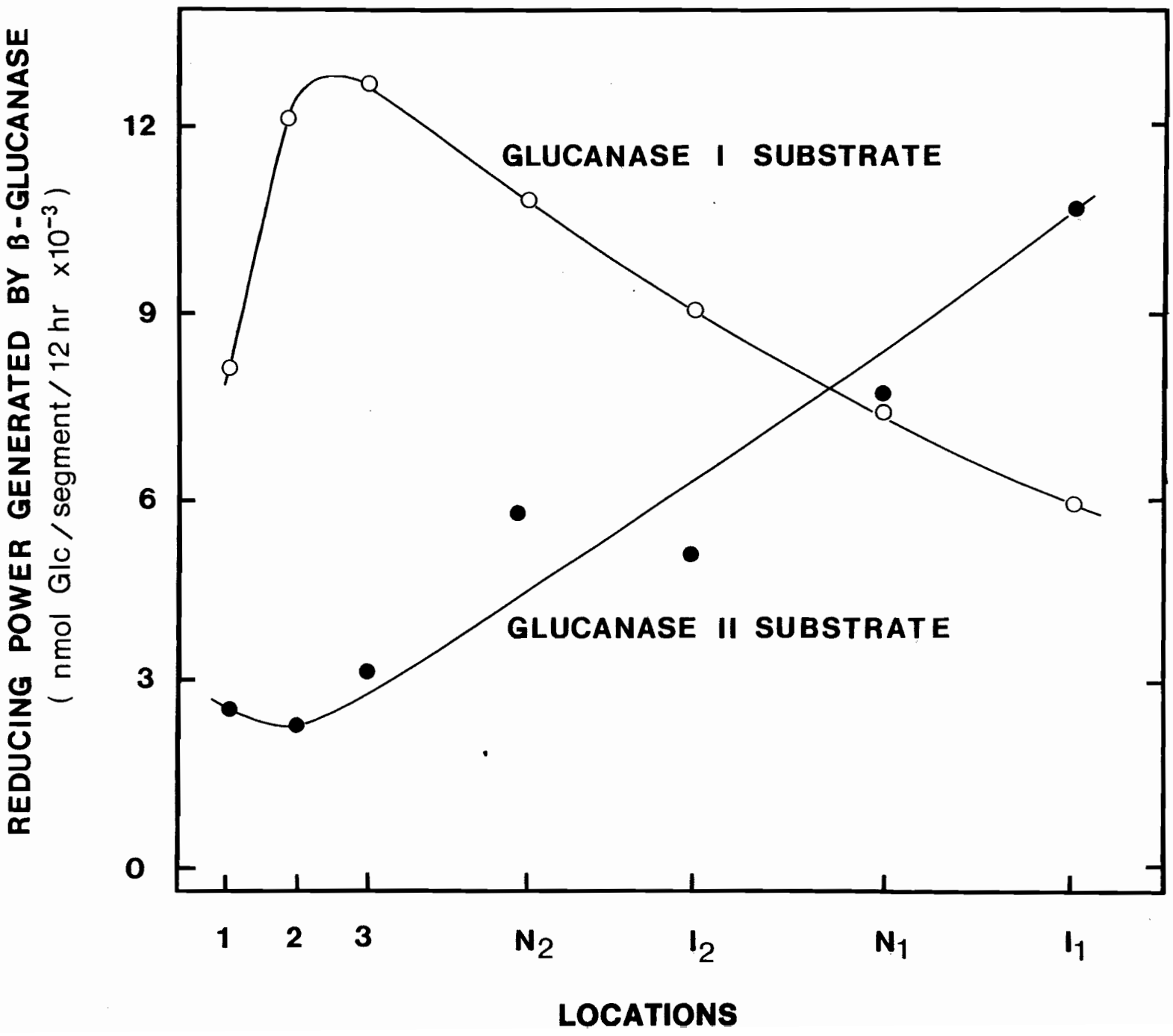

REDUCING POWER GENERATED BY β-GLUCANASE
(nmol Glc / segment / 12 hr x10⁻³)
12
9
6
3
0
GLUCANASE I SUBSTRATE
GLUCANASE II SUBSTRATE
1
2
3
N2
I2
N1
I1
LOCATIONS

Fig. 7. Products formed by ~~glucanase~~ action of glucanase I and II on endogenous water-soluble substrates. Purified glucanases (0.05 ml, 0.4 μmol) were incubated at 35°C for 12 hr with the water-soluble fractions derived from apical and basal tissue, respectively. The hydrolysates were loaded onto Bio-Gel P-2 columns(0.7 x 30 cm) and fractionated (0.12 ml/min, 0.2 ml/fraction) with 20 mM sodium acetate buffer, pH 5.5. The fractions were assayed for carbohydrate content by the phenol-H_2SO_4 method (38). On this column, laminarin (D.P. 20) elutes in the void volume (V_o) and glucose in the bed volume (V_t). Laminaridextrin standards elute in loci corresponding to the peaks between V_o and V_t. ---- before enzyme hydrolysis

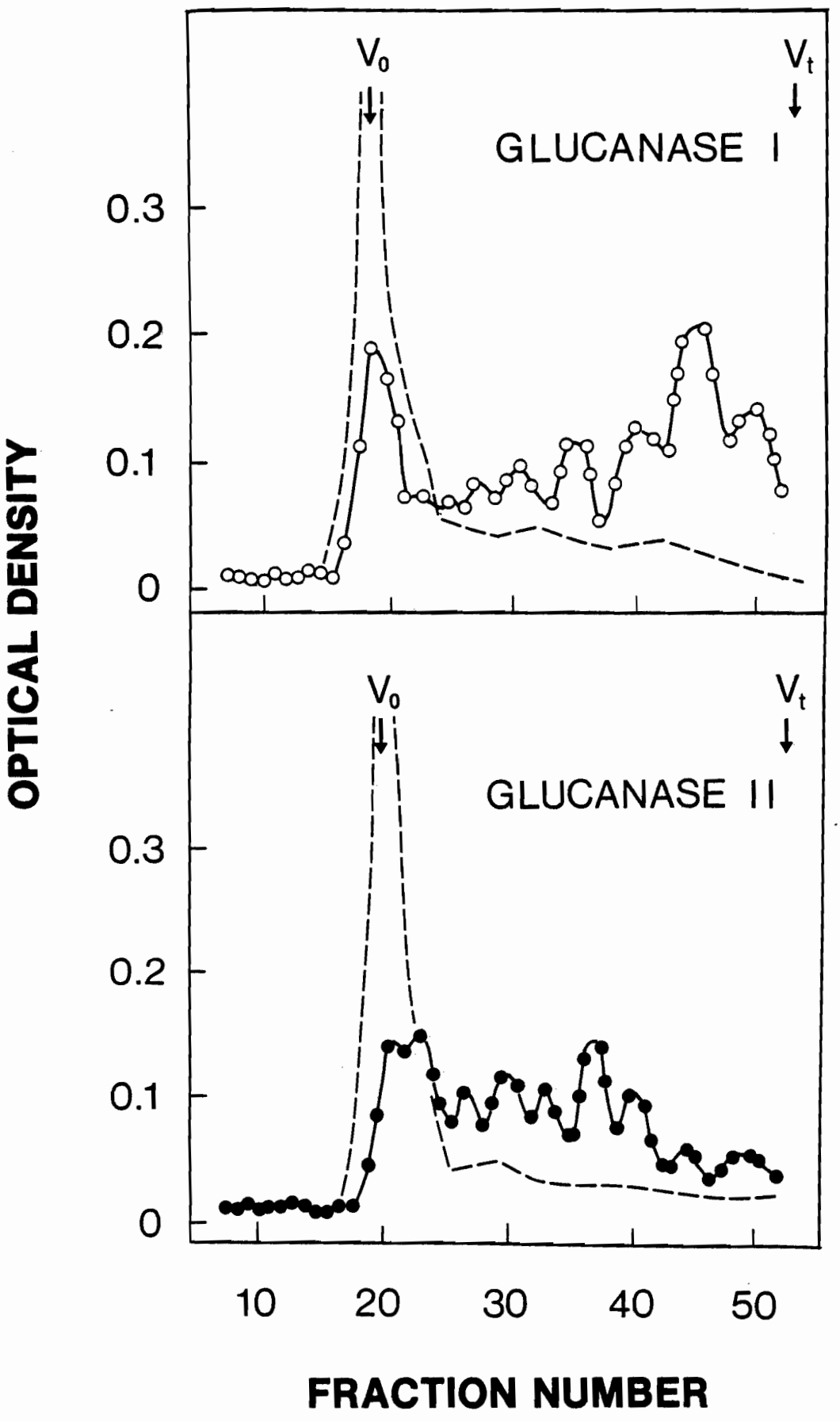
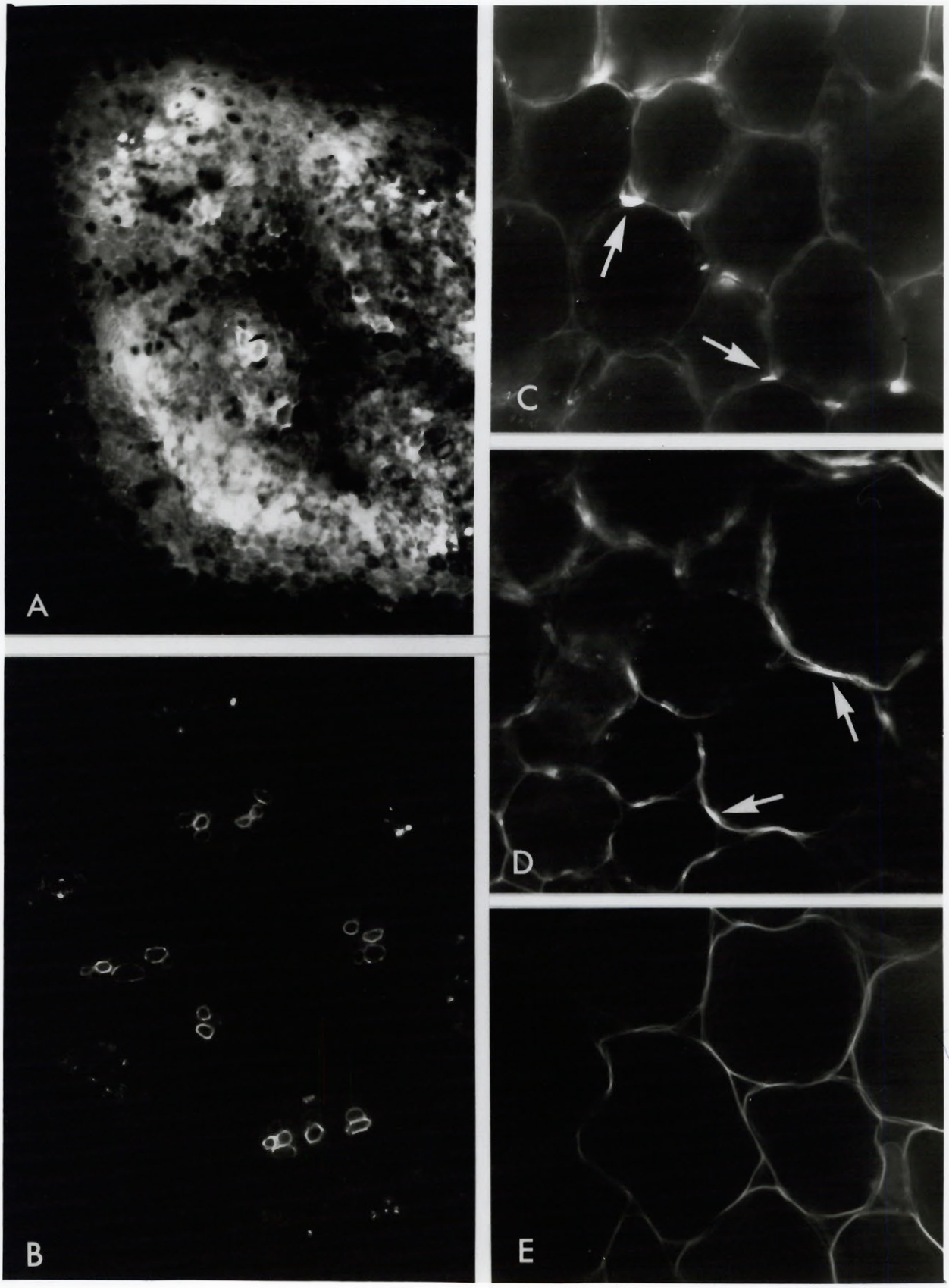

V0
Vt
GLUCANASE I
0.3
0.2
0.1
0
V0
Vt
GLUCANASE II
0.3
0.2
0.1
0
OPTICAL DENSITY
10
20
30
40
50
FRACTION NUMBER

Fig. 8. Localization of substrates of 1,3-β-D-glucanases by fluorescence microscropy in aniline blue. Transverse hand sections showing: (A) the stele of apical tissue with dense fluorescence, x 100, (B) the same tissue treated with purified glucanase I before staining with aniline blue, x 100, (C) and (D) cortical parenchyma cells in basal tissue, with fluorescent regions concentrated in cell walls (arrows), x 400, (E) basal tissue pre-treated with purified glucanase II, x 400.


A
B
C
D
E

APPENDIX IIIA

EFFECT OF 2,4-D CONCENTRATIONS ON DEVELOPMENT OF CM-PACHYMANASE ACTIVITIES IN INTACT PEA SEEDLINGS.

The apices (500 seedlings per tray) of seven day old dark-grown peas were sprayed with 10 ml solution containing 0.1 M NaCl, 0.1% Tween 80 and 2,4-D at concentration indicated below. Crude enzyme preparations were obtained from 50 segments (10 mn each) excised three days after treatment. Enzyme extraction and assay procedures were described in Materials and Methods of Chapter I and III. Protein was estimated by the Lowry method.

Treatments*	Length	Fr. wt. / length	Protein	CM-pachymanase activity Total	Specific
	mm/seg	mg/mm	µg/seg	units/seg	units/mg prot.
Zero day control	105	1.5	58	9	124
3 day control	215	1.4	85	17	205
+ 0.001% 2,4-D	200	1.4	88	36	420
+ 0.01% 2,4-D	125	4.1	125	235	1750
+ 0.1% 2,4-D	130	3.7	109	298	2450

* Pea seedlings sprayed with 1% 2,4-D turned brown, wilted and collapsed within 24 hr.

APPENDIX IIIB

EFFECT OF IAA AND GA ON DEVELOPMENT OF CM-PACHYMANASE ACTIVITIES IN DECAPITATED PEA EPICOTYLS.

Six-day etiolated pea seedlings were decapitated (i.e., plumule and hook removed) and the apical 10 mm region of the epicotyl was marked to delineate a "segment". The apices were painted with lanolin ± IAA (0.5%, w/w) or GA_3 (0.5%, w/w). Treated peas were left in darkness for 5 days. Thirty "segments" of each treatment were harvested at daily intervals, and lanolin was wiped off before analysis. The enzyme extraction procedure and CM-pachymanase assays were conducted as described in Materials and Methods of Chapters I and III. Protein levels were estimated by the Lowry method.

Treatments	Length	Fr. wt. length	Protein	CM-pachymanase activity Total	Specific
	mm/seg	mg/mm	μg/seg	units/seg	units/mg prot.
Zero day control	106	1.7	67	6	93
5 day control	185	1.6	72	7	57
+ IAA	165	4.6	112	172	1640
+ GA	255	1.8	98	36	420

APPENDIX IIIC

DEVELOPMENT OF 1,3-β-D-GLUCANASE ACTIVITIES WITH TIME AFTER TREATMENT OF DECAPITATAED EPICOTYLS WITH IAA ± FUdR.

Pea seedlings were decapitated and treated with 0.5% (w/w) IAA ± 0.1% FUdR (w/w) in lanolin paste as described in Appendix IIIB. Segments were harvested at daily intervals up to day 5 following treatment. Laminarinase and CM-pachymanase activities were measured reductometrically and viscometrically respectively, as described in Materials and Methods of Chapter I.

Time	Fr. wt. length			Laminarinase activity			CM-pachymanase activity		
day	mg/mm			μg glc equiv/seg			units/seg		
	Cont.	+IAA	+IAA, FUdR	Cont.	+IAA	+IAA, FUdR	Cont.	+IAA	+IAA, FUdR
0	2.1	2.1	2.1	30	30	30	25	25	25
1	2.8	4.9	5.5	29	25	28	18	48	17
2	3.2	6.6	6.4	29	88	76	80	319	278
3	2.9	7.0	6.9	67	78	97	158	547	491
4	2.6	7.2	6.9	75	65	74	145	692	493
5	2.8	7.9	6.3	90	84	86	160	729	566

APPENDIX IIID

EFFECT OF WOUNDING ON CM-PACHYMANASE ACTIVITIES FROM BASAL TISSUE OF PEA EPICOTYLS.

Segments (10 mm) of first internodes (basal tissue) were excised from 7 day old etiolated pea seedlings, and each of these segments was sliced into various number of sections as shown below. These sections were floated on 2 ml of 20 mM sodium acetate buffer, pH 5.5, in petri dishes of 5 cm in diameter at room temperature for 10 min or 2 hr before crude enzyme preparations were extracted from them. CM-pachymanase activities were measured viscometrically as described in Materials and Methods of Chapter I.

Treatment	CM-pachymanase activity	
slices/cm	units/seg	
	10 min pre-incubation	2 hr pre-incubation
1	598	654
4	553	634
10	636	716

CONCLUDING REMARKS

The enzymic properties of purified 1,3-β-D-glucanases from apical and basal tissues of Pisum sativum seedlings have been investigated in the present study and described in the three chapters of this thesis. These properties are now classified and summarized in the below table.

Summary of Enzymic Properties

Property	Glucanase I	Glucanase II	References
Location of Enzymes and Endogenous Substrates			
Cellular location of enzyme	apex	base	Figs. 3 & 8, Ch. III
Subcellular location of enzyme			
% soluble enzyme	69	95	Table II, Ch. III
% particulate and wall bound	31	5	Table II, Ch. III
Main endogenous substrate	water sol., apex	water sol., base	Figs. 3 & 6, Ch. III
Cellular location of substrate	stele	cortical cell wall	Fig. 8, Ch. III
Kinetics			
pH optima, Laminarin	6.0	6.0	Fig. 6, Ch. I
CM-pachyman	5.5	5.5	Fig. 6, Ch. I
K_m, various substrates (mg/ml)	1.5 - 4.2	0.6 - 7.4	Table IV, Ch. I
Thermalstability (% enzyme activity retained at 50°C for 60 min)	62	40	Fig. 7, Ch. I
Reaction Rates			
Sp. viscosity loss/μg red. power generated from CM-pachyman	2.9	62.5	Fig. 1, Ch. III

Summary of Enzymic Properties

(Cont'd)

Property	Glucanase I	Glucanase II	References
µg red. power generated from laminarin/unit viscosity loss of CM-pachyman	1930	9.4	Tables I & V, Ch. II
Reaction Products			
Distribution of intermediates: lower dextrins (D.P. 1 - 4) / higher dextrins (D.P. >4)			
Endogenous substrates	>1	<1	Fig. 7, Ch. III
Fucus [^{14}C] 1,3-β-D-glucan	>1	<1	Fig. 2, Ch. III
Laminarin	>1	<1	Fig. 1, Ch. II
Products of action on barley β-glucan	3-O-β-cellobiosyl-D-glucose		Fig. 3, Ch. II
Mode of Action			
Hydrolysis of laminaridextrins	linkages close to non-reducing terminal	random at internal linkages	Fig. 4, Ch. II
Action pattern	"multiple attack"	"multi-chain"	Ch. II
Effect of Growth Regulators			
IAA or 2,4-D	none	increase sp. act.	Table III. Figs. 3 & 5, Ch. III
GA	none	none	Appendix III
Ethylene ± 2,4-D	suppress	suppress	Table III, Ch. III
Inhibition			
% inhibition by L_6 of CM-Pachymanase activity	21	60	Fig. 6, Ch. II
Specific inhibitors	N-bromosuccinimide	Succinic anhydride	Table III; Ch. II Table I, Ch. I

CONTRIBUTION TO ORIGINAL KNOWLEDGE

This is the first study to show that two endo-1,3-β-D-glucanases (E.C. 3.2.1.6) are elaborated in a simple plant species. They have distinct physical and biochemical properties and very different loci in which they accumulate. Biochemical and histochemical studies indicate that the distribution patterns of these glucanases is paralleled by the distribution of their water-soluble substrates, suggesting that the hydrolases and their substrates normally exist *in vivo* in separate cellular compartments. Only one of them (glucanase II) appears to be subject to hormonal (auxin) regulation.

Both enzymes have been purified, characterized and their enzymic properties extensively studied. Commercially unavailable substrates have been employed (mostly prepared in this laboratory), with defined structures, chain lengths and properties, e.g., a homologous series of laminaridextrins (D.P. 2 - 7) and their derivatives, [^{3}H]-reduced laminaridextrins, CM-pachyman mixed-linkage barley β-glucan, [^{14}C]-labelled *Fucus* β-glucan, etc., in order to define the action pattern of these enzymes. No 1,3-β-D-glucanase from any source has been tested with such a range of substrates or in the detail that is recorded in the present work.

The studies on the substrate specificities and mode of action of the two purified β-glucanases show that their active sites differ in such a way that I catalyses endo-hydrolysis according to a "multiple attack" pattern (similar to that of some α-amylases), whereas II appears to act via a more random "multi-chain" action pattern (like acid hydrolysis). This is the first demonstration that action patterns well-established with α-amylases may also apply to β-glucanases.