Modes of Action of Exo-and Endo-β-1, 3-Glucanases from Irpex lacteus (Polyporus tulipiferae)

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Summary

An experimental investigation was made on action patterns of two highly purified β-1, 3-glucanase components (named L-I and -IV), obtained from Driselase, a commercial enzyme preparation from *Irpex lacteus (Polyporus tulipiferae)*, on various substrates.

L-I and -IV exhibited a high substrate specificity in hydrolyzing β-1, 3-gluco-oligosaccharides with a degree of polymerization higher than 3, although their action patterns were different from each other, but they were inactive toward laminaribiose. L-I did not attack modified pachyman in which the nonreducing terminal residue had been altered by periodate oxidation, whereas L-IV attacked the modified pachyman at a rate of hydrolysis comparable with that of the original pachyman. These facts indicate that L-I cleaves the substrate molecule in an exo-wise manner, successively removing the glucosyl residue from the nonreducing terminal, whereas L-IV cleaves it in a random fashion. L-I can also bypass the β-1, 6-linked branches to cleave β-1, 3-linkages, producing glucose and gentiobiose when scleroglucan was used as substrate, but cannot cleave mixed-linkage β-1, 3-; β-1, 4-glucans such as lichenan and reduced, pneumococcal polysaccharide RS III. On the other hand, L-IV can cleave preferentially the β-1, 4-linkages adjacent to the β-1, 3-linked glucose residue in the mixed-linkage β-glucans, but scarcely have action on β-1, 3-glucans containing β-1, 6-linked branch.

Introduction

As already reported,[1] the author found four kinds of β-1, 3-glucanase (E. C. 3. 2. 1. 6) components in Driselase, which is a commercial enzyme prep-

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Abbreviations ; G_1, D-glucose ; G_2, laminaribiose ; G_3, laminaritriose ; G_4, laminaritetraose ; G_5, laminaripentaose ; Gb, gentiobiose ; CMC, sodium carboxymethyl-cellulose ; DS, degree of substitution

aration from a wood-rottening fungus *Irpex lacteus (Polyporus tulipiferae)*, and isolated two major $\beta-1,3$-glucanase components of them as essentially homogeneous materials. Enzymatic properties of these highly purified $\beta-1,3$-glucanases from this fungus have been examined in detail and their action patterns have been compared with each other. One of them is exo-$\beta-1,3$-glucanase L-I, which has been demonstrated to split off glucosyl residues from nonreducing terminals of a substrate molecule, whereas the other is endo-$\beta-1,3$-glucanase L-IV of a smaller-oligosaccharide producing type, which attacks at random glucosidic linkages of a substrate molecule. However, action patterns of their purified $\beta-1,3$-glucanases on various kinds of substrates have not fully been elucidated.

The present paper describes modes of action of these two kinds of $\beta-1,3$-glucanases on several laminari-oligosaccharides and various polysaccharides containing $\beta-1,3$-glucosidic linkages.

Materials and Methods

Enzyme Preparations. The exo- and endo-$\beta-1,3$-glucanases (named L-I and -IV, respectively) used were obtained by previously reported procedures[1] from Driselase, a commercial product of *Irpex lacteus (Polyporus tulipiferae)* manufactured by Kyowa Hakko Co., Ltd. Each of them gave a single protein peak upon polyacrylamide gel disc electrophoresis. The molecular weight of the former was 58,000 and that of the latter 28,000.

Substrates. a) Pachyman was prepared from a Chinese medicine air-dried *Bukuryo (Poria cocos)* following the method described by Saito et al.[2]

b) Sodium carboxymethyl pachyman (CM-pachyman, DS=0.4-0.6) was prepared from pachyman by the method of Clarke and Stone.[3]

c) Laminari-oligosaccharides (G_2-G_5) were prepared from a partial hydrolysate of *Bukuryo (Poria cocos)* according to the method described by Whelan.[4] The preparations were found to be pure on inspection by paper chromatography.

d) Curdlan and scleroglucan were gifted from Professor Tokuya Harada of Osaka University and from Dr. Junko Ebata of Osaka City University, respectively.

e) Laminaran I (from *Eisenia arborea*) used as substrate was purchased from Tokyo Chemical Industry Co., Ltd., and Laminaran II (from *Eisenia bicyclis*) and Laminaran III (from *Laminaria angustata*) were gifted from Professor Kazutosi Nisizawa of Nihon University.

f) Barley glucan and reduced, pneumococcal polysaccharide RS III were generously presented by professor B. A. Stone (Department of Biochemistry, La Trobe University, Australia).

g) Lichenan (from *Cetraria icelandica*) was obtained from Sigma Chemical

Co. (St. Louis, Mo., U. S. A.).

h) Yeast glucan was prepared from dried baker's yeast from Wako Pure Chemical Industries Co., Ltd. by the method described by Misaki *et al.*[5]

i) IO_4^--oxidized pachyman, BH_4^--reduced pachyman, and IO_4^--oxidized and BH_4^--reduced pachyman were prepared according to the method described by Nelson *et al.*[6]

j) Carboxymethyl-cllulose (CMC) was a product of Daiichi Industrial and Pharmaceutical Co. The degree of substitution was 0.62.

k) Cellooligosaccharides ranging from cellobiose to cellohexaose used as standards were prepared from cellulose powder by the method of Miller *et al.*[7]

The other chemicals used were of reagent grade, commercially available.

Enzyme Assays and Analytical Methods. a) β-1, 3-Glucanase Activity: The activity of β-1, 3-glucanase was determined as follows. The reaction mixture used consisted of 0.5 ml of 0.5 % substrate solution, 1 ml of 0.1 M sodium acetate buffer (pH 5.0), and 0.5 ml of diluted enzyme solution. After incubation at 30°C for a suitable time, the reducing sugars produced were determined per ml of the reaction mixture by the colorimetric method of Somogyi[8] and Nelson.[9]

b) Paper Chromatography: Enzymatic hydrolysates from various substrates were identified by paper chromatography. After an appropriate period of incubation at 30°C, a 0.1 ml aliquot of reaction mixture was inactivated by heat and then spotted on a Whatman No. 3 MM filter paper together with a solution of authentic sugars as standards. Chromatograms were developed twice by the ascending technique with *n*-butanol : pyridine : water (6 : 4 : 3 v/v) at room temperature for an appropriate period. Spots of hydrolysis products on the paper chromatograms were detected by the acetone-silver nitrate procedure.[10] The approximate relative amounts of hydrolysis products on the chromatograms were estimated from the color intensity and size of the spots.

c) Determination of Protein: The protein in an enzyme solution was determined from the absorbance at 280 nm, using crystalline bovine serum albumin (from Miles Laboratories, Inc.) as a standard.

Results

Action Patterns of β-1, 3-Glucanases L-I and -IV. (A) Actions on Laminari-oligosaccharides: The present study was undertaken to obtain information on the action pattern of both the enzymes on a homologous series from G_2 to G_5. Approximately 5 mM of each of laminari-oligosaccharides from G_2 to G_5 was dissolved in 0.4 ml of 0.1 M sodium acetate buffer (pH 5.0), and 0.1 ml of enzyme solution (0.4 μM) was added. After an appropriate period

of incubation at 30°C, 0.1 ml aliquots of the enzymatic hydrolysates were withdrawn, and, with 0.1 ml of ethanol added, heated in a boiling water bath for 5 min. The hydrolysis products were examined by paper chromatography.

Figs. 1 and 2, show the paper chromatograms of the products formed in course of hydrolysis of laminari-oligosaccharides (G_2-G_5) by L−I and −IV, respectively. Neither of both the enzymes could attack laminaribiose even after a prolonged incubation, but they hydrolyzed the other oligosaccharides tested to produce various saccharides. During the reaction with G_3 by L−I, almost equal amounts of G_1 and G_2 were produced. The products from G_4 were a mixture of $G_3=G_1>G_2$ in the earlier stage of hydrolysis, and as reaction proceeded, G_1 and G_2 gradually increased. G_5 gave a mixture of $G_1>G_3\geqq G_4>G_2$ in the earlier stage and that of $G_1\gg G_2>G_3\gg G_4$ in the later stage of digestion (Fig. 1). On the other hand, the

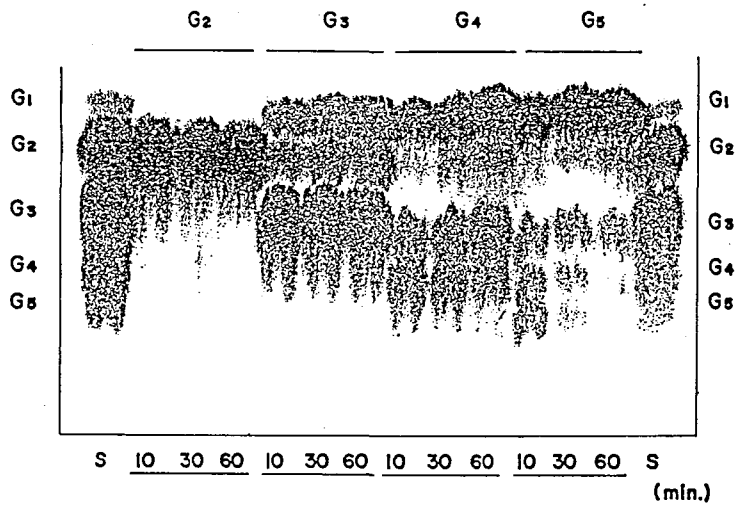


Fig. 1. Paper chromatograms showing products formed in course of hydrolysis of laminari-oligosaccharides by L−I.
S, standard ; G_1, glucose ; G_2, laminaribiose ; G_3, laminaritriose ; G_4, laminaritetraose ; G_5, laminaripentaose.
Development : twice by the ascending technique for 24 hr.

hydrolysis products from G_3 formed by L−IV were found to be a mixture of G_1, G_2, and small amounts of two unknown oligosaccharides located under the spot of G_3. G_4 was finally hydrolyzed to G_1, G_2, and a small amount of G_3. with slight amounts of two unknown oligosaccharides located under the spot of G_4. From G_5, all the four possible products were formed in a mixture of $G_2=G_1>G_3>G_4$, with a slight amount of unknown

oligosaccharide in the earlier stage (Fig. 2). From these product patterns of hydrolysis products, as expected, it is evident that L-I and -IV hydrolyze the oligosaccharides by exo-action and by random fashion with transglucosylation action, respectively.

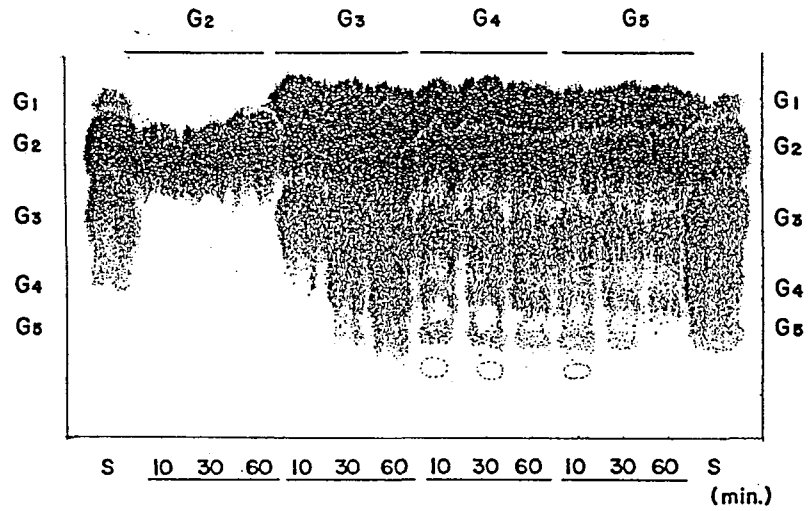


Fig. 2. Paper chromatograms showing products formed in course of hydrolysis of laminari-oligosaccharides by L-IV.
The symbols are the same as in Fig. 1.
Development : twice by the ascending technique for 24 hr.

(B) Actions on Various Glucans: Relative rates of hydrolysis of various polysaccharides by β-1, 3-glucanases L-I and L-IV were determined under the standard assay conditions. Paper chromatography of the sugars formed in the reaction mixture was carried out by the multiple ascending technique described in the "Materials and Methods" section.

(1) Actions on Chemically Modified Pachymans: In order to establish the action pattern of β-1, 3-glucanases on pachyman, actions of both the enzymes on various derivatives of pachyman were investigated. As can be seen from Table 1, the pachyman which had been modified only at its reducing terminal residue by sodium borohydride, was attacked more rapidly than the original pachyman by L-I. In contrast, L-1 could attack neither the pachyman which had been oxidized by periodate nor the one which had been oxidized by periodate and subsequently reduced by sodium borohydride, thus having had both the original reducing and nonreducing terminals modified. On the other hand, these modified pachymans were attacked by L-IV at a rate of hydrolysis only somewhat lower than the original pachyman. CM-pachyman (DS=0.4) was more slowly degraded by

both the enzymes than the original pachyman. The rate of increase in reducing value of CM−pachyman attacked by L−IV was high as compared with that by L−I.

Table 1. Relative rates of hydrolysis of pachyman derivatives by L−I and −IV.

Substrate	Relative rate of hydrolysis A)	
	L-I	L-IV
Pachyman	(100)	(100)
IO_4-oxidized pachyman	-	73
IO_4-oxidized and BH_4-reduced pachyman	-	89
BH_4-reduced pachyman	117	86
CM-pachyman	1	11

The method is described in the "Materials and Methods" section. After incubation for 30 min, the reducing sugars formed were measured by the method of Somogyi[8] and Nelson.[9]
IO_4, periodate ; BH_4, sodium borohydride.
A) The rate for pachyman was arbitrarily set as 100.

(2) Actions on Pachyman and Curdlan: Actions of both the enzymes on pachyman and curdlan are shown in Table 2. L−IV was active for the $\beta-1, 3-$glucosidic linkage of polysaccharides such as pachyman and curdlan. However, the rate of hydrolysis of curdlan by L−I was only 4% of that of pachyman. Fig. 3 shows a progressive hydrolysis of curdlan by both the enzymes. The hydrolysis products formed from curdlan by L−IV were only G_1, G_2, and G_3.

(3) Actions on $\beta-1, 3-$ Glucans Containing $\beta-1, 6-$ Linked Branches: $\beta-1, 3-$Glucans containing $\beta-1, 6-$linked branches, scleroglucan and laminaran III (L. a.), were attacked by L−I at a rate comparable with that of pachyman, whereas they showed considerable resistance to attack by L−IV (Table 2). Fig. 4 shows paper chromatograms of products formed during the course of hydrolysis of scleroglucan by L−I. Glucose and a minor amount of gentiobiose were produced from the glucan by L−I.

(4) Actions on the Mixed−Linkage $\beta-1, 3-$; $\beta-1, 4-$ Glucans: Actions of both the enzymes on the $\beta-1, 3-$glucans containing the $\beta-1, 4-$linkage were also investigated. L−IV attacked the mixed-linkage $\beta-1, 3-$; $\beta-1, 4-$glucans such as lichenan and barley glucan (Table 2). As can be

seen in Figs. 5 and 6, from their paper chromatographic mobilities, the products formed during the course of hydrolysis of lichenan (β−1, 3−; β−1, 4−D−glucan containing 3−O−β−cellotriosyl−D−glucose) by L−IV were concluded to be 3−O−β−cellobiosyl−D−glucose, having a mobility similar to that of G_4, together with minor amounts of G_1 and G_2. L−IV hydrolyzed also reduced, pneumococcal polysaccharide RS III (β−1, 3−; β−1, 4−D−glucan containing alternating 1, 3−and 1, 4−linkages). The paper chromatograms of hydrolysis products revealed that the major product was laminaribiose, and that no cellobiose was released. However, the ability of L−I to attack these mixed-linkage β−glucans was extremely low.

Toble 2. Relative rates of hydrolysis of various polysaccharides by L−I and−IV.

Substrate	Predominant glucosidic linkage	Relative rate of hydrolysis A)	
		L−I	L−IV
Pachyman	β − 1, 3 −	100	100
Curdlan	β − 1, 3 −	4.0	42.6
Scleroglucan	β − 1, 3 − ; β − 1 , 6 −	89.3	1.9
Yeast glucan	β − 1, 3 − ; β − 1 , 6 −	44.7	32.3
Laminaran I (E. a.)[a]	β − 1, 3 − ; β − 1 , 6 −	45.2	4.1
Laminaran II (E. b.)[b]	β − 1, 3 − ; β − 1 , 6 −	37.9	1.9
Laminaran III (L. a.)[c]	β − 1, 3 − ; β − 1 , 6 −	88.1	35.6
Lichenan	β − 1, 3 − ; β − 1 , 4 −	3.3	48.0
Barley glucan	β − 1, 3 − ; β − 1 , 4 −	6.5	88.0
C M C	β − 1, 4 −	0	0

a) Eisenia arborea b) Eisenia bicyclis c) Laminaria angustata

The method is described in the "Materials and Methods" section. After incubation for 480 min, the reducing sugars formed were measured by the method of Somogyi[8] and Nelson.[9]

A) The rate for pachyman was arbitrarily set as 100.

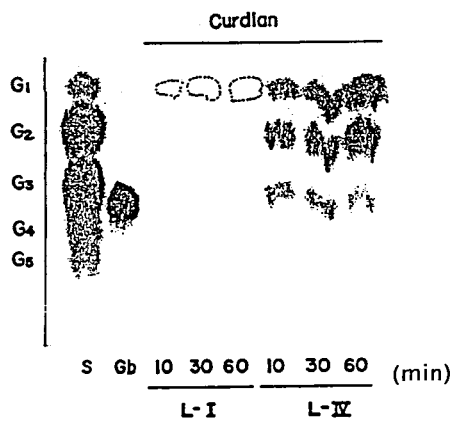


Fig. 3. Paper chromatograms of the hydrolysis products from curdlan by L−I and−IV.
The symbols are the same as in Fig. 1, except for Gb, gentiobiose.
Development ; twice by the ascending technique for 24 hr.

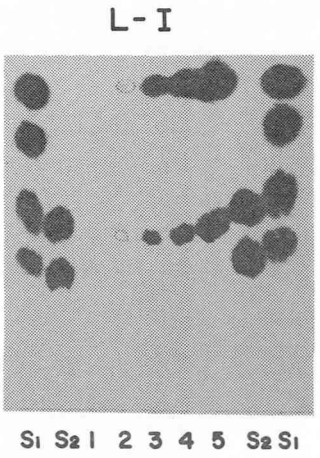


Fig. 4. Paper chromatograms showing products formed in course of hydrolysis of scleroglucan by L-I.
S_1, standard ; glucose, laminaribiose, laminaritriose, and laminaritetraose from the top.
S_2, standard ; gentiobiose and $\beta-1$, 3- ; $\beta-1$, 6-triose from the top.
Incubation times : 1, 0 min ; 2, 10 min ; 3, 30 min ; 4, 60 min ; 5, 24 hr.
Development : twice by the ascending technique for 24 hr.

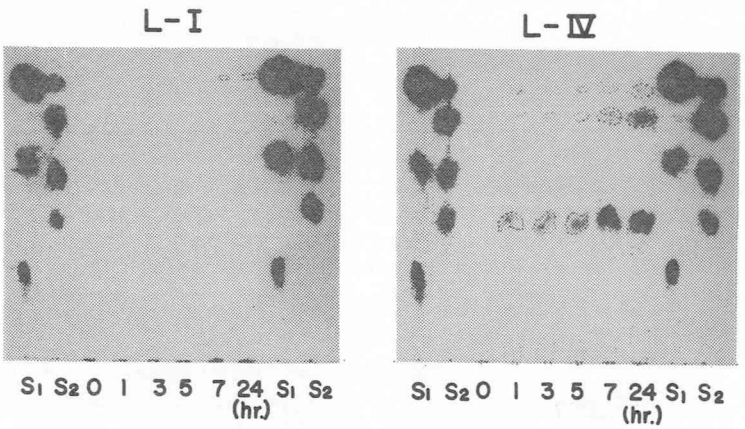


Fig. 5. Paper chromatograms of the hydrolysis products from lichenan by L-I and -IV.
S_1, standard of cellooligosaccharides ; glucose, cellobiose, and cellotriose from the top.
S_2, standard of laminari-oligosaccharides ; glucose, laminaribiose, laminaritriose, and laminaritetraose from the top.
Development : twice by the ascending technique for 24 hr.

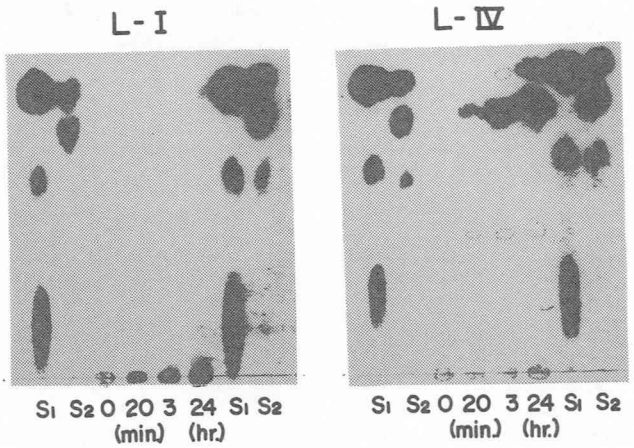
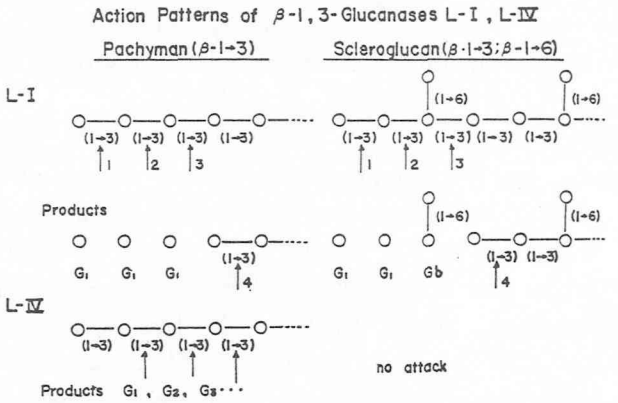
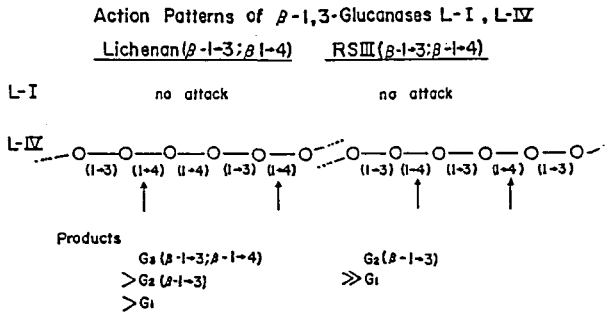


Fig. 6. Paper chromatograms of the hydrolysis products from reduced, pneumococcal polysaccharide RS III by L-I and -IV.
S_1, standard of cellooligosaccharides ; glucose, cellobiose, and cellotriose from the top.
S_2, standard of laminari-oligosaccharides ; glucose, laminaribiose, and laminaritriose from the top.
Development : twice by the ascending technique for 24 hr.



Scheme 1. Action patterns of L-I and L-IV on pachyman and scleroglucan.
○ and ↑ in the partial structures of pachyman and scleroglucan indicate glucosyl residue and the site of main cleavage, respectively.



Scheme 2. Action patterns of L−I and −IV on lichenan and reduced, pneumococcal polysaccharide RS III.
○ and ↑ in the partial structures of lichenan and RS III indicate glucosyl residue and the site of main cleavage, respectively.

Discussion

In a previous work,[1] the author obtained at least four $\beta-1,3-$glucanase components from *Irpex lacteus (Polyporus tulipiferae)*. Two major $\beta-1,3-$glucanase components (L−I and −IV) of them were each in a highly purified state and behaved practically as a single protein on polyacrylamide gel disc electrophoresis. Based on the hydrolysis patterns of those purified enzymes on the pachyman or CM-pachyman, $\beta-1,3-$glucanases L−I and −IV were classified into an exo- and an endo-$\beta-1,3-$glucanase, respectively.

To obtain further information on action patterns of both the enzymes, products of hydrolysis by the enzymes of various kinds of substrates containing the $\beta-1,3-$glucosidic linkage were examined by paper chromatography.

A homologous series from G_2 to G_5 was first investigated as substrates. Neither of both the enzymes could attack laminaribiose, even after prolonged incubation, but they hydrolyzed the other oligosaccharides to produce saccharides. From G_3, almost equal amounts of G_1 and G_2 were produced by both the enzymes. As expected, G_4 was hydrolyzed into G_1, G_2, and G_3 and from G_5 all four possible products (G_1, G_2, G_3 and G_4) were formed although the amounts of products formed from these substrates by both the enzymes were appreciably different from one another in the earlier stage. Furthermore, small amounts of unknown oligosaccharides located under the spots of G_3, G_4, and G_5, were observed during the reactions of G_3, G_4, and G_5 with L−IV, respectively (Fig. 2). These results indicate that endo-$\beta-1,3-$glucanase L−IV from *Irpex lacteus* has a glucosyl transfer action, like enzymes from *Rizopus arrhizus*[11] and *Rizoctonia*

solani[12] which have a significant transglycosylase activity. Thus, β-1, 3-glucanases L-I and -IV from *Irpex lacteus* hydrolyze β-1, 3-gluco-oligosaccharides with a degree of polymerization more than 3, with a preference for substrates with longer chains, although action patterns of both the enzymes on them are different from each other (Figs. 1 and 2). Moreover, the patterns, as observed by paper chromatography, of the products formed by the actions of L-I and -IV on the oligosaccharides, confirmed also the previous evidence[1] for exo- and endo-manner, respectively.

The actions of both the enzymes on various polysaccharides also were examined. L-I did not attack the modified pachyman in which nonreducing terminals were altered by periodate oxidation. However, the modified pachymans were as susceptible to the attack by L-IV as the original pachyman. These facts indicate that β-1, 3-glucanase L-I from *Irpex lacteus* cleaves pachyman, in an exo-wise fashion to remove glucose upon sequential hydrolysis, from the nonreducing terminals of the chains, whereas β-1, 3-glucanase L-IV attacks at random the inner glucosidic linkages of the pachyman including laminarioligomer. Thus, an untreated glucose residue of β-1, 3-glucosidic linkage at the nonreducing terminal of the substrate appears necessary for the action of L-I.

Pachyman was hydrolyzed at a relatively high rate by both the enzymes, whereas curdlan was not so readily hydrolyzed as had been expected, as shown in Table 2.

L-I acts on scleroglucan,[13] yeast glucan, and laminarans (I, II, and III) containing β-1, 6-linked branches, although the rates of hydrolysis of the glucans are different from one another. The enzymatic hydrolysis products, when scleroglucan was used as substrate, comprized only two components corresponding to glucose and gentiobiose, as revealed by paper chromatography during the entire course of hydrolysis (Fig. 4). However, L-IV has little action on the glucans, except for yeast glucan and laminaran III (L. a.). It is indicated, therefore, that L-I is capable of bypassing the β-1, 6-branch chains in predominantly β-1, 3-linked glucans to cleave β-1, 3-linkages from the nonreducing terminal, producing glucose and gentiobiose[14] until it approaches the reducing terminal, whereas L-IV is apparently incapable of bypassing the β-1, 6-linkages of the β-glucans (Scheme 1).

L-IV attacks lichenan, which contains both β-1, 3- and β-1, 4-glucosidic linkages in an endo manner, at a rate less than half that for pachyman. Although it will eventually produces glucose and laminaribiose as main products from lichenan, the release of 3-O-β-cellobiosyl-D-glucose as an intermediate product of hydrolysis appears to cleave preferentially such internal β-1, 4-linkages as are adjacent to the β-1, 3-linked glucose residue, as deduced according to the hydrolytic process

illustrated in Scheme 2 (Fig. 5). L-IV attacks also reduced, pneumococcal polysaccharide RS III ($\beta-1, 3-$; $\beta-1, 4-$D-glucan) to release laminaribiose as a main product in a similar manner as barley $\beta-1, 3-$; $\beta-1, 4-$glucan endo-hydrolases.[15] As shown in Fig. 6 and Scheme 2, the presence of laminaribiose and the absence of cellobiose in the hydrolysis products reveal that L-IV cleaved only the $\beta-1, 4-$linkages in the polysaccharide RS III. But the enzyme did not show any activity on CM-cellulose ($\beta-1, 4-$linked glucose polymer) lacking $\beta-1, 3-$glucosidic linkage (Table 2). However, L-I had no demonstrable action on these $\beta-$glucans containing $\beta-1, 4-$linkages.

Thus, both the enzymes from this fungus, having such specific action patterns as have been described, should be useful for the structural analysis of glucans containing $\beta-1, 3-$linkages.

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