Carbohydrate Research, 148 (1986) 57–62
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

MODE OF ACTION OF ENDO-(1→3)-β-D-GLUCANASES FROM MARINE MOLLUSCS ON THE LAMINARIN FROM *Laminaria cichorioides*: THE STRUCTURE AND THE INHIBITORY EFFECT OF THE RESULTING (1→3;1→6)-β-D-GLUCO-OLIGOSACCHARIDES

NATALIA M. SHEVCHENKO, TATIANA N. ZVYAGINTSEVA, AND LJUDMILA A. ELYAKOVA
Pacific Institute of Bioorganic Chemistry, Far East Science Centre, Academy of Sciences of the U.S.S.R., Vladivostok (U.S.S.R.)

(Received May 2nd, 1985; accepted for publication, October 15th, 1985)

ABSTRACT

The oligosaccharides released by the action of endo-(1→3)-β-D-glucanases from the marine molluscs *Chlamys albidus* (laminarinase Lo) and *Spisula sachalinensis* (laminarinase LIV) on *Laminaria* laminarin have been studied. For laminarinase Lo, the branched products were shown to be 6^2-β-D-glucopyranosyl-laminaribiose and 6^3- and 6^2-β-D-glucopyranosyl-laminaritrioses by methylation analysis and ^{13}C-n.m.r. spectroscopy. It is suggested that one or two (1→3) linkages adjacent to (1→6) branch-points result in resistance to enzymic attack. 6^3-β-D-Glucopyranosyl-laminaritriose inhibited laminarinases Lo and LIV (I_{50} 1.2×10^{-3} M and 1.5×10^{-3} M, respectively).

INTRODUCTION

Hydrolysis of (1→3;1→6)-β-D-glucans by various (1→3)-β-D-glucanases affords laminarisaccharides and oligosaccharides containing both β-(1→6) and β-(1→3) linkages[1,2]. The patterns of oligosaccharides produced by different (1→3)-β-D-glucanases vary, indicating that there are differences in the modes of action of the enzymes. The *Rhizopus sp.* glucanase catalyses hydrolysis of a β-D-glucosyl linkage at C-1 of the 3-substituted glucosyl residues[3] and this enzyme has been used extensively to study the structure of different (1→3)-β-D-glucans[4–6].

For the enzymic hydrolysis of a branched (1→3)-β-D-glucan from *Grifora umbellata* by exo-(1→3)-β-D-glucanase, the molar ratio of released gentiobiose to glucose (Gen/Glc) was constant (0.5) throughout the hydrolysis[7]. On the other hand, in the early stage of enzymic hydrolysis of a β-D-glucan from *Dictyophora indusiata* with exo-(1→3)-β-D-glucanase from *Basidiomycetes*, mainly gentiobiose was liberated (Gen/Glc 5.5)[8]. The ratio gradually decreased and reached a constant value[8]. These findings indicate that the branching in the glucan occurs irregularly and that the (1→6)-linked β-D-glucosyl groups are mainly near the non-reducing end of the main chain.

We now report the characterisation of di- and branched tri- and tetra-

0008-6215/86/$ 03.50

saccharides isolated from hydrolysates of laminarin from *Laminaria cichorioides*[9–11] which had been degraded by purified endo-(1→3)-β-D-glucanases (Lo and LIV) obtained from the marine molluscs *Chlamys albidus*[12] and *Spisula sachalinensis*[13], and the inhibitory effect of the oligosaccharides on the hydrolysis.

RESULTS AND DISCUSSION

Some aspects of the specificity of glucanases Lo and LIV towards β-D-glucans, including laminarin, have been discussed[9,10]. 6-*O*-β-D-Glucosyl- and 6-*O*-β-laminaribiosyl-D-mannitol were found amongst the products of degradation of laminarin by each enzyme. Velocity constants for the hydrolysis of laminarisaccharides and different glucans were determined for each enzyme.

Laminarin from *Laminaria cichorioides* is a (1→3)-β-D-glucan (d.p. 30) containing about three (1→6) branches per molecule, and the reducing ends are terminated by mannitol (up to 80%)[11]. Each branch contains one glucosyl group. The yields of oligosaccharides released by hydrolysis with glucanases Lo and LIV of laminarin and the (1→3)-β-D-glucan obtained therefrom by Smith degradation are given in Table I. The enzyme hydrolysates were fractionated on Bio-Gel P-2[14].

The major products of prolonged hydrolysis of the (1→3)-β-D-glucan by homogeneous glucanases Lo and LIV were mono- (I), di- (II), and tri-saccharides (III), together with smaller amounts of tetra- (IV), penta- (V), and higher (VI) oligo-saccharides. Most of the (1→3)-β-D-glucan was converted by glucanase LIV into glucose (up to ~60%), but glucanase Lo gave only ~30% conversion into glucose and there was a much larger trisaccharide fraction (III). Laminarin gave more of the fraction of higher molecular weight (Table I). Although each enzyme produced the same array of oligosaccharides from laminarin, the relative amounts in each hydrolysate varied significantly. As seen from the data in Table I, the action

TABLE I

DISTRIBUTION OF THE ENZYMIC HYDROLYSIS PRODUCTS OF (1→3)-β-D-GLUCAN AND LAMINARIN BY GLUCANASES Lo AND LIV

Products[a]	*Contents (%)*			
	Lo		*LIV*	
	(1→3)-β-D-Glucan	*Laminarin*	*(1→3)-β-D-Glucan*	*Laminarin*
Glucose (I)	32.9	22.5	56.6	23.9
Disaccharides (II)	26.1	21.6	26.6	16.5
Trisaccharides (III)	20.0	11.25	7.4	15.0
Tetrasaccharides (IV)	8.1	15.75	5.2	12.2
Pentasaccharides (V)	5.9	8.9	2.2	10.4
D.p. >5 (VI)	7.0	20.0	2.0	22.0

[a]See Fig. 1.

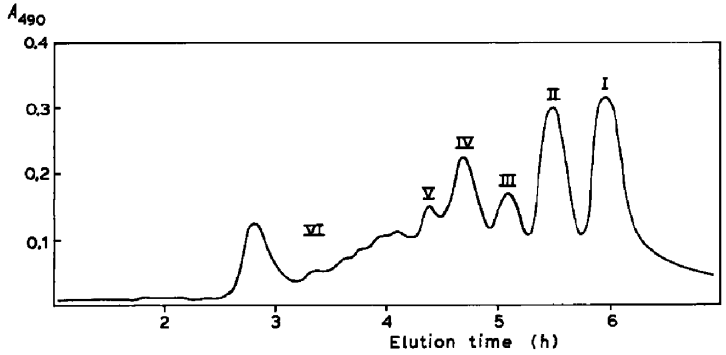

Fig. 1. Gel-permeation chromatography on Bio-Gel P-2 of the hydrolysis products of laminarin by glucanase Lo.

of glucanase Lo on laminarin resulted in the accumulation of tetrasaccharide products, but glucanase LIV gave mainly trisaccharides. Approximately one third of the laminarin was converted into products of d.p. >4 by each enzyme. Laminaritetraose and higher laminarisaccharides are substrates[9] for glucanases Lo and LIV. Therefore, the products of hydrolysis of laminarin by each enzyme are believed to consist of (1→3;1→6)-β-linked oligosaccharides together with traces of laminarisaccharides.

The mixture of di-, tri-, and tetra-saccharides generated by the action of laminarinase Lo on laminarin was resolved on Bio-Gel P-2 (Fig. 1) to give fractions I–V (Table I). P.c. of fraction II revealed laminaribiose [(R_{Glc} 0.8); gentiobiose (R_{Glc} 0.48) was absent]; fraction III contained laminaritriose (R_{Glc} 0.46) and branched trisaccharides (R_{Glc} 0.41) in about equal proportions, and fraction IV appeared to contain mainly branched structures (R_{Glc} 0.27) together with a trace of laminaritetraose (R_{Glc} 0.35).

The ^{13}C-n.m.r. spectra of fractions III and IV contained signals at 85.5 and 69.5 p.p.m. (Table II) attributed to C-3 and C-6 in (1→3)- and (1→6)-β-linked glucans, respectively[2,15]. Resonances of the oligosaccharides were assigned by comparing their spectra with those[2] of known oligosaccharides (Table II). For fraction III, the assignments of the reducing units were made by analogy with the data for laminaritriose, and the internal and non-reducing units with those for the gentiobiosyl unit in 3^2-β-D-gentiobiosylgentiobiose. Therefore, the branched trisaccharide present in fraction III, together with laminaratriose, was identified as 6^2-β-D-glucosyl-laminaribiose. Methylation analysis also indicated the presence of this structure in fraction III; 2,4-di-*O*-methyl-D-glucose, corresponding to 6^1-β-D-glucosyl-laminaribiose, was not detected in the hydrolysate of the fully methylated trisaccharide fraction.

Methylation analysis of the components of fraction IV gave 2,3,4,6-tetra-, 2,4,6-tri-, 2,3,4-tri-, and 2,4-di-*O*-methyl-D-glucose in the molar ratios 3:3:1:1, indicating that 6^3-, 6^2-, and 6^1-β-D-glucopyranosyl-laminaritriose could be present.

TABLE II

^{13}C-N.M.R. DATA FOR GLUCO-OLIGOSACCHARIDES

Compound	*Unit*[c]	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
Fraction III[a]	Rα	92.5	71.6	84.0	68.9	72.3	61.3
	Rβ	96.2	73.8	85.2	69.4	76.2	61.3
	S	103.1	73.8	76.2	70.2	75.3	69.9
	N	103.3	73.8	76.2	70.2	76.2	61.3
Tetrasaccharide (R_F 0.27)[b]	Rα	92.7	71.7	83.2	68.9	72.0	61.5
	Rβ	96.4	74.5	85.5	68.9	76.3	61.5
	S	103.4	73.6	85.9	68.9	76.3	61.5
	T	103.5	74.1	76.3	70.4	75.5	69.5
	N	103.5	74.0	76.3	70.4	76.7	61.5
Laminaritriose[2]	Rα	92.6	71.7	82.9	68.8	71.9	61.4
	Rβ	96.4	74.5	84.9	68.8	76.2	61.4
	S	103.2	73.9	85.2	68.8	76.2	61.4
	N	103.5	74.1	76.2	70.0	76.7	61.4
3^2-β-D-gentiobiosylgentiobiose[2]	Rα	92.8	72.1	73.4	70.2	71.1	69.1
	Rβ	96.6	74.7	76.3	70.2	75.5	69.5
	S	103.2	73.5	84.8	68.6	76.3	61.4
	T	103.5	74.1	76.3	70.2	75.3	69.7
	N	103.5	73.8	76.3	70.3	76.6	61.4

[a]6^2-β-D-Glucosyl-laminaribiose. [b]6^3-β-D-Glucosyl-laminaritriose. [c]Key: R, reducing terminal; N, non-reducing unit; S, second unit; T, third unit.

Methylation analysis of the tetrasaccharide-alditols gave 2,3,4-tri- and 2,4-di-*O*-methyl-D-glucose in the ratio 1:1, and therefore 6^1-β-D-glucopyranosyl-laminaritriose was not present.

Thus, glucanase Lo hydrolyses exclusively the (1→3) linkages of β-D-glucans. The nature of the oligosaccharides produced by glucanases Lo and LIV indicates that not all β-(1→3) linkages can be hydrolysed by these enzymes.

The formation of 6^2-β-D-glucosyl-laminaritriose indicates that substitution of HO-6 of the central residue of laminaritriose hinders hydrolysis of the (1→3) linkages. Modification of the non-reducing end of laminaribiose and laminaritriose, as in 6^2-β-D-glucosyl-laminaribiose and 6^3-β-D-glucosyl-laminaritriose, further complicates hydrolysis of the (1→3) linkages of these oligosaccharides. Thus, glucanase Lo can hydrolyse the (1→3) linkages of laminarin next to a (1→6) branch at the non-reducing end but not at the reducing end.

The fractions III and IV (at concentrations up to 2×10^{-3}M) did not inhibit the action of endo-laminarinases on laminarin and coloured lichenan. However, 6^3-β-D-glucopyranosyl-laminaritriose (R_{Glc} 0.27), isolated from fraction IV, inhibited both glucanase Lo and LIV (I_{50} values[16] 1.2×10^{-3}M and 1.5×10^{-3}M, respectively). This tetrasaccharide, the structure of which was established by methylation analysis and ^{13}C-n.m.r. spectroscopy (Table II), may play a regulatory

role in the degradation of (1→3;1→6)-β-D-glucans by endo-laminarinases and the accompanying (1→6)-β-D-glucanases[17].

The structures of the oligosaccharides produced by the action of glucanase LIV on laminarin and the branched oligosaccharides of d.p. >4 formed by glucanase Lo are being investigated.

EXPERIMENTAL

Enzymes. — Homogeneous endo-(1→3)-β-D-glucanases, LIV from *S. sachalinensis* and Lo from *Ch. albidus*, were purified by published methods[12,13,17] and shown[17] to be free of (1→6)-β-D-glucanases.

Substrates. — A laminarin was prepared[11] from the marine alga *L. cichorioides*, and the corresponding (1→3)-β-D-glucan was obtained by Smith degradation[11]. Coloured lichenan was a commercial preparation.

General methods. — All evaporations were carried out under diminished pressure at 30–40°. Total sugars were determined by the phenol–sulphuric acid method[18] and reducing sugars by the Nelson method[19]. P.c. was performed on FN-15 paper, using 6:4:3 1-butanol–pyridine–water and detection with alkaline silver nitrate[20]. G.l.c. was performed on a Pye–Unicam 104 apparatus fitted with a glass column (0.4 × 150 cm) packed with 3% of QF-1 on Gas-Chrom (100–120 mesh) and programmed from 125° to 225° at 5°/min.

^{13}C-N.m.r. spectra were recorded with an HX-90E Bruker spectrometer for solutions in D_2O (internal methanol, δ 50.1). I.r. spectra were recorded with a Carl Zeiss IR-75 spectrometer.

Enzymic hydrolysis. — Laminarin (2.5 g) (4 mg/mL) was incubated with glucanase Lo or LIV (21 U; 1 unit was defined as the amount of enzyme catalysing the formation of 1 μmol of Glc per min) in 0.05M acetate buffer (pH 5.4) at 25°. The reducing power of the reaction mixture was monitored and reached a maximum value of 63% for glucanase Lo and 70% for glucanase LIV (of total sugar). After inactivation of the enzymes by heating for 10 min at 100°, the digestion mixtures were analysed by liquid chromatography, using a Jeol model JLC-6-AH apparatus[14] (Table I).

Isolation of oligosaccharides. — The mixture (80 mg) was applied to a column (1.6 × 114 cm) of Bio-Gel P-2 (200–400 mesh) and eluted with distilled water at 13 mL/h at 50°. The sugar content in each fraction (2.5 mL) was determined by the phenol–sulphuric acid method[18] (Fig. 1). The procedure was repeated ten times. The fractions were combined as appropriate and concentrated. The resulting oligosaccharides were rechromatographed on Bio-Gel P-2.

Methylation analysis. — Each oligosaccharide (5 mg) was methylated (Hakomori[21]) until the product showed no i.r. absorption for hydroxyl, and then hydrolysed (methanolic 1.5M HCl, 90°, 4 h), and the products were acetylated with acetic anhydride–pyridine (1:1, 1 h, 100°) and subjected to g.l.c.

The tetrasaccharide-alditols (10 mg) formed by reduction with sodium

borohydride (10 mg/mL, 24 h, 25°) were methylated and then hydrolysed, and the acetylated products were analysed by g.l.c.

Inhibition of (1→3)-β-D-glucanases. — Mixtures containing laminarin (1 mg/mL) or coloured lichenan (1.5 mg/mL), oligosaccharide (0–1 mg/mL), and glucanase (LIV or Lo; 5×10^{-2} U) were incubated for 15 min at 35° in 0.05M acetate buffer (pH 5.2). The controls contained no enzyme. The reaction was stopped by the addition of Nelson's reagent or acetone. The residual enzyme activity was assayed by the Nelson method or by measuring the absorbance at 490 nm of the acetone supernatants.

REFERENCES

1 R. YAMAMOTO AND D. J. NEVINS, *Carbohydr. Res.*, 122 (1983) 217–226.
2 F. NANJO, T. USUI, AND T. SUZUKI, *Agric. Biol. Chem.*, 48 (1984) 1523–1532.
3 J. J. MARSHALL, *Carbohydr. Res.*, 34 (1974) 289–305.
4 R. YAMAMOTO AND D. J. NEVINS, *Carbohydr. Res.*, 67 (1978) 275–280.
5 D. J. NEVINS, R. YAMAMOTO, AND D. J. HUBER, *Phytochemistry*, 17 (1978) 1503–1505.
6 R. YAMAMOTO AND D. J. NEVINS, *Phytochemistry*, 22 (1983) 2699–2701.
7 Y. UENO, M. ABE, R. YAMAUCHI, AND K. KATO, *Carbohydr. Res.*, 87 (1980) 257–264.
8 C. HARA, T. KIHO, AND S. UKAI, *Carbohydr. Res.*, 117 (1983) 201–213.
9 L. A. ELYAKOVA, T. N. ZVYAGINTSEVA, AND N. M. PRIVALOVA, *Bioorg. Khim.*, 4 (1978) 1553–1559.
10 T. N. ZVYAGINTSEVA AND L. A. ELYAKOVA, *Abstr. Special Symp. FEBS, Greece*, 1982, p. 285.
11 L. A. ELYAKOVA AND T. N. ZVYAGINTSEVA, *Carbohydr. Res.*, 34 (1974) 241–248.
12 L. A. ELYAKOVA AND N. M. PRIVALOVA, *Comp. Biochem. Physiol. B*, 60 (1978) 225–228.
13 V. V. SOVA, L. A. ELYAKOVA, AND V. E. VASKOVSKY, *Biochim. Biophys. Acta*, 212 (1970) 111–115.
14 T. N. ZVYAGINTSEVA, N. M. DENISENKO, AND L. A. ELYAKOVA, *Khim. Prirod. Soedin.*, 5 (1977) 697–699.
15 H. SAITO, T. OHKI, N. TAKASUKA, AND T. SASAKI, *Carbohydr. Res.*, 58 (1977) 293–305.
16 J. WITWICKI AND M. V. CHIDAMBARAM, *Enzyme*, 29 (1983) 131–132.
17 V. YA. RUDAKOVA, N. M. SHEVCHENKO, AND L. A. ELYAKOVA, *Comp. Biochem. Physiol. B*, 81 (1985) 677–682.
18 M. DUBOIS, K. A. GILLES, J. R. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1965) 350–355.
19 N. NELSON, *J. Biol. Chem.*, 153 (1944) 375–377.
20 J. ROBYT AND D. FRENCH, *Arch. Biochem. Biophys.*, 100 (1963) 451–454.
21 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.