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Transglycosylation and multiple attack of endo-(1→3)-β-D-glucanase L-IV from *Spisula sachalinensis*: a new approach to the evaluation of the degree of multiple attack on polysaccharides

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ABSTRACT

The marked capability for transglycosylation by the endo-(1→3)-β-D-glucanase L-IV from *Spisula sachalinensis* to glycerol, D-glucose, methyl α- and β-D-glucopyranoside, and methyl cellobioside as acceptors was employed to study the multiple attack of the enzyme on laminarin. The enzyme hydrolyses, on average, ~4 glycosidic bonds during enzyme–substrate interaction. The relative transfer constants for each acceptor have been calculated.

INTRODUCTION

There has been much discussion concerning the ability of amylases to hydrolyse several glycosidic bonds during one enzyme–substrate interaction, which resulted in the design of experimental tests for the so-called multiple or repetitive attack[1–5].

These methods showed that porcine pancreatic alpha-amylase is characterized by a high degree of repetitive attack; as for other amylases, different methods may lead to different results.

Enzymes that cleave β-D-glycosidic linkages have been studied little. Evidence that the endo-(1→3)-β-D-glucanase L-IV from the marine mollusc *Spisula sachalinensis* acts by a multiple attack mechanism has been obtained[6–7], and the degree of multiple attack[8] (number of secondary bond cleavages assigned to the number of primary cleavages) on (1→3)-β-D-glucan with an average d.p. of 25 was shown to be ~3.

The enzyme L-IV possessed a relatively high transglycosylating ability in reactions where some aryl glycosides[9] and D-glucose[10] were the acceptors. We now report on the action of L-IV on laminarin in the presence of various acceptors and on the degree of repetitive attack

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EXPERIMENTAL

General. — The method of Sova and Elyakova[11] was used to obtain L-IV that was homogeneous in electrophoresis. [1-^{14}C]Glycerol (specific radioactivity, 1.2 MBq.μmol^{-1}) was obtained from Amersham International. Laminarin was isolated from *Laminaria cichorioides*[12]. Smith-degraded laminarin was obtained as described[8].

The reaction conditions and the analysis of radioactive mixtures were as described[10]. Gel filtration was carried out on a column (0.6 × 100 cm) of Bio-Gel P-2 (−400 mesh) at 55° using an autoanalyser Jeol-JLC 6AH[9], in which sugars were detected by the orcinol–H_2SO_4 reaction. Mass spectrometry involving ERIAD (Russian abbreviation for the extraction of ions from solution under atmospheric pressure) of reaction mixtures was performed at the Institute of Analytical Instrumentation, Academy of Sciences of the U.S.S.R., Leningrad[13]. ERIAD-m.s. can be applied to mixtures of oligosaccharides and their derivatives without their preliminary modification. The unfractionated reaction mixtures in 10^{-3}M CH_3COONa with $<10^{-1}$M cations and $\geq 10^{-5}$M oligosaccharides were analysed directly after the addition of 10 μL of methanol to 5 μL of the mixture, and on the basis of $[M + Na]^+$ ions. There were practically no peaks for fragment ions. The continuous process enables semiquantitative ratios between the relative concentration of oligosaccharides in solution and the areas (heights) of the peaks of the $[M + Na]^+$ ions to be obtained.

RESULTS AND DISCUSSION

The endo-(1→3)-β-D-glucanase L-IV catalyses transglycosylation[9] to such acceptors as D-glucose[10], to give laminari-oligosaccharides with the acceptor glucose at the reducing end.

Fig. 1 shows that the accumulation of the products of transfer to D-[1-^{14}C]glucose[10] depends on the degree of reaction d.r., where d.r. = $1 - RA_1/RA_0$, RA_1 is the radioactivity in the glucose, and RA_0 is the total radioactivity. Up to d.r. 0.12 (the proportion of degraded linkages in the substrate as determined by accumulation of reducing sugars was ~0.1), the dependence of product accumulation on d.r. was linear. These dependences (RA_1/RA_0 on d.r.) are described by the following relationships (obtained by the method of least squares): [^{14}C]laminaribiose, $RA_2/RA_0 = 0.449 \times$ d.r. $+ 11 \times 10^{-4}$; [^{14}C]laminaritriose, $RA_3/RA_0 = 0.154 \times$ d.r. $+ 2 \times 10^{-4}$; [^{14}C]laminaritetraose, $RA_4/RA_0 = 0.105 \times$ d.r. $- 5 \times 10^{-4}$; [^{14}C]laminaripentaose, $RA_5/RA_0 = 0.057 \times$ d.r. $- 4 \times 10^{-4}$; and [^{14}C]lanimarihexaose, $RA_6/RA_0 = 0.039 \times$ d.r $- 4 \times 10^{-4}$. [^{14}C]Laminari-biose, -triose, and -tetraose were present in the initial phase of the reaction at d.r. 0.0236 (the proportion of decomposed linkages was ~0.01). Since L-IV effects an insignificant proportion of the total primary cleavages[8,14] at the non-reducing end of laminarin, this finding may be explained in two ways (Scheme 1).

Scheme 1 A shows the so-called displaced enzyme–substrate complex which forms when D-glucose, added as an acceptor, can bind to the subsites (-2,-3,-4) of the glycon

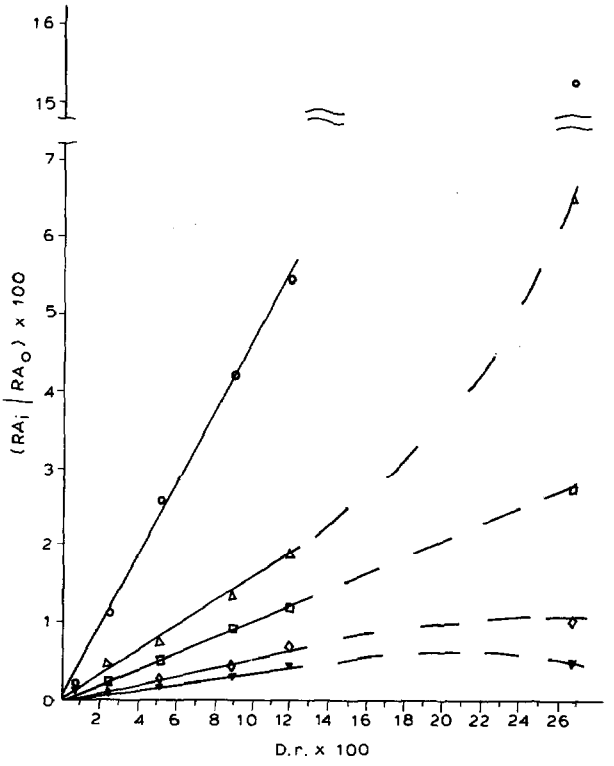
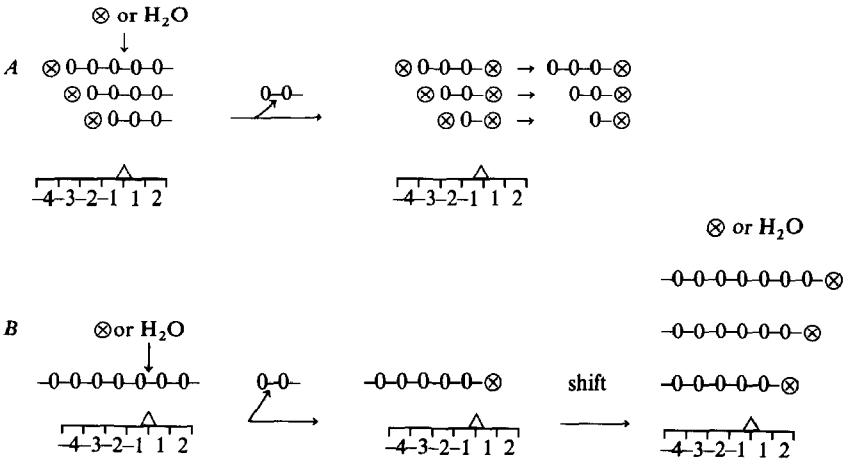


Fig. 1. Accumulation of the products of transglycosylation to D-[^{14}C]glucose[10]: RA_i, radioactivity of the i-mer (i = 2–6); L-IV, 0.08 U.mL^{-1}; [D-glucose] 1.14 × 10^{-3}M; Smith-degraded laminarin, 0.9 × 10^{-3}M; 0, laminaribiose; Δ, laminaritriose; □, laminaritetraose; ◇, laminaripentaose; ▽, laminarihexaose. Points at d.r. 0.0088 correspond to the background of the reaction mixture without enzyme. Straight lines drawn by the method of least squares.



Scheme 1. Degradation of laminarin (-0-0-0-) by L-IV in the presence of D-glucose (⊗). *A*, Transglycosylation involving the displaced enzyme–substrate complex; *B*, transglycosylation accompanied by multiple attack with substrate shift by 1, 2, or 3 D-glucose residues and with the subsequent formation of labelled dimer, trimer, or tetramer: −2 or 2 *etc.* represent the subsites of the glycon or aglycon moieties, and △ indicates the location of the active site.

part of the active centre of the enzyme. In order to estimate the binding of D-glucose to L-IV, the L-IV–D-[^{14}C]glucose dissociation constant (K_d) was determined by equilibrium dialysis[15] and found to be 5×10^{-4}M. According to Elyakova and Zvyagintseva[6], part of the active centre of the enzyme. In order to estimate the binding of D-glucose to L-IV, the L-IV–D-[^{14}C]glucose dissociation constant (K_d) was determined by equilibrium dialysis[15] and found to be 5×10^{-4}M. According to Elyakova and Zvyagintseva[6], K_M for laminarin is 5×10^{-5}M; hence, it may be inferred that the effect of the displaced enzyme–substrate complex on the yield of products when the concentrations of D-glucose and substrate are approximately equal is not substantial (see legend to Fig. 1). Scheme 1B shows the transglycosylation mechanism with subsequent multiple attack on the labelled product–substrate. The low-molecular-weight oligosaccharides are also formed without a lag. The ratio of the sum of the concentration of the labelled dimer, trimer, or tetramer to the concentration of the remaining transfer products after slight degradation of the substrate may be used to estimate the degree of multiple attack (Θ). Since the molar concentrations of the labelled oligosaccharides are proportional to their radioactivity, the following relationship applies.

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The corresponding values of the degree of multiplicity are given in Table I (see ref. 10). Extrapolation to zero degree of cleavage results in $\Theta = 3.6$ (*cf.* 3 ± 1 obtained previously[8]). The deviation for a reaction of <3 min is probably connected with the error of measurement of low levels of radioactivity.

Thus, independent methods confirm that L-IV acts on laminarin by multiple attack. Therefore, the effect of added D-glucose on the total distribution of products, formed from laminarin by L-IV, was examined. Fig. 2 shows that, when D-glucose is added, the ratio of the concentrations of dimer and trimer products is changed substantially. From the experiment with D-[^{14}C]glucose, the ratio of $k_{tr,g}$ (transfer to glucose) to k_{hydr} (constant for transfer to water) can be estimated as

$$\frac{k_{tr,g}}{k_{hydr}} = \frac{[P_{trans}]}{[P_{hydr}]} \times \frac{[H_2O]}{[glucose]}, \qquad (1)$$

where P_{trans} and P_{hydr} are the products of transglycosylation (^{14}C-labelled oligosaccharides) and hydrolysis, respectively, their accumulation being dependent on the increase of the reducing ability of the reaction mixture. Hence, $k_{tr,g}/k_{hydr} = 10^4$, the order of magnitude of which coincides with the reported ratio for *p*-nitrophenyl β-D-glucopyranoside[16]. From equation *1*, it can be calculated that, at 40mM added D-glucose, ~90% of the linkages cleaved in the substrate would be associated with transglycosylation. The molar ratio dimer/trimer for L-IV is 3.8 (Fig. 2B) whereas, in the absence of the acceptor, it varies from 1.4 to 2, depending on the extent of the reaction. Such an increase in the yield is indicative of a substrate shift of one glucose residue during multiple attack.

TABLE I

The evaluation of the degree of multiple attack of the endo-(1→3)-β-D-glucanase L-IV on laminarin based on the products of transglycosylation to D-[1-^{14}C]glucose[10]

Time of reaction (min)	3	9	18	30
Bonds cleaved in the substrate (%)	1	3	6	9
Degree of reaction (d.r.)[a]	1.48	4.29	7.91	11.05
$\sum_{i=2}^{4} RA_i/RA_0$[a]	1.23	3.28	5.9	7.96
Θ	3.8[b]	3.25	2.9	2.6

[a] Allowing for the background, as %.

[b] Determined as $\sum_{i=2}^{4} RA_i \Big/ \sum_{i=8}^{\infty} RA_i$.

In the absence of an acceptor other than water, this shift results in glucose being the main product of multiple attack[8]. In the presence of an acceptor, the main product of multiple attack is the glucosyl acceptor.

This conclusion was confirmed in the study of L-IV-catalysed transfer to glycerol, methyl α- and β-D-glucopyranoside, and methyl cellobioside. The proportion of degraded linkages in the substrate was estimated using a carbohydrate analyser[9] (see Figs. 2–4), with the exception of experiments involving radioactivity. The concentration of the products of hydrolysis was estimated by the increase in reducing sugars. The proportion of transfer products was calculated either as the difference between the first and the

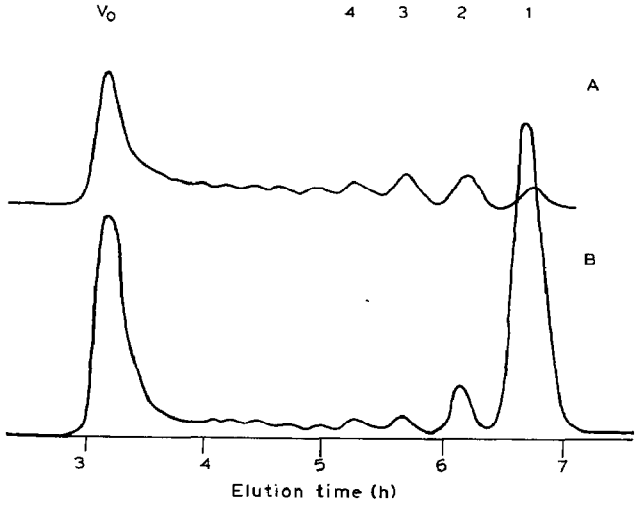

Fig. 2. Formation of products from laminarin (10 mg.mL^{-1}) under the action of L-IV alone (*A*) and in the presence of D-glucose (7.2 mg.mL^{-1}) (*B*), based on the use of a carbohydrate analyser[9]: 1–4 and V_0 correspond to glucose, biose, triose, and tetraose, and void volume, respectively.

TABLE II

The accumulation of glucose as shown by the increase of the absorbance[a] in the glucose oxidizing assay during the degradation of laminarin (1 mg.mL^{-1}) by the endo-(1→3)-β-D-glucanase L-IV in the presence of glycerol

	Time of reaction (min)					
	0	*5*	*10*	*15*	*20*	*25*
	Absorbance (A)					
Control	0.065	0.14	0.21	0.28	0.375	0.45
	0.07	0.14	0.215	0.30	0.375	0.45
ΔA	–	0.07	0.145	0.22	0.305	0.38
0.5M Glycerol	0.09	0.15	0.19	0.24	0.3	0.33
	0.10	0.15	0.195	0.24	0.295	0.335
ΔA	–	0.55	0.1	0.145	0.2	0.235
M Glycerol	0.09	0.125	0.155	0.19	0.22	0.25
	0.085	0.12	0.16	0.195	0.225	0.25
ΔA	–	0.035	0.07	0.105	0.135	0.16

[a] At 450 nm, duplicate readings.

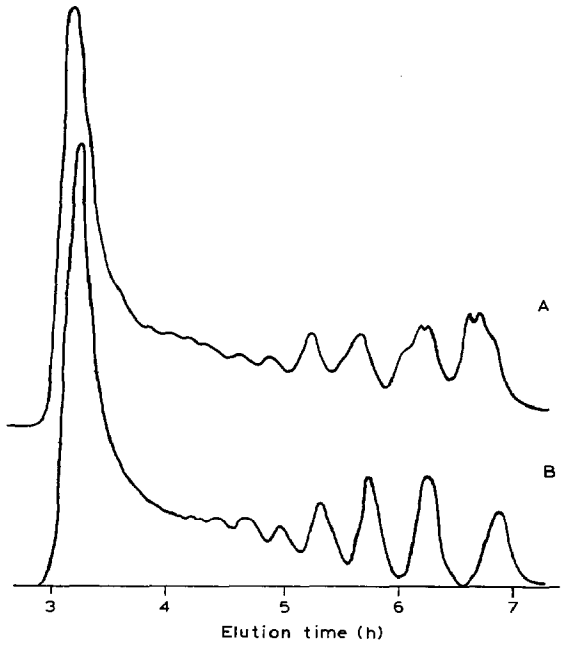

Fig. 3. Chromatograms of products of degradation of laminarin by L-IV: *A*, in the presence of M glycerol (apparent degree of the hydrolysis: reducing sugar as a percentage of total sugars is 4.7%); *B*, control (8% hydrolysis).

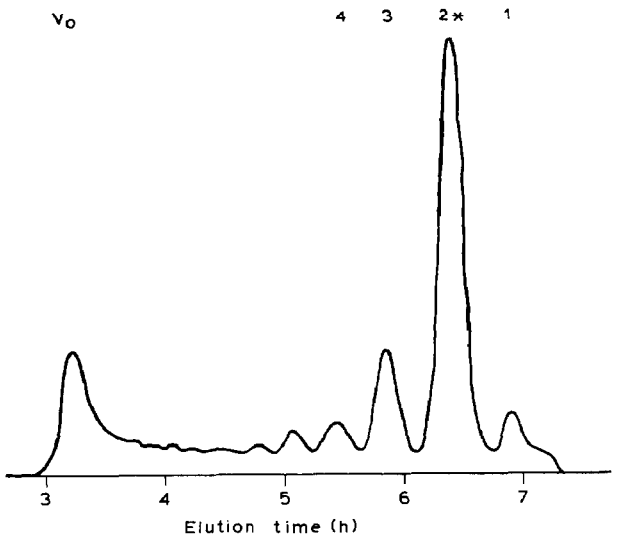

Fig. 4. Formation of products from laminarin (1 mg.mL^{-1}) under the action of L-IV in the presence of methyl α-D-glucopyranoside (1 mg.mL^{-1}). Peaks as in Fig. 2; peak 2* contains laminaribiose and methyl α-D-glucopyranoside.

second products or by radioactivity when the acceptor was [^{14}C]glycerol. No acceptors were found to produce an effect on the total number of bond cleavages in laminarin, in agreement with earlier data[16]. The hydrolysis of laminarin (8 mg.mL^{-1}) in the presence of [^{14}C]glycerol (38mM) yielded radioactive products. Table II shows the effect of glycerol on the yields of glucose during degradation by L-IV. M Glycerol reduced the yields by 50%. Calculation using equation *1* shows that $k_{tr} = 50k_{hydr}$. The effects of methyl α- and β-D-glucopyranoside and methyl cellobioside were similar to that of glycerol at noticeably lower concentrations. All of these have virtually the same k_{tr}/k_{hydr} value $(3 \pm 2) \times 10^3$. On gel filtration on Biogel P-2, methyl α- and β-D-glucopyranoside are not separated from laminaribiose; consequently, the chromatogram (Fig. 4) has altered ratios of the areas of the third and fourth peak, not the second and third peaks as with the glucose acceptor and where the ratio of the area of the glucose peak to those of the oligosaccharides markedly declines compared with that in the control hydrolysis (Fig. 2A).

The addition of glycosidic bond acceptors having no reducing ends decreased the reducing value of the reaction mixture. A similar experiment was performed by Banks *et al.*[5], who studied the degradation of amylose by porcine pancreatic alpha-amylase in the presence of glycerol (40%), erythritol (0.3M), and methyl α-D-glucopyranoside (0.3M). However, in interpreting the dependence of the blue value/reducing value, the authors[5] inferred the disappearance of multiple attack. It appears that the effect of these substances as acceptors in the transglycosylation reaction has not been taken into consideration, which makes this interpretation an ambiguous one.

The study of distribution of transfer products to methyl cellobioside is difficult by

TABLE III

Relative areas of the peaks for $[M + Na]^+$ ions of the products of reaction of laminarin with L-IV in the presence of methyl cellobioside[a]

m/z	Compound[b]	Time of reaction (h)					
		0.5	1	1.5	2.5	4	6
541	MeG_3	15.2±1.4	20.6±0.7	36.0±0.4	34.1±0.3	36.8±9.3	58.6±1.0
703	MeG_4	4.7±0.7	5.5±0.1	9.8±0.8	9.2±0.8	16.3±3.0	19.6±0.6
865	MeG_5	2.1±0.3	2.6±0.4	5.0±0.5	3.5±0.3	2.5±0.7	8.8±0.4
1026	MeG_6	—	0.3±0.3	1.4±0.1	1.2±0.1	2.2±0.5	2.4±0.3
203	G	5.4±0.2	8.4±0.9	5.4±0.2	7.3±0.6	11.1±2.2	18.2±0.5
365	LG_2	1.4±0.2	3.0±0.1	7.7±0.6	4.5±0.4	7.3±1.4	16.3±0.5
527	LG_3	3.3±0.1	6.9±0.5	11.6±0.1	9.5±0.6	11.7±2.1	29.2±0.8
689	LG_4	2.4±0.4	3.0±0.2	6.2±0.1	4.4±0.1	10.1±3.0	15.1±0.3
851	LG_5	0.9±0.4	0.8±0.2	3.0±0.6	1.6±0.1	1.7±0.3	6.2±0.3
1012	LG_6	—	0.3±0.3	1.4±0.6	0.9±0.2	2.3±0.6	3.5±0.9

[a] [laminarin] 10 mg.mL^{-1}, [methyl cellobioside] 5 mg. mL^{-1}. Peak area m/z 379 [methyl cellobioside + Na]$^+$ is taken as 100. [b] MeG_3, MeG_4, MeG_5, and MeG_6 are transfer products of glucose, biose, triose, and tetraose, respectively to methyl cellobioside; G, glucose; LG_2, laminaribiose, LG_3–LG_6, laminari-triose–hexaose.

the gel filtration method. Mass spectrometry involving ERIAD was used (see Experimental). The data are shown in Table III. It has been shown that the ratio of the peak areas of $[M + Na]^+$ of laminaribiose and laminaritriose was close to that of their molar concentrations in the solutions studied[13]. The sensitivity declines by a factor of 1.5–2.5 with each increase of m/z by 150–200 (*i.e.*, an extra sugar unit), depending on the composition of the mixture and the characteristics of the instruments used. However, similar molar ratios for $[LG_2]/[LG_3]$ were obtained with the carbohydrate analyser and ERIAD. $[LG_4 + Na]^+$ was recorded 1.5–2 times less effectively than $[LG_3 + Na]^+$. Comparison of the data in Table III and Fig. 1 shows that the appearance of the products of laminaritetraose transfer to the acceptor (MeG_6 in Table III and ^{14}C-LG_5 in Fig. 1) involves a lag phase which indicates that the laminaritetraose–acceptor complex is mainly a product of secondary hydrolysis. The proportion of the product of multiple attack in the total yield of this compound is too small to be recorded in the initial stage of reaction, hence, the approximation used in estimating Θ. The ERIAD data indicate that the ratio of molar concentration of the basic products of transfer and multiple attack, MeG_3 and MeG_4, does not exceed 2.5 (*cf.* 2.9 for the $[^{14}C\text{-}LG_2]/[^{14}C\text{-}LG_3]$ ratio; Fig. 1). It is possible that the shift of the enzyme in relation to substrate by a given number of monomer units has little dependence on the nature (size) of the added acceptor. Preliminary experiments[17] involving *p*-nitrophenyl β-D-glucopyranoside as an acceptor support this hypothesis.

Thus, the relatively high transglycosylation ability of L-IV allowed independent confirmation of its action on laminarin by the multiple attack mechanism. The extent of multiple attack accords with the view[8] that the main products of the repetitive attacks are formed by substrate shifts of one, two, or three glucose residues.

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