Carbohydrate Research, 152 (1986) 261–273
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

A STUDY OF THE PATTERN OF ACTION OF ENDO-(1→3)-β-D-GLUCANASES FROM MARINE BIVALVES ON A POLYMER SUBSTRATE LABELLED AT THE REDUCING END

PETER W. BEZUKLADNIKOV AND LYUDMILA A. ELYAKOVA*

Pacific Institute of Bioorganic Chemistry, Far East Science Centre, Academy of Sciences of the U.S.S.R., Vladivostok 690022 (U.S.S.R.)

(Received September 21st, 1984; accepted for publication, November 14th, 1985)

ABSTRACT

The pattern of action of L-IV and L-O endo-laminarinases (EC 3.2.1.6) from the crystalline style of the marine bivalves *Spisula sachalinensis* and *Chlamys albidus*, respectively, on Smith-degraded laminarin labelled with ^3H at the reducing end has been studied. The average d.p. of the substrate was 25. On the basis of relative bond-cleavage frequencies, the L-IV enzyme was shown to attack the substrate molecule more than once per encounter. The degree of this repetitive attack and the subsite affinities of the aglycon part of the active centre of both (1→3)-β-D-glucanases have been evaluated.

INTRODUCTION

In the early 1970's, a quantitative theory of the pattern of action of amylases in terms of subsite affinities was proposed independently by Hiromi[1] and by Thoma *et al.*[2]. Once the subsite affinities of the enzyme have been determined, it is possible to calculate the time courses of various products formed from a given substrate by simple hydrolysis. However, the situation may be complicated[3] by repetitive attack and transglycosylation reactions. The influence of the latter on the distribution of the reaction products would decrease as the initial substrate concentration decreased.

Multiple (repetitive) attack in the action pattern of alpha-amylases has been widely discussed[4–6]. Thus far, the multiple attack mechanism has been demonstrated for pancreatic alpha-amylase[6–9]. The question of the occurrence of the multiple-attack mechanism among the endo-carbohydrases which cleave other than α-(1→4) bonds has not been considered yet due to the lack of experimental data.

To date, several experimental and data-processing procedures for the evaluation of the degree of multiple attack have been proposed for the degradation of the large molecules (average d.p. of several hundred glucose residues)[6,7,9] and for the

*To whom correspondence should be addressed.

0008-6215/86/$ 03.50

hydrolysis of oligosaccharides[8,10]. However, there are no naturally occurring, large, water-soluble, homo-linked substrates for glucanases other than amylases. The degrees of multiple attack obtained by study of the action of the enzyme on oligosaccharides are less than those for polymer substrates. Therefore, the most suitable substrates for assessing the multiple-attack mechanism of (1→3)-β- and (1→4)-β-D-glucanases are small, water-soluble polymers which are markedly larger than the active site of the enzyme.

The main problem in evaluating the number of repetitive attacks during the enzymic degradation of small polymers is the determination of the proportion of the products of repetitive attacks in the total products. If repetitive attack does not occur, or if it occurs towards the non-reducing end of a substrate, as with porcine pancreatic alpha-amylase[8], the distribution of the initial cleavage products may be derived by analysis of the products of hydrolysis of substrates labelled at the reducing end[2,3]

We now report on the action of L-O and L-IV endo-(1→3)-β-D-glucanases (EC 3.2.1.6) from the crystalline styles of the marine bivalves *Chlamys albidus* and *Spisula sachalinensis*, respectively, on a linear (1→3)-β-D-glucan (d.p. 25) labelled at the reducing end with ^{3}H. L-IV and L-O are similar in some of their features as endo-depolymerases, and some aspects of their specificity have been considered[11-17]. Particular interest has been focused on the very high yield of D-glucose observed during the early stage of the hydrolysis of the laminarin[15]. The kinetics of the release of D-glucose for L-O (the sigmoid form of the glucose accumulation curve) may be described in terms of preferred attack[17], but it was thought that L-IV effected some repetitive attack in the degradation of laminarin[17].

EXPERIMENTAL

Enzymes. — Homogeneous L-IV and L-O were prepared[12,13] from the crystalline styles of *Spisula sachalinensis* and *Chlamys albidus*, respectively. The mixture of enzymes from *Eulota maakii*, which contained β-D-glucosidase and exo-(1→3)-β-D-glucanase activities, was prepared in our laboratory[18].

General methods. — D-Glucose was determined by the D-glucose oxidase method[19]. The percentage of M-chains (*i.e.*, those that have D-mannitol at the "reducing" end) was determined by the modified Nash method[20]. The phenol–sulfuric acid procedure[21] and Nelson method[22] were used for determining the concentrations of polysaccharides and reducing sugars, respectively. ^{13}C-N.m.r. spectra were recorded with a Bruker HX-90E instrument. G.l.c.–m.s. of glycoside acetates[23] was carried out on a LKB-9000S instrument.

Radioactive substrate. — (*a*) *Preparation.* Soluble laminarin, isolated[24] from *Laminaria cichorioides*, contained 14% of β-(1→6) linkages (^{13}C-n.m.r. analysis), 82% of M-chains, and had d.p. 30. To a solution of laminarin (500 mg) in distilled water (25 mL, used throughout) was added an aqueous solution (25 mL) of $NaIO_4$ (1 g), and the mixture was kept in the dark at 4° for 72 h. An excess of ethanolic

ethylene glycol was then added and the polyaldehyde was dialysed against water until free from formaldehyde. The dialysed solution was concentrated to dryness, and to a solution of the polyaldehyde (400 mg) in water (2.5 mL) was added immediately an aqueous solution (2.5 mL) of NaB^3H_4 (400 mg). The mixture was stored for 24 h at room temperature, the pH was then adjusted to 7, and the polyalcohol was dialysed against water until the radioactivity of the solution was constant. The specific activity of the polyalcohol was 4.5×10^3 d.p.m./μg of glucose. Sulfuric acid (conc., 1 mL) was added to the dialysed solution (90 mL), and the mixture was kept at 12° for 60 h and then dialysed against water until the radioactivity of the solution was constant. Concentration then gave labelled glucan (98 mg). The specific radioactivity of this Smith-degraded glucan was 0.8×10^3 d.p.m./μg of glucose which corresponded to the loss of ~4 non-reducing ends per laminarin molecule. The unlabelled glucan (prepared similarly) contained <3% of β-(1→6) linkages, that is, ~0.6 β-(1→6) linkage per molecule with a d.p. of 25 according to the ^{13}C-n.m.r. data.

(*b*) *Enzymic hydrolysis.* — A typical reaction mixture contained substrate (4 mg) dissolved in mM acetate buffer (4 mL, pH 5.2) containing 5mM NaCl and the enzyme concentration was 0.01 unit/mL*. The L-O reaction mixture contained 0.1 mg/mL of bovine serum albumin in addition to the above components. The reaction temperature was 25°. Aliquots were taken at intervals and heated at 100° for 5 min, and the reducing power (as glucose) and glucose content were determined. The degree of hydrolysis (reducing value/sugar content, as μmol of glucose/μmol of glucose) was ⩾0.1.

P.c. of each aliquot was performed on Whatman No. 3 paper with 1-butanol–pyridine–water (6:4:3) and the multiple ascending technique at 37°. The location of the products was ascertained by using unlabelled compounds, and the corresponding zones were excised and their radioactivity was determined.

Gel filtration was carried out on a column (0.6 × 100 cm) of Bio-Gel P-2 (−400 mesh) using an autoanalyser[16], in which sugars were detected by the orcinol–H_2SO_4 reaction.

Radioactivity was determined with a toluene cocktail for paper chromatogram sections and a dioxane liquid scintillator for aqueous solutions, using a Mark II counter. The proportions of the components in the mixture were calculated as the percentages of the radioactivity in a particular section of the radioactivity of the whole chromatogram.

Analysis of bond-cleavage frequencies. — Allen[25] suggested the data-processing procedure that enabled the frequencies of bond-cleavage of the oligosaccharides to be obtained independently of background radioactivity and the total radioactivity. The separation of all components is of value in this procedure. For a polymer substrate, this method cannot be used without transformation, as the

*One unit is the amount of enzyme which catalyses the formation of 1 μmol of reducing sugar (as glucose)/min under the conditions described.

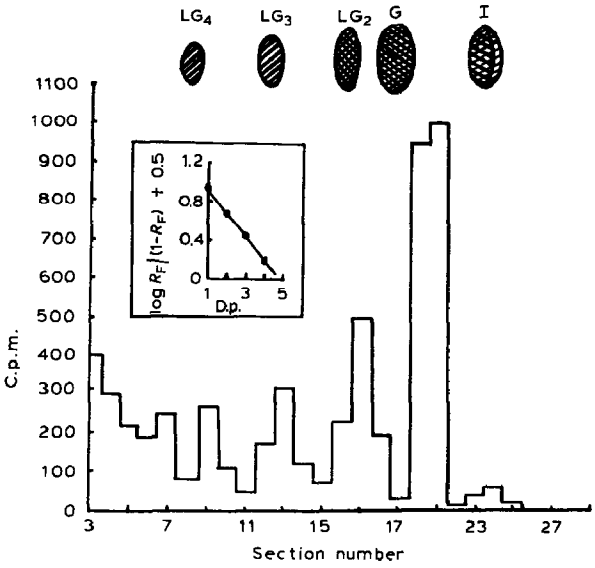


Fig. 1. Paper chromatogram (see Experimental) of the hydrolysate of reducing end-radiolabelled (1→3)-β-D-glucan by L-O. The inset shows a plot of log $R_F/(1 - R_F) + 0.5$ *versus* d.p. of G_iX: I, glycerol and ethylene glycol; G, D-glucose; LG_2, laminaribiose, *etc.*; d.r., 0.36; $RA_o = 15 \times 10^3$ c.p.m., 3 ascents. Sections 20–21, GX; 16–18, G_2X; 12–14, G_3X; 8–10, G_4X; 7, G_5X; 1 and 2, polysaccharides.

experimental possibilities do not allow separation of all of the products of enzymic hydrolysis of such a substrate. If the relative amounts of labelled products beyond the 1st and 2nd sections of a paper chromatogram (see Fig. 1) are assumed to be equal to the degree of reaction (d.r.), then the following interpretation, analogous to that suggested[25], is possible (see Appendix for the details):

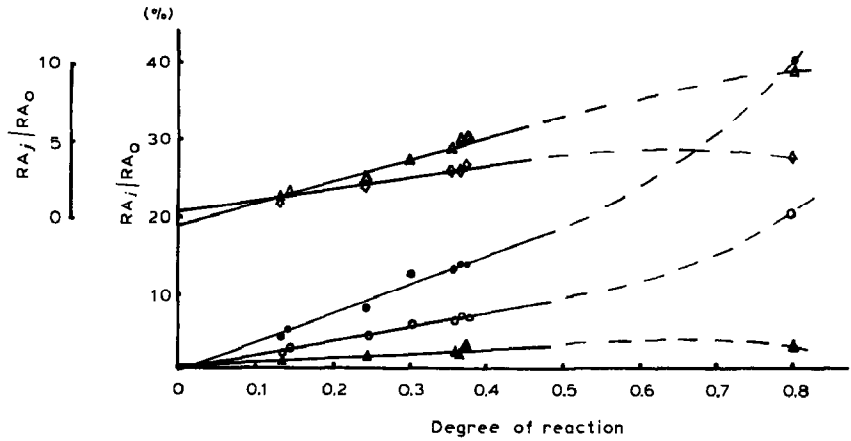


Fig. 2. Analysis of the frequencies of bond cleavage for the hydrolysis of labelled (1→3)-β-D-glucan by L-O: ●, GX; ○, G_2X; △, G_3X; ◇, G_4X; ▲, G_5X; G_iX (G_jX), labelled laminari-oligosaccharides of *i* (*j*) glucose residues. The degree of reaction (d.r.) was calculated by using equation *1*. The lines are weighted least-squares fits to the first points, where d.r. <0.4. The standard deviations in the slopes were <2; $i = 1, 2, 5$; $j = 3, 4$.

$$\text{d.r.} = RA_p/RA_o = 1 - RA_{1+2}/RA_o, \quad (1)$$

where RA_p is the radioactivity of sections 3, 4, *etc.*, RA_o is the total radioactivity, and RA_{1+2} is the radioactivity of the 1st and 2nd paper sections of the chromatogram.

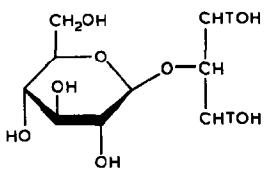
The percentage of each component in the reaction mixture was analysed as a function of the d.r. (see Fig. 2) for determining the relative mole fractions of the hydrolysis products. The slope of each plot in Fig. 2 is the relative mole fraction of the corresponding product (G_iX), which is equal to the relative cleavage frequency of the *i*-bond from the reducing end.

RESULTS AND DISCUSSION

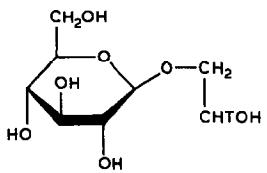
Identification of radioactive products. — A typical distribution of the radioactive products of the hydrolysis of ^{3}H-labelled (1→3)-β-D-glucan is depicted in Fig. 1. The main product GX had an R_F value greater than that of D-glucose. GX reacted weakly with the silver nitrate reagent, was insoluble in toluene, and gave a positive reaction with the phenol–sulfuric reagents, indicating that it contained glucose. The presence of β linkages was proved by hydrolysis by the mixture of enzymes from *Eulota maakii*. P.c. of the digest revealed the radioactivity to be in the region where ethylene glycol and glycerol usually migrated (see Fig. 1) and to be a toluene-soluble product. Analogous hydrolysis of the other product G_2X (see Fig. 1) to the same extent of substrate consumption gave the main peak of radioactivity in the GX-region.

The partial acid hydrolysis of the polyalcohol is the reliable stage of the Smith degradation[26]. It is evident both from the method of substrate preparation and from the above data that GX is a 1-*O*-substituted derivative of D-glucose. The g.l.c.–m.s. data showed that GX contained two 1-*O*-substituted glucose derivatives, and the structures are suggested to be **1** and **2**, so that G_2X, G_3X, *etc.* are, respectively, the radioactive derivatives of laminaribiose, laminaritriose, and higher oligosaccharides.

Evaluation of subsite affinities. — On the assumption that, in the complexes in which the polymer substrate suffers bond cleavage not far from the reducing end, the whole glycon part of the enzyme binding-region is occupied, the known equation (see ref. 3, for example) may be rewritten in such way (equation 2) that the affinity of the subsite *i* is related to the relative mole fractions of oligomers [G_iX]:



1



2

$$A_i = RT \times \ln([G_iX]/[G_{i-1}X]), \quad (2)$$

where A_i is the affinity of the subsite *i* of the aglycon part of the active centre, R is the gas constant, and T is the absolute temperature. Equation *2* is valid only when the rate coefficients for the hydrolysis are the same for various productive complexes, and the subsite interactions with ethylene glycol and glycerol residues are negligible in comparison with those of the substrate monomer.

Typical data for hydrolysis with L-O are illustrated in Fig. 2. The experimental points are the results of processing of the data, such as presented in Fig. 1. The data for L-IV were similar, but the paper chromatogram was irrigated five times and resolution of the homologous series of G_iX up to G_7X was achieved.

The relative frequencies of bond cleavage near the reducing end of the substrate are shown in Fig. 3 for both laminarinases. The aglycon part of the active-centre subsite affinities (A_i) computed by equation *2* are presented in Table I.

Calculation of the degree of multiple attack. — The degree of multiple attack (θ) is equal to the average number of repetitive attacks per initial attack. If the total number of reaction products is N_t and the number of initial attacks is N, then

$$N_t = 2N + \theta \times N = (2 + \theta)N. \quad (3)$$

($2N$: product gain due to initial attack; $\theta \times N$: product gain due to repetitive attack)

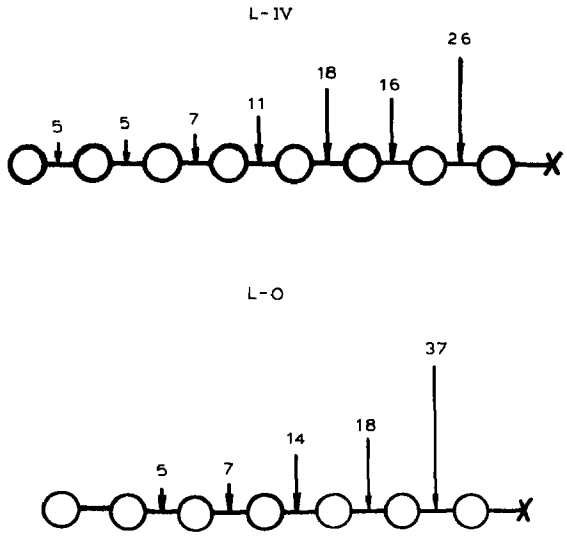


Fig. 3. The relative bond-cleavage frequencies of the labelled (1→3)-β-D-glucan by L-IV and L-O endo-laminarinases; ○, D-Glc residue; ×, ethylene glycol or glycerol residue.

TABLE I

EVALUATION OF THE SUBSITE AFFINITIES OF THE AGLYCON PART OF THE ACTIVE CENTRE AND DEGREE OF REPETITIVE ATTACK OF ENDO-(1→3)-β-D-GLUCANASES L-O AND L-IV.

Substrate	*Enzyme*												
		L-O					*L-IV*						
Labelled	i	1	2	3	4	5	1	2	3	4	5	6	7
	A_i[a]	...[d]	1.8	0.6	1.7	0.8	...	1.2	−0.3	1.2	1.1	0.8	0
	P_i (%)	21	10	8	4	3	15	9	10	6	4	3	3
	[G](%)			28 ±4						42 ±7			
Usual	b_i[b]	1	0.43	0.30	0.18	...	1	0.5	0.4	0.24			
	m	1	2	3			1	2	3	4			
	$\theta(m)$[c]	0.34	0.55	0.7			0.7	1.5	3.2	7			
	$\sigma(\theta)$	0.08	0.18	0.36			0.15	0.6	2.3	11			

[a]Subsite affinities A_i were computed using equation *2* and the relative bond-cleavage frequencies depicted in Fig. 3. Standard deviation $\sigma(A_i)$ was $<\pm 0.3$ kJ/mol. [b]Averaged using the two for L-O, and four for L-IV, carbohydrate analyser chromatograms. [c]The degree of multiple attack θ was computed, using equation *6*, as a function of m. [d]..., Not determined.

If the probability of i-mer appearance from the reducing end of the substrate at the initial attack is P_i and from the non-reducing end is P_{-i}, then the probability of i-mer release, when multiple attack is absent, is equal to $P_i + P_{-i}$, with

$$\sum_{i=1}^{[d.p./2]} (P_i + P_{-i}) = 1.$$

If the maximum d.p. of the products of multiple attack is designated as m and the number of i-mer molecules in the reaction mixture as G_i, then equation *4* is obtained.

$$\sum_{i=1}^{m} G_i = \sum_{i=1}^{m} (P_i + P_{-i}) \times N + \theta \times N \qquad (4)$$

Since the mole fraction (f_i) of i-mer of the total products is G_i/N_t, with

$$\sum_{i=1}^{d.p.=1} f_i = 1,$$

then, from equations *3* and *4*,

$$(2 + \theta) \sum_{i=1}^{m} f_i = \theta + \sum_{i=1}^{m} (P_i + P_{-i})$$

is obtained and finally equation *5*.

$$\theta = \frac{2\sum_{i=1}^{m} f_i - \sum_{i=1}^{m} (P_i + P_{-i})}{1 - \sum_{i=1}^{m} f_i} \qquad (5)$$

The bond-cleavage frequencies P_i can be estimated from the relative bond-cleavage frequencies $[G_iX]$ in the following way. In accordance with current notions about the enzymic depolymerisation of unbranched polysaccharides, the bonds distant from the substrate ends are cleaved with the same frequency. The results presented indicate (see Fig. 3) that the frequencies of formation of G_6X and G_7X during hydrolysis with L-IV are nearly equal. Hence, it is assumed that the

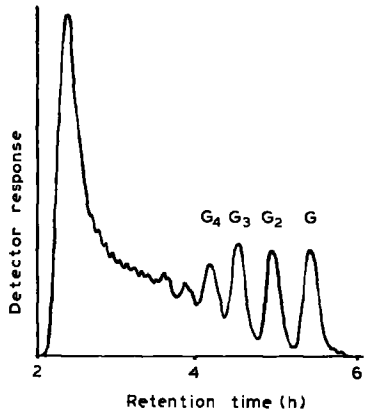


Fig. 4. Chromatogram (see Experimental) of the L-IV hydrolysate of laminarin on Bio-Gel P-2: G, D-glucose; G_2, laminaribiose; *etc.* The degree of hydrolysis was 0.094.

unknown, relative cleavage-frequencies of the internal bonds are equal to the relative bond-cleavage frequency G_7X. It has been shown[27] that the influence of the non-reducing end on the appearance of oligosaccharides of d.p. ≤4 during hydrolysis with L–IV was minor. Based on this result, the approximation that cleavage frequencies of the bonds near the non-reducing end of the substrate are equal to those of the internal bonds was used. Normalisation to 100% of the relative bond-cleavage frequencies $[G_iX]$ then gave the bond-cleavage frequencies P_i shown in Table I.

For f_i with $i \leq 4$, they would be obtained by using the parameters [G] [the ratio of glucose concentration (mol) to the total reducing power of the reaction mixture (as glucose)] and b_i [the relative mole fractions of the oligosaccharides in comparison with the monomer fraction, obtained from the carbohydrate auto-analyser chromatograms (see Fig. 4)]; [G] and b_i are given in Table I for both laminarinases.

Taking into account the existence of the modified reducing-end of the substrate molecule, equation *6* can be used to evaluate the degree of multiple attack (see Appendix).

$$\theta = \frac{(P_1 + [G]) \sum_{i=1}^{m} b_i - \sum_{i=1}^{m} (P_i + P_{-i})}{100\% - [G] \sum_{i=1}^{m} b_i}, \qquad (6)$$

where P_1 is the probability (%) of the appearance of the labelled D-glucose residue GX after initial attack; [G] and P_i are expressed as percentages.

The degree of multiple attack for L-IV and L-O were calculated using equation 6, as a function of m with the values P_i, [G], and b_i obtained. The θ values in Table I indicate that the formation of D-glucose and oligosaccharides during the hydrolysis of laminarin with L-O may be explained mainly by an initial enzyme attack on the basis of the subsite theory. This explanation is unsatisfactory for L-IV, the action pattern of which may be rationalised on the basis of the multiple-attack model with $\theta = 3$, assuming that the products of multiple attack are D-glucose, laminaribiose, and laminaritriose. The standard deviations of θ are shown in Table I as $\sigma(\theta)$.

Thus, it is concluded that, for the endo-(1→3)-β-D-glucanase L-IV, there is a measurable extent of repetitive attack in the degradation of laminarin, which accords with the suppositions made by Mazur and Elyakova[17] based on the data obtained by Elyakova and Zvyagintseva[15].

The subsite affinities in Table I are positive (excluding A_3 of L-IV) and relatively small compared to those of mapped enzymes[3]; consequently, they are not responsible for the substrate binding. Substrate can bind to L-IV[14] and L-O (unpublished data) by interacting with two subsites adjacent to the catalytic amino acid and most probably with subsites of the glycon part of the active centre. Although the nature of "barrier" aglycon subsites is unknown, they account (to some extent) for the high yield of glucose during hydrolysis of natural small-polymer substrates by L-O and L-IV, since exo-glucanases are absent from the crystalline style of bivalvia[28]. Such a composition of the active centre is responsible for the unusual enzyme properties: the main part of the bond ruptures (~50%, see P_i in Table I) are internal, but the probability of bond cleavage near the reducing terminal of the substrate is great.

APPENDIX

Analysis of the relative bond-cleavage frequencies. — The assumptions are those given by Allen[25]; n is the length of the substrate, i is the length of the product, q is the d.p. of the largest product which leaves the origin of the paper chromatogram, G_i^* is the radioactivity of the section of the paper chromatogram containing i-mer, B_i is the background of the section where i-mer usually migrates, $(G_i^* - B_i)$ is the radioactivity of the product, and $[G_iX]$ is the fraction of i-mer among the products q.

From the definitions, we may write

$$[G_iX] = (G_i^* - B_i)/\sum_{j=1}^{q} (G_j^* - B_j), \qquad (IA)$$

and

$$B_i = k_i \sum_{j=1}^{n} G_j^*, \tag{2A}$$

where k_i is a proportionality coefficient for i-mer background.

Substituting equation *2A* in equation *1A* gives

$$[G_iX] = \frac{G_i^* - k_i \sum_{j=1}^{n} G_j^*}{\sum_{j=1}^{q} (G_j^* - k_j \sum_{j=1}^{n} G_j^*)}. \tag{3A}$$

Dividing equation *3A* by

$$\sum_{j=1}^{n} G_j^* \text{ and rearranging gives}$$

$$\frac{G_i^*}{\sum_{j=1}^{n} G_j^*} = [G_iX] \frac{\sum_{j=1}^{q} G_j^*}{\sum_{j=1}^{n} G_j^*} - [G_iX] \sum_{j=1}^{q} k_j + k_i. \tag{4A}$$

The slope of the plot of the left-hand part of equation *4A versus*

$$\sum_{j=1}^{q} G_j^* / \sum_{j=1}^{n} G_j^*$$

gives $[G_iX]$, which equals the relative bond-cleavage frequency.

Combined effect of repetitive attack after initial non-random attack on the yield of oligosaccharides: evaluation of the degree of multiple attack. — When θ_i is the average number of repetitive attacks per initial attack, which gives rise to release of i-mer with

$$\sum_{i=1}^{m} \theta_i = \theta, \tag{5A}$$

then the number of i-mer molecules in the reaction mixture is described by

$$G_i = (P_i + P_{-i} + \theta_i)N, \tag{6A}$$

which is true only when the probability of i-mer re-attack is zero.

Substituting N from equation *3* gives

$$f_i = (P_i + P_{-i} + 100\theta_i)/(\theta + 2)\ (\%), \tag{7A}$$

where P_i and P_{-i} are expressed as percentages.

The mole fraction of monomer (f_i) contains a number of D-glucose molecules (G_1) and some of the labelled D-glucose residues (G_1X); thus,

$$f_i = (G_1 + G_1X)/N_t \times 100\%. \tag{8A}$$

By definition, $[G] = G_1/N_t' \times 100\%$, where N_t' is the total number of reducing ends. As each initial attack at the early stage of hydrolysis gives G_iX in addition to reducing sugars, then $N_t' = N_t - N$; from equation *3*, this gives

$$G_1 = [G]N(\theta + 1)/100\%. \tag{9A}$$

The definition of P_1 gives

$$G_1X = P_1N/100\%. \tag{10A}$$

Substituting equations *9A*, *10A*, and *3* in equation *8A* gives

$$f_i = \{[G](\theta + 1) + P_1\}/(\theta + 2)\ (\%). \tag{11A}$$

By definition, $b_i = f_i/f_1$, and it follows that

$$f_i = b_i\ \{[G](\theta + 1) + P_1\}/(\theta + 2)\ (\%). \tag{12A}$$

Substituting equation *12A* into equation *7A*, and noting equation *5A*, gives

$$\sum_{i=1}^{m} \{[G](\theta + 1) + P_1\}b_i = \sum_{i=1}^{m} (P_i + P_{-i}) + \theta\, 100\%,$$

and hence

$$\theta = \frac{(P_1 + [G]) \sum_{i=1}^{m} b_i - \sum_{i=1}^{m} (P_1 + P_{-i})}{100\% - [G] \sum_{i=1}^{m} b_i},$$

which is identical to equation *6*.

ACKNOWLEDGMENT

The authors thank Dr. A. K. Mazur for helpful criticism.

REFERENCES

1 K. HIROMI, *Biochem. Biophys. Res. Commun.*, 40 (1970) 1–6.
2 J. A. THOMA, C. BROTHERS, AND J. SPRADLIN, *Biochemistry*, 9 (1970) 1768–1775.
3 J. D. ALLEN, *Methods Enzymol.*, 64 (1980) 248–277.
4 J. A. THOMA, *Biopolymers*, 15 (1976) 729–746.
5 W. BANKS AND C. T. GREENWOOD, *Carbohydr. Res.*, 57 (1977) 301–315.
6 A. K. MAZUR, *Biopolymers*, 23 (1984) 1735–1756.
7 J. F. ROBYT AND D. FRENCH, *Arch. Biochem. Biophys.*, 122 (1967) 8–16.
8 J. F. ROBYT AND D. FRENCH, *Arch. Biochem. Biophys.*, 138 (1970) 662–670.
9 W. BANKS, C. T. GREENWOOD, AND K. M. KHAN, *Carbohydr. Res.*, 12 (1970) 79–87.
10 J. D. ALLEN AND J. A. THOMA, *Carbohydr. Res.*, 61 (1978) 377–385.
11 V. V. SOVA, L. A. ELYAKOVA, AND V. E. VASKOVSKY, *Biochim. Biophys. Acta*, 212 (1970) 111–115.
12 V. V. SOVA AND L. A. ELYAKOVA, *Biochim. Biophys. Acta*, 258 (1972) 219–227.
13 N. M. PRIVALOVA AND L. A. ELYAKOVA, *Comp. Biochem. Physiol., B*, 61 (1978) 225–228.
14 L. A. ELYAKOVA, T. N. ZVYAGINTSEVA, AND N. M. PRIVALOVA, *Bioorg. Khim.*, 4 (1978) 1553–1559.
15 L. A. ELYAKOVA AND T. N. ZVYAGINTSEVA, *Bioorg. Khim.*, 7 (1981) 680–685.
16 N. I. NAZAROVA AND L. A. ELYAKOVA, *Bioorg. Khim.*, 8 (1982) 1189–1196.
17 A. K. MAZUR AND L. A. ELYAKOVA, *Mol. Biol. (USSR)*, 17 (1983) 101–112.
18 N. I. SHIROKOVA, N. I. UVAROVA, AND L. A. ELYAKOVA, *Khim. Prir. Soedin.*, 2 (1974) 222–226.
19 A. KESTON, *Abstr. Pap. Am. Chem. Soc. Meet.*, 129 (1956) 31.
20 V. E. VASKOVSKY AND S. V. ISAY, *Anal. Biochem.*, 30 (1969) 25–29.
21 M. DUBOIS, K. A. GILLES, J. R. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
22 N. NELSON, *J. Biol. Chem.*, 153 (1944) 375–381.
23 M. L. WOLFROM AND A. THOMPSON, *Methods Carbohydr. Chem.*, 2 (1963) 211–213.
24 L. A. ELYAKOVA AND T. N. ZVYAGINTSEVA, *Carbohydr. Res.*, 34 (1974) 241–248.
25 J. D. ALLEN, *Carbohydr. Res.*, 39 (1975) 313–315.
26 N. K. KOCHETKOV, A. F. BOCHKOV, B. A. DMITRIEV, A. I. USOV, O. S. CHIZHOV, AND V. N. SHIBAEV, *Carbohydrate Chemistry*, 1967, p. 500 (in Russian).
27 A. A. ARTYUKOV, E. V. SUNDUKOVA, P. W. BEZUKLADNIKOV, AND L. A. ELYAKOVA, *Abstr. Pap. Allunion Symp. Liquid Chromatography, 2nd*, 1982, p. 35.
28 L. A. ELYAKOVA AND T. G. SHILOVA, *Comp. Biochem. Physiol.*, 64 (1979) 245–248.