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STUDY OF CARBOHYDRASE-CATALYZED REACTIONS WITH THE AID OF EDIAP MASS SPECTROMETRY

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The distribution of the products of the enzymatic transformation of various substrates under the action of the endo-β-(1 → 3)-glucanase L IV from the mollusc Spisula sachalinensis has been investigated and the possibility has been shown of the simultaneous recording, with the aid of EDIAP mass spectrometry, of the products of hydrolysis and of transglycosylation. It has been shown that methyl gentiobioside and methyl cellobioside are acceptors in the transglycosylation reaction, as a result of which glucooligosaccharides with a mixed type of glycosidic bonds are formed. The ratio of mono-, di-, tri-, and tetrasaccharides labeled at the ends with ethylene glycol formed in the enzymatic hydroysis of laminarin previously subjected to Smith degradation has been determined.

Workers investigating the mechanism of the action of carbohydrases and, in particular, endo-glycanases suffer from a deficiency of methods permitting the rapid evaluation of the composition and structure of the oligosaccharides formed in reactions catalyzed by these enzymes.

Since the beginning of the eighties, in addition to the method of field desorption, the methods of fast-atom bombardment and of secondary ion emission by ion bombardment have begun to be applied to oligosaccharides and glycolipids [2-5]. A common disadvantage of these methods, apart from fragmentation, in the analysis of mixtures of oligosaccharides is the fact that after the beginning of analysis the growth of the peaks of the quasimolecular ions in the mass spectrum and their subsequent disappearance take place at different rates, depending on the degree of polymerization, which complicates the evaluation of the relative concentrations of oligosaccharides in the mixture. Thus, there are practically no examples in the literature of the solution with the aid of mass-spectrometric methods of any problems connected with the study of the distribution of oligosaccharides and/or their derivatives in mixtures.

As has been shown previously, [6], the EDIAP method permits the analysis of the products of the enzymatic hydrolysis of laminarin ((1 → 3), (1 → 6)-β-D-glucan), consisting of a mixture of glucooligosaccharides, by direct introduction with no preliminary

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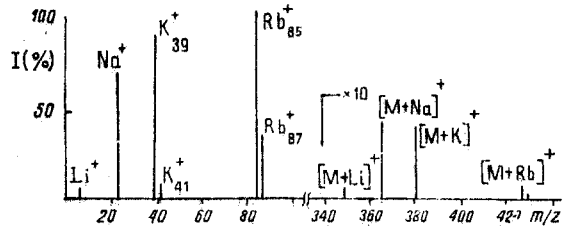

Fig. 1. EDIAP mass spectrum of laminaribiose (10^{-4} M) in the presence of NaCl and KCl (10^{-4} M each) and of Li_2SO_4 and Rb_2CO_3 ($5 \cdot 10^{-5}$ M each).

TABLE 1. Dependence of the Intensity of the Peak of the Quasimolecular Ion of Methyl Gentiobioside on Its Concentration in the Sample Being Analyzed

Intensity of the peak, mm	Concentration of methyl gentiobioside, M				
	10^{-5}	$5 \cdot 10^{-5}$	$2 \cdot 10^{-4}$	$17 \cdot 10^{-4}$	$17 \cdot 10^{-3}$
Maximum	10	20	530	460	130
Mean	8	13	400	172	100
Minimum	5	7	280	100	55

*The mean intensity was calculated from four values without taking the maximum and minimum into account.

modification whatever. Under these conditions there is practically no fragmentation of the oligosaccharides, and the mass spectra only of their quasimolecular ions in complexes with Na^+ are obtained.

In the present investigation, EDIAP mass spectrometry was used for the analysis of the distribution of the by-products of reactions catalyzed by the endo-(1 → 3)-β-D-glucanase L IV from the marine mollusc Spisula sachalinensis (EC 3.2.1.6) [7]. For this purpose, certain laws of the recording of the quasimolecular ions of sugars as functions of their degree of polymerization and structure and the type of cation were determined.

To find the conditions to achieve the maximum sensitivity of the method, we studied the formation of the quasimolecular ions of laminaribiose in the presence of an equimolar amount of salts of various alkali metals in the sequence Li, Na, K, Rb, and at various concentrations of them (from 10^{-6} to 10^{-3} M). It was found that the intensity of the peaks of the alkali-metal cations scarcely changed on the addition of an equimolar amount of laminaribiose and rose in the sequence Li, Na, K, Rb. A typical mass spectrum of laminaribiose with the addition of salts of all four metals is shown in Fig. 1. The peak with the maximum intensity was that of the quasimolecular ion $[M + Na]^+$. The intensities of the other quasimolecular ions fell in the sequence K, Rb, Li. It is just for this reason, and also in connection with the facts that Na^+ is a common cation of buffer solutions and has no isotopes, that this was selected for the experiments.

In order to have the possibility of obtaining quantitative characteristics of the distribution of oligosaccharides by the EDIAP method it is necessary to know the following characteristic features: 1) the dependence of the intensity of the peak of the quasimolecular ion (below, IPQMI) of a given oligosaccharide on the concentration at fixed levels of detection; and 2) the dependence of the IPQMIs of oligosaccharides on their degree of polymerization.

The dependence of the IPQMI on the concentration of the substance is shown in Table 1 for the case of the 1-O-methyl derivative of gentiobiose (6-O-β-D-glucopyranosyl-D-glucopyranose). In all the experiments [NaCl] = 10^{-4} M, it can be seen that with a change in the concentration from 10^{-5} to $2 \cdot 10^{-4}$ M there was a rise in the IPQMI $[M + Na]^+$. Similar results were obtained for laminaribiose. It was found that for the reliable recording of the spectrum (ratio of signal to noise > 3) 50-100 picomoles of a sugar was sufficient.

The dependence of the IPQMIs of sugars on their nature and degrees of polymerization was studied for a number of mixtures containing the following components in various

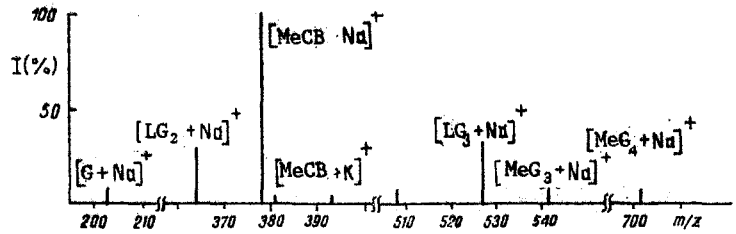

Fig. 2. EDIAP mass spectrum of a reaction mixture: laminaritriose (10^{-3} M); L IV; acceptor – methyl cellobioside ($3 \cdot 10^{-3}$ M). Degree of conversion of the substrate LG_3 ~ 50%.

TABLE 2. Relative Areas of the Peaks of the Quasimolecular Ions of the Products of the Transformation of Laminarin Under the Action of L IV in the Presence of Methyl Cellobioside*

m/z	Compound**	Reaction time, h					
		0,5	1	1,5	2,5	4	6
541	MeG_3	15,2±1,4	20,6±0,7	36±0,4	34,1±0,3	36,8±9,3	58,6±1
703	MeG_4	4,7±0,7	5,5±0,1	9,8±0,8	9,2±0,8	16,3±3	19,6±0,6
865	MeG_5	2,1±0,3	2,6±0,4	5±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±0,1	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±0,2	6,2±0,1	4,4±0,1	10,1±3	15,1±0,3
851	LG_5	0,9±0,4	0,8±0,2	3±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

*[Laminarin] = 10 mg/ml; [MeCB] = 5 mg/ml. Area of the peak with m/z 379 ($[MeCB + Na]^+$) – 100%.
**MeG_3, MeG_4, MeG_5, and MeG_6 are the products of transfer to methyl cellobioside.

combinations: glucose (G), mannitol (Mn), laminaribiose (LG_2), methyl gentiobioside (MeGB), methyl cellobioside (MeCB), laminaritriose (LG_3), and laminaripentaose (PG_5). The concentrations of these substances in the mixture did not exceed 10^{-4} M, while [NaCl] was $5 \cdot 10^{-4}$ M. It was found that with a change in the parameters of the analysis of the sample and the composition of the mixtures being analyzed the quantitative ratios between the IPQMI $[M + Na]^+$ changed, but qualitatively the IPQMIs at various molar concentrations of sugars decreased in the sequence MeCB ≈ MeGB ≈ LG_2 ≈ LG_3 > Mn ≈ G ≥ LG_5. On the whole, a decrease in the efficiency of recording the ions was observed with a rise in m/z, but the peaks of the monomers were weak (results not given). A glycosidic bond is perhaps important for binding with Na^+.

On the basis of the results obtained, we studied the possibility of the formation of oligosaccharides with a mixed type of bond with reactions involving the transfer of residues of a substrate [8] to various potential acceptors catalyzed by the endoglucanase L IV. The acceptors – MeGB and MeCB – had masses different from those of the glucooligosaccharides that were the products of the hydrolysis reaction. Figure 2 shows a typical mass spectrum of a LG_3 + MeCB + L IV reaction mixture. The results for MeGB were similar. In such a reaction mixture only two main products of the transglycosylation reaction could appear: in the case of the transfer of glucose – the trimer MeG_3 methylated at the reducing end, and in the case of the transfer of laminaribiose the tetramer MeG_4. It follows from the mass spectra that both methylated sugars were capable of serving as acceptors in this reaction. The appearance of the transfer products was recorded from the rise in the corresponding peaks in the mass spectrum with an increase in the depth of the reaction. The appearance of both possible transglycosylation products was detected, but the main product of the transfer reaction, taking into consideration the decrease in the size of the peaks with a rise in m/z, was MeG_4. Consequently, it may be assumed that in laminaritriose the enzyme catalyzes the cleavage of the bond close to the reducing end more frequently than at the nonreducing end.

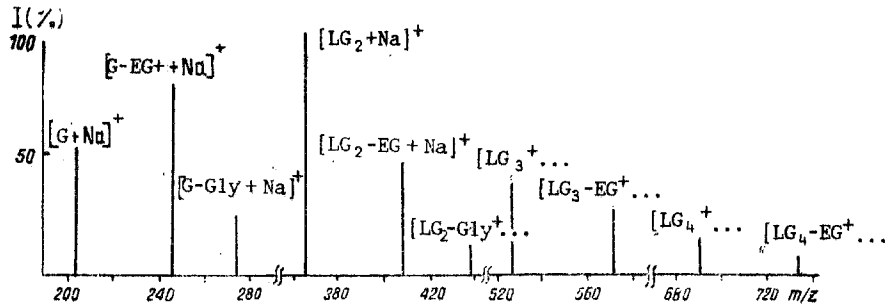

Fig. 3. EDIAP mass spectra of hydrolysis products of Smith degradation of laminarin (1 mg/ml) by glucanase L IV. G-Gly, LG_2-Gly, G-EG, LG_2-EG, etc.: Peaks of quasimolecular ions $[M + Na]^+$ glycosyl-(glycerol), laminaribiosyl(glycerol), glycosyl(ethylene glycol), and laminaribiosyl(ethylene glycol), etc.

When laminarin was used as substrate and methyl cellobioside as acceptor, four new peaks, in comparison with control (a mixture of solutions of MeCB and a laminarin hydrolysate) appeared in the mass spectrum of the reaction products. Their m/z values corresponded to those of the quasimolecular ions with Na^+ of the products of the transfer to MeCB of, respectively, glucose, a biose, a triose, and a tetraose. The changes in the relative areas of the peaks with an increase in the depth of the reaction are shown in Table 2. It must be stated that the acquisition of similar information on the composition of these reactions by other methods is an extremely laborious problem, while the use of the EDIAP method, thanks to the absence of fragmentation and the continuous feed of the sample being analyzed, permits the selection of the best conditions for recording and the recording of readily interpretable mass spectra in a few minutes.

To study hydrolysis reactions catalyzed by carbohydrases, one of the most effective methods of evaluating the distribution of the products formed is that in which a substrate radioactively labeled at its end is used [9, 10]. In this case, the distribution of the unlabeled products is determined in independent experiments. The natural substrate β-(1 → 3)-glucanase – laminarin from Laminaria cichorioides – contains at its "reducing" end a considerable proportion of molecules of a natural label – a mannitol residue with a molecular mass 2 amu greater than for a glucose residue [11]. As has been shown previously [6], the EDIAP MS method permits the recording, against a background of the peaks of laminarioligosaccharides, of the distribution of the mannitol-containing oligosaccharides formed in the hydrolysis of the substrate. In order to evaluate such distributions, with the aid of EDIAP MS we have studied the kinetics of the accumulation of the product of the enzymatic hydrolysis by the β-(1 → 3) glucanase L IV of laminarin that had been subjected to Smith degradation (a linear β-(1 → 3)-glucan with DP ~ 25, containing at the reducing end ethylene glycol (EG) and glycerol (Gly) residues in place of the mannitol and glucose residues, respectively, present in the initial laminarin [10]). In the typical mass spectrum of the reaction mixture shown in Fig. 3, the peaks of the quasimolecular ions of mono-, di-, tri-, and tetrasaccharides containing ethylene glycol residues can be clearly seen. In view of the characteristics of the EDIAP mass spectra described above, the ratio of glycosyl(ethylene glycol), laminaribiosyl(ethylene glycol), laminaritriosyl(ethylene glycol), and laminaritetraosyl(ethylene glycol) observed with the aid of this method was 26:16:18:11, respectively, which is what was obtained previously in a study of the kinetics of the hydrolysis of just the same substrate containing an end-residue labeled with tritium [10].

Thus, the EDIAP method, permitting semiquantitative estimates to be obtained of the distribution of oligosaccharides and/or their derivatives in mixtures being analyzed, can be used successfully for recording the products both of the reactions of transglycosylation to acceptors with molecular masses different from the molecular masses of the hydrolysis products and of the hydrolysis products themselves in one and the same sample.

EXPERIMENTAL

The endo-β-(1 → 3)-glucanase L IV was isolated as described in [7]. Methyl gentiobioside and methyl cellobioside were synthesized from commercial gentiobiose and cellobiose preparations (Fluka), by a standard method [12]. The laminarin from Laminaria cichorioides

and the Smith-degraded laminarin were obtained by the methods of [11] and [10], respectively. Laminarioligosaccharides with degrees of polymerization from 2 to 5 were obtained by the formolysis of the linear β-(1 → 3)-glucan pachyman from Poria cocos followed by chromatography on Bio-Gel P-2. The other reagents were of kh.ch. ["chemically pure"] grade.

Reaction mixtures were obtained by mixing aqueous solutions of their components in the appropriate proportions. To initiate the enzymatic reaction, an aliquot of the initial solution of L IV was added such that the final enzymatic activity was 10^{-2} U/ml (U is the amount of enzyme catalyzing the formation from laminarin of 1 μmole of reducing sugars per minute), and NaCl and Na acetate buffer were added to a concentration of 1 mM. The activity of the glucanase was determined by Nelson's method [13] from the increase in the amount of reducing sugars in the reaction mixture. The reaction was performed at 25°C. It was stopped by boiling an aliquot for 5 min.

Mass spectra were obtained on an experimental specimen of a mass spectrometer with an EDIAP attachment constructed in the Scientific and Technical Division of the USSR Academy of Sciences [14] and fitted with a dialogue-computing complex (DCC). From an aliquot was taken 5-10 μl of solution and it was added to a double volume of methanol, and samples were introduced into the mass spectrometer in this form. The stability of admission was checked by the constancy of the monitor current and the distribution of the areas of the peaks obtained was checked with the aid of the DCC by not less than five scannings of each spectrum. The rate of feed of the samples was 0.2-0.4 μl/min.

SUMMARY

1. It has been shown that methyl gentiobioside and methyl cellobioside are acceptors in the transglycosylation reaction catalyzed by the endo-β-(1 → 3)-glucanase L IV.

2. The ratio of mono-, di-, tri-, and tetrasaccharides labeled at the end with an ethylene glycol residue formed on the enzymatic hydrolysis of Smith-degraded laminarin has been determined and in this way it has been shown that the EDIAP MS method permits a semiquantitative estimate of the distribution of the products of the transformation of a β-(1 → 3)-glucan.

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