PARTIAL CHARACTERIZATION OF THE ACTIVE SITE OF CARROT POLY(GALACTURONATE) HYDROLASE AND DEGRADATION OF OLIGO(D-GALACTOSIDURONATES)

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Received November 12, 1993
Accepted October 31, 1994

The dimension and arrangement of the active center in carrot poly(1→4–α-D-galacturonide)galacturonohydrolase (E.C.3.2.1.67) is studied. Molecular activities k_0 and parameters k_0/K_m were calculated from the experimentally determined Michaelis constants K_m and maximum rates of catalytic hydrolysis of linear oligo(D-galactosiduronates) (polymerization degree n = 2 to 7) at pH 5.0 and 30 °C. From the dependence of log k_0 and log k_0/K_m on n we derived that the active center consists of six subsites, the catalytic site being situated between the first and second subsite. In accord with the theory of Hiromi [Hiromi K.: Biochem. Biophys. Res. Commun. *40*, 1 (1970)], the kinetic data were used for calculation of affinities (A_i) of the third (A_3) to the sixth (A_6) subsite. Two possible models were studied for the action of carrot poly(galacturonate)hydrolase which catalyzes the gradual terminal hydrolytic cleavage of oligo(D-galactosiduronates) from the non-reducing end of the molecule. The distribution of products in monomolecular hydrolysis of penta(D-galactosiduronate) under optimal conditions (pH and temperature) indicates a multi-chain enzymatic attack with predominant single collision. The kinetic results of the enzyme degradation are in good accord with the above-mentioned assumption.

The character of the active center in enzymes catalyzing the degradation of oligo- and polysaccharides determines their specificity and the mode of action. Therefore, an investigation of enzymatic degradation of defined substrates may afford information on the character of the active site and on the action of the enzyme. Many endopolygalacturonases were studied in this respect and were classified[1] into three groups (A, B and C) according to the mode and rate of degradation of oligo(D-galactosiduronates). Detailed studies of the active site, i.e. its size, localization of the catalytic groups and affinities of the subsites, have contributed to elucidation of the existing substantial differences in the mode of action of endopolygalacturonases. The active center in endopolygalacturonase from the surface culture *Aspergillus niger* (mode of action A) consists of four subsites, the catalytic site being situated between the first and second subsite from the reducing end of the substrate molecule[2]. In endopolygalacturonases

with the degradation mode B the active center comprizes three subsites, in those with the mode C it consists of five subsites, with different location of the catalytic sites[2–4].

Compared with endopolygalacturonases, much less is known about pectolytic enzymes with terminal mode of action. The reason is their low content in plants, meagre production by microorganisms, and difficult purification. The common feature of this group of enzymes is the (α, 1→4) cleavage of the substrates, predominantly from the non-reducing end, the primary product being D-galactopyranuronate. Differences in the preference and rate of degradation of oligomeric and polymeric substrates, and also in the propensity to cleave modified D-galactosiduronates have been found[1]. Most of these enzymes do not degrade pectin; they degrade polygalacturonic acid and sodium pectate with various velocities and to various degrees[5,6]. More pronounced are the differences in action of these enzymes on oligomeric substrates. For most of plant exopolygalacturonases the reaction rate increases with increasing chain length of the degraded oligosaccharide[7–10]. Other exopolygalacturonases, such as those isolated from surface culture *Aspergillus niger*[11] or pectinase Pectinex ultra[12], prefer tetra(D-galactosiduronate). A marked difference in the mode of action was observed with galacturonases isolated from an *Aspergillus niger* micelium extract. These enzymes highly preferred the lowest degradable substrate, di(D-galactosiduronate)[13–15]. Similarly, a disaccharide was the preferred substrate for the galacturonase from bacterium *Butyrvibrio fibrisolvens*[16]. For the exopolygalacturonase produced in vivo by *Penicilium digitatum*, the chain length of the substrate was not the rate-limiting factor[17].

The found substantial differences in the action of terminally degrading galacturonases indicate a different character of the active sites. However, the active sites in this group of enzymes have not been investigated; so far, only the active site in poly(galacturonosidase) of *Selenomonas ruminantium* has been partially characterized[18].

At present, two methods are available for the study of the mode of action of glycanohydrolases: these are based on the concept of subsites of the active center. Hiromi[19,20] suggested a kinetic model for characterization of the active site and calculation of the affinity of subsites, based on the dependence of degradation rate on the polymerization degree of the substrate. Thoma and collaborators[21,22] suggested a method based on the study of terminally marked reaction products. For terminally active enzymes, which mostly form a single productive complex, the kinetic method seems to be more suitable[23]. The studied enzyme, poly(1→4-α-D-galacturonide)galacturonohydrolase (E.C.3.2.1.67) (exoPg) isolated from carrot, represents an enzyme of typically terminal mode of action. It catalyzes specifically the (α, 1→4) degradation of sodium pectate, affording monosaccharide as the only product. The substrates are attacked at the non-reducing end of the molecule and the hydrolysis rate depends on the chain length of the degraded substrate[7,24]. Because basic characteristics of the investigated exoPg are similar to those of α-glucoamylase, we characterized its active site using the kinetic model of Hiromi and collaborators[19,20,25].

For the degradation of poly- and oligosaccharides by enzymes with terminal hydrolysis mechanism, two different limit models exist: a single-chain and a multi-chain mechanism. Whereas for the action of plant β-amylases on high-molecular amylose (n = 100) some authors assume only the single-chain mechanism[26,27], other authors are inclined unequivocally to a multi-chain process[28,29]. Later on, in the studies of the effect of β-amylase on low-molecular amylose (n = 44) and maltodextrins (n = 6 and 7), French and collaborators[30,31] as well as Bailey and coworkers[32,33] have found that the degradation of all these substrates lies between the single- and multi-chain mechanisms. Because of these different results, we decided to study the mode of action of carrot exoPg in hydrolytic cleavage of oligo(D-galactosiduronate) with polymerization degree 5. Quantitative product analysis, together with kinetic studies of the enzymatic reaction, makes it possible to determine the predominant mechanism in reactions of glycanohydrolases proceeding with a terminal mechanism[34–36].

EXPERIMENTAL

Materials

Carrot exoPg was isolated and purified as described previously[24]. Residues from pressing of *Daucus carota* subsp. *sativus* cv. Karotka were extracted with 5% NaCl solution at pH 5.0 and room temperature. The isolation comprised: salting out with ammonium sulfate up to 90% saturation, separation of the protein complex by chromatography on DEAE Sephadex (A-50), desalting on Sephadex G-25 (Medium), subsequent separation on Sephadex G-100 (Fine), desalting on Sephadex G-25 (Medium) and freeze-drying. The thus-prepared enzyme, specific exoPG 70-fold purified, had relative molecular weight 50 000. At pH 5.0 and 30 °C, the specific activity of the purified enzyme in the reaction with sodium pectate amounted to 0.032 μcat/mg. The specificity of the enzyme was proved by product analysis using polymeric and oligomeric substrates[24].

Oligo(D-galactosiduronates), [α-D-(1→4)] linked units of sodium salt of D-galactopyranuronic acid (polymerization degree n = 2 to 7), were prepared by hydrolysis of sodium pectate (poly(1→4)-α-D-galactosidepyranuronate), separation of the complex of oligosaccharides on Sephadex G-25 (Fine) and subsequent desalting on Sephadex G-10 (ref.[37]). The homogeneity of the oligo(D-galactosiduronates) was proved by thin-layer chromatography on silica gel (Silufol, The Czech Republic) in 1-butanol–formic acid–water (2 : 3 : 1), log [R_F/(1 − R_F)] values being linearly dependent on the polymerization degree[38]. The purity was determined from the ratio of reducing groups to carboxyl groups[39].

Methods

The activity of exoPg was determined by measuring the increase in reducing groups using the method of Somogyi[40] and a D-galactopyranuronic acid calibration graph. As substrate we used 0.5% solution of sodium pectate (molecular weight 27 000, determined viscosimetrically) in 0.1 M acetate buffer pH 5.0. The enzyme solution (0.5 ml) was incubated with the same volume of substrate solution at 30 °C.

The mode and degree of degradation of penta(D-galactosiduronate) were determined as follows. The samples of the reaction mixture contained 37.5 μmol of the oligosaccharide and 0.07 mg of the enzyme in 3.5 ml of 0.1 M acetate buffer pH 5.0. After incubation for 30, 60 and 180 min at 30 °C,

an aliquot (0.1 ml) was taken and analyzed for the increase of reducing groups by the method of Somogyi[40]. Fractions (2 ml) of the sample (inactivated by boiling for 20 min) were applied onto two 20 × 120 cm Sephadex G-25 (Fine) columns connected in series, that had been equilibrated and washed with 0.1 M acetate buffer pH 4.4. Fractions (4 ml) were taken in 30 min intervals. Each fraction was subjected to TLC to identify the individual oligo(D-galactosiduronates); their population was determined on the basis of the reducing power by the Somogyi method[40].

RESULTS

Kinetic Data

To obtain the kinetic parameters K_m and V for the individual oligo(D-galactosiduronates) we determined the initial rates at six or eight concentrations of the substrate s in the region 0.025 – 0.8 mmol/l at pH 5.0 and 30 °C. The individual sets of the experimental values of v and s were subjected to direct nonlinear regression analysis with the regression function $v = Vs/(K_m + s)$, using a personal computer[41]. The resulting K_m and V, together with the statistical characteristics, are given in Table I. The table also gives molar concentrations of the enzyme, e_0, molecular activities, $k_0 = V/e_0$, and ratios k_0/K_m.

As seen from the dependence of log k_0 and log k_0/K_m on the polymerization degree (Fig. 1), the rate constants increase with increasing degree of polymerization when $n < 6$, and remain constant when $n \geq 6$; this shows that the active site of carrot exoPg contains six subsites. As follows from the cleavage of the polymeric substrate (monosaccharide as the only reaction product) and of the oligo(D-galactosiduronates), n = 3 to 7 (monogalacturonate as the primary product in the region 0.025 – 0.8 mmol/l), the catalytic site is situated between the first and the second subsite.

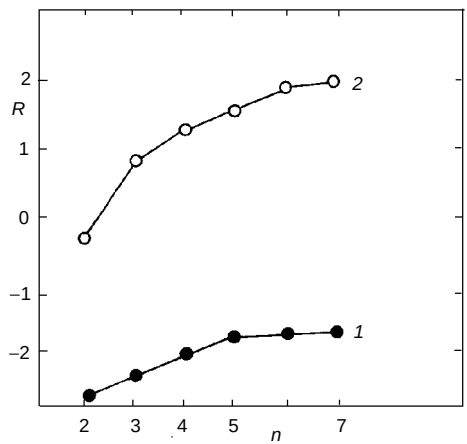


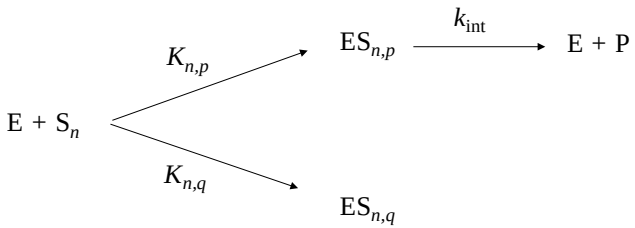
FIG. 1
Dependence of kinetic properties of carrot poly(galacturonate)hydrolase on polymerization degree, n, of oligo(D-galactosiduronates). *1* R = log k_0, *2* R = log k_0/K_m

Calculation of Affinity of Subsites

The affinity of subsites on the reducing side of the catalytic site was calculated using the algorithm described for amylases[19,20,23]. Its main features are described below.

The mechanism of hydrolysis of oligo(D-galactosiduronates) by carrot exoPg is described by Scheme 1.

Symbol E is the enzyme, S_n is the substrate of polymerization degree n, P are the products, $ES_{n,p}$ and $ES_{n,q}$ denote the productive and nonproductive complex, respec-



SCHEME 1

TABLE I
Experimental values of kinetic parameters with standard deviations and regression parameters for hydrolysis of oligo(D-galactosiduronates), catalyzed by poly(galacturonate)hydrolase at 30 °C and pH 5.0 (n degree of polymerization, K_m Michaelis constant, V maximum reaction rate, k_0 molecular activity, e_0 enzyme concentration)

Substrate	n	K_m, μmol l^{-1} V, nmol s^{-1}	Determinaton coefficient	Variation coefficient of regression %	e_0, nmol l^{-1} k_0 s^{-1}	k_0/K_m l mol^{-1} s^{-1}
Di(D-g)[a]	2	4 580 ± 1 100 2.23 ± 0.48	0.998	1.92	1 086 0.00206 ± 0.00037	0.449 ± 0.080
Tri(D-g)	3	498 ± 49 3.09 ± 0.17	0.997	2.92	1 086 0.00285 ± 0.00016	5.72 ± 0.31
Tetra(D-g)	4	400 ± 39 3.68 ± 0.18	0.996	3.05	543 0.00617 ± 0.00033	15.4 ± 0.83
Penta(D-g)	5	413 ± 27 6.01 ± 0.21	0.998	2.06	543 0.0111 ± 0.0004	26.9 ± 0.94
Hexa(D-g)	6	325 ± 23 7.58 ± 0.25	0.997	2.38	543 0.0139 ± 0.0005	42.7 ± 1.4
Hepta(D-g)	7	309 ± 22 8.16 ± 0.26	0.997	2.35	543 0.0150 ± 0.0005	48.6 ± 1.6

[a] (D-g) (D-galactosiduronate).

tively, and k_{int} is the intrinsic rate constant of hydrolysis in each productive complex. $K_{n,p}$ and $K_{n,q}$ are the rate constants of the formation of productive and nonproductive complex, respectively. The symbols p and q denote the kind of the bond and are equal to the number of the subsite at which, at the given bond kind, the nonreducing end of the monosaccharide is situated.

Since carrot exoPg is an enzyme with typical terminal action[8,24], we can assume a model of the active site that is schematically depicted in Fig. 2. The active site consists of six subsites (m = 6). The subsites are numbered from left to right, starting from the non-reducing substrate unit situated in the productive bonding. The catalytic site at which the bond is broken is localized between the first and second subsite. The affinities of the subsites are denoted A_i (i = 1, 2,…m). As follows from the kind of bonding of the pentasaccharide molecule to the active site (Fig. 2), the only possible productive kind of the bond is j = 1 or p = 1. Then the binding constant for the enzyme E and substrate S_n, which is bonded by the j-th kind, is given by relationship (*1*).

$$k_0/K_m = k_{int}K_{n,j} = k_{int}K_{n,1} \qquad (1)$$

The binding constant $K_{n,j}$ is connected with the molar bond affinity, $B_{n,j}$ which is a sum of the subsite affinities A_i (i.e. subsites occupied by the substrate molecule by the bond kind j). It follows that

$$\boldsymbol{R}T \ln K_{n,j} = -\Delta G_{n,j} = B_{n,j} - Z = (\sum_{1}^{cov} A_i)_{n,j} - Z \ , \qquad (2)$$

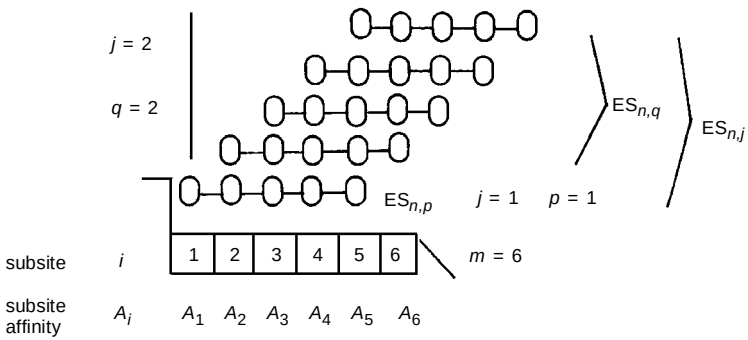


FIG. 2

Schematic model of the active site of carrot poly(galacturonate)hydrolase and kind of the bonding of penta(D-galactosiduronate). The active site consists of m = 6 subsites. The catalytic site is denoted by a wedge, A_i (i = 1, 2, 3…m) are affinities of subsites, $ES_{n,j}$ denotes enzyme–substrate complex, $ES_{n,p}$ is productive complex, $ES_{n,q}$ unproductive complex. Indices j, p and q are numbers specifying the bond kind, –O– represents a galacturonate unit

where $\Delta G_{n,j}$ is the change of standard molar free energy in the formation of the complex $ES_{n,j}$, $\boldsymbol{R}$ is the gas constant, T absolute temperature and the correction member Z relates to the molar free energy of mixing in water; its value amounts to 10.38 kJ/mol at 30 °C. For the binding constant we get

$$K_{n,j} = 0.0186 \exp \left(\sum_{1}^{\text{cov}} A_i / \boldsymbol{R}T\right)_{n,j} \quad . \tag{3}$$

Using Eqs (*1*) and (*2*), we can write for k_0/K_m

$$k_0/K_m = 0.0186 k_{int} \exp \left(\sum_{1}^{\text{cov}} A_i/\boldsymbol{R}T\right)_{n,j} \quad , \tag{4}$$

where k_{int} is the intrinsic rate constant of hydrolysis of the substrate bond in the productive complex. The affinities of subsites A_3 to A_6 can be then calculated in the following manner[19,20,23]:

$$A_{n+1} = \boldsymbol{R}T[\ln (k_0/K_m)_{n+1} - \ln (k_0/K_m)_n] \quad . \tag{5}$$

After introducing experimental values of k_0/K_m from Table I into Eq. (*5*), we get for the subsite activities A_3 to A_6 the following values: A_3 = 1.53 kJ/mol, A_4 = 0.59 kJ/mol, A_5 = 0.35 kJ/mol, and A_6 = 0.27 kJ/mol. The calculated values are given in the histogram depicted in Fig. 3.

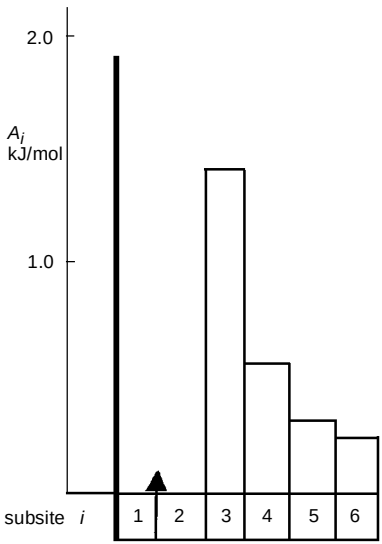


FIG. 3
Histogram of affinities of subsites in carrot poly(galacturonate)hydrolase. The active site consists of six subsites *i* and the catalytic site (denoted by a wedge) is situated between the first and second subsite. The subsites are numbered from the nonreducing end. The respective affinities A_i of the third to the sixth subsite are A_3 = 1.53 kJ/mol, A_4 = 0.59 kJ/mol, A_5 = 0.35 kJ/mol and A_6 = 0.27 kJ/mol

Mechanism of Cleavage of Penta(D-galactosiduronate) by Carrot Poly(galacturonate)hydrolase

The mechanism of hydrolysis by carrot exoPg was investigated (i) by analysis of products arising in hydrolysis of penta(D-galactosiduronate) under conditions of monomolecular reaction, and (ii) by kinetics of degradation of penta(D-galactosiduronate) under simultaneous quantitative determination of the reaction products. We first used a small amount of the pentamer to estimate the incubation time suitable for achieving the desired conversion of the substrate by the given amount of enzyme. On the basis of these experiments we then chose the reaction conditions described in the Experimental. Under the optimum reaction conditions (pH 5.0, 30 °C, 30 min), carrot poly(galacturonate)hydrolase degraded penta(D-galactosiduronate) to give exclusively tetra(D-galactosiduronate) and monosaccharide. After 60 and 180 min the reaction mixture contained also tri(D-galactosiduronate) in addition to the two above-mentioned products. The kinetic data, together with the quantitative data on the product distribution, are given in Table II.

DISCUSSION

The substrate binding site of poly(galacturonate)hydrolase was studied on the basis of kinetics of degradation of linear oligo(D-galactosiduronates) (polymerization degree 2 to 7) under conditions of monomolecular hydrolysis. To this end we determined experimentally the Michaelis constants and maximum reaction rates for the individual substrates. From them we calculated the rate constants k_0 and parameters k_0/K_m. From the logarithmic dependence of k_0 and k_0/K_m on the polymerization degree of the substrates we derived that the active site of carrot exoPg consists of six subsites with the catalytic one situated between the first and second subsite. The affinity of subsites on the right-hand side from the catalytic site was calculated on the basis of the kinetic

TABLE II
Kinetics of hydrolysis of penta(D-galactosiduronate) by exopolygalacturonase

Incubation min	D-Galactopyranuronate μmol	Tetra(D-galactosiduronate), μmol		Tri(D-galactosiduronate), μmol	
		calculated[a]	found	calculated[a]	found
30	9.80	8.61	8.57	0	0
60	22.50	20.50	17.70	0	2.78
180	30.26	20.90	25.90	0	3.84

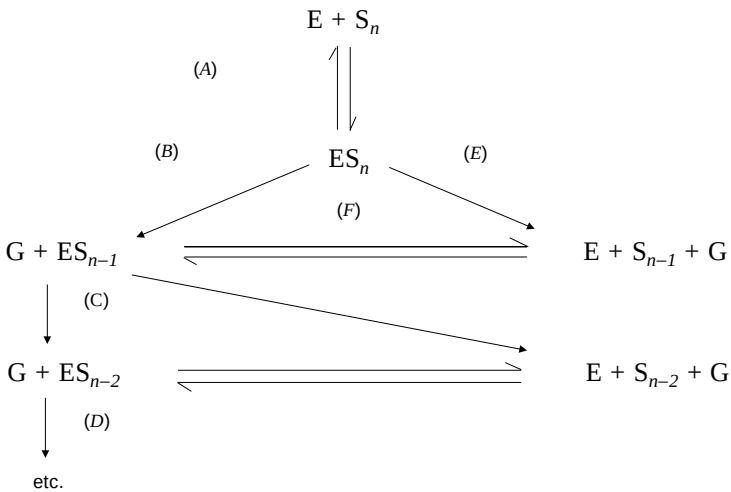
[a] Calculated for a completely multi-chain process from the yield of D-galactopyranuronate after fractionation on a column of Sephadex G-25 (Fine).

theory of Hiromi and coworkers[19,20]. The affinities of the third, fourth, fifth and sixth subsite from the non-reducing end were the following: A_3 = 1.53 kJ/mol, A_4 = 0.59 kJ/mol, A_5 = 0.33 kJ/mol and A_6 = 0.27 kJ/mol. Although affinities of the two subsites adjacent to the catalytic site could not be determined by the described procedure, they may be assessed from the product analysis and the molecular activity values k_0 found for the oligosaccharides investigated. Since the rate constants for di(D-galactosiduronate) and tri(D-galactosiduronate) differ only very sligthly $(k_0)_2$ = 0.0021 s^{-1}, $(k_0)_3$ = 0.0028 s^{-1}), we can assume that the affinity A_2 of the second subsite (the first from the catalytic site at the reducing end) is approximately the same as that of the third subsite (from the non-reducing end), A_3. We assume that the contribution of A_1 and A_2 is indispensable for productive bonding of the substrates. The fact that D-(galactosiduronate) is formed as the primary product even from longer substrate molecules indicates a positive value of A_1. These assumptions are in good accord with the results obtained in the mechanistic studies.

If the activity of carrot exoPg is determined by formation and subsequent decomposition of the ES complex, then single-chain and multi-chain action of the enzyme can be distinguished on the basis of the Scheme 2.

Symbol E denotes the enzyme, S_n a substrate of polymerization degree *n*, and G galactopyranuronate.

The single-chain action results from subsequent reactions (*A*), (*B*), (*C*) and (*D*) in which the same enzyme molecule interacts with the same molecule of the substrate; the multi-chain action employs either reactions (*A*) and (*F*), or (*A*), (*B*) and (*F*) under recombination with the same or a different substrate[32–34].



SCHEME 2

The mechanism of hydrolysis was followed in three experiments with penta(D-galactosiduronate) at various incubation times. We correlated the kinetics of hydrolysis with the quantitative detection of products. For a fully single-chain process (with excess of the substrate – pentasaccharide) a short action of the enzyme should give rise to a single reaction product: D-galactopyranuronate. In an exclusively multi-chain process the amount of the liberated monosaccharide should be identical with that of the tetrasaccharide (provided there is sufficient amount of the original substrate in the reaction medium). Since after 30 min penta(D-galactosiduronate) afforded only the mono- and the tetrasaccharide (for quantitative analysis see Table II), an exclusive or predominant single-chain mechanism can be excluded. Also the quantitative analysis of the hydrolysis products after 60 and 180 min (Table II) indicates a predominant multi-chain mechanism. Comparison of the found contents of products with those calculated for the given time intervals under assumption of a fully multi-chain process shows that up to 30 min the reaction proceeds exclusively by a multi-chain mechanism. On the other hand, after 60 and 180 min the reaction mixture contained also the trisaccharide, the deviation from the fully multi-chain process being about 14% (Table II). This may have three alternative reasons: (i) in addition to the multi-chain process there is minor concurrent single-chain process, (ii) an exclusive multi-chain process with multiple attack takes place (as seen from Table II, maximum two units of the monomer are split off in one enzyme–substrate collision), (iii) there is an exclusive multi-chain process with random attack, in which the formed tetra(D-galactosiduronate) becomes the competing substrate. Which of these alternatives participates to the observed 14% deviation can be find out by using kinetic relationships of Michaelis and Menten, on the basis of experimental determination of reaction rates k_1, k_{-1} and k_2 that are responsible for the formation of the ES complex, its dissociation, and reaction leading to the product[34–36]. The lifetime of the enzyme–substrate complex in a simple collision attack is given by the ratio k_2/k_{-1}. The multiplicity of the attack may be assessed by the relation between the Michaelis constant and the rate constants. However, a solution of this problem exceeds the scope of this work devoted to determination of the predominant mechanism. Undoubtedly, it is a multi-chain process. Pressey and Arants[42] assume a multi-chain process in the reaction of peach exopolygalacturonase with a polymeric substrate and, similarly, Mill[14] concluded that also exopolygalacturonase from *Aspergillus niger* reacts mainly by the multi-chain mechanism as found in the cleavage of tri(D-galactosiduronate) with this enzyme.

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