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Mechanism of action of D-galacturonan digalacturonohydrolase of *Selenomonas ruminantium* on oligogalactosiduronic acids

Kvetoslava Heinrichová, Mária Dzúrová and Lubomíra Rexová-Benková
Institute of Chemistry, Slovak Academy of Sciences, 842 38 Bratislava (Czechoslovakia)

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ABSTRACT

The mechanism of action of the specific D-galacturonan digalacturonohydrolase [poly-(1 → 4)-α-D-galactosiduronate digalacturonohydrolase, EC 3.2.1.82] of *Selenomonas ruminantium* was investigated by using reducing-end [1-^{3}H]-labeled oligogalactosiduronates having degree of polymerization 3–5 as the substrates. The reaction products, incorporation and distribution of radioactivity in products, and the frequency of oligogalactosiduronate bond-cleavage were quantitatively estimated as functions of the substrate concentration. An alternative cleavage of tri(D-galactosiduronate) occurred during the enzyme reaction, indicating the participation of some bimolecular mechanism in addition to unimolecular hydrolysis in the action of the enzyme. Unimolecular hydrolysis takes place at low initial concentration of the substrate. The shifted termolecular enzyme–substrate complex formation and the subsequent galactosyluronic transfer is the predominant mechanism in degradation of tri(D-galactosiduronate) at high concentration. Tetra(D-galactosiduronate) and penta(D-galactosiduronate) are degraded by unimolecular hydrolysis at low, as well as high concentration of the substrate.

INTRODUCTION

Some glycanohydrolases catalyze not only hydrolysis of glycosidic bonds but also such reactions as transglycosylation or condensation[1–9]. These atypical activities offered new information on the mode of action, kinetic properties, and the active sites of these enzymes[1–3]. A typical mechanisms are common with glycanohydrolases, degrading substrates by an endo-mechanism[4], namely, a random hydrolytic splitting of glycosidic linkages in high-molecular substrates. This is connected with distribution of the affinity of the binding subsites and localization of catalytic sites in the active center of the enzyme. The active sites of most endo-glycanohydrolases are characterized by higher affinities of the binding subsites interacting with sugar units situated at the nonreducing-end side from the cleaved glycosidic linkage, and

Correspondence to: Dr. K. Heinrichová, Institute of Chemistry, Slovak Academy of Sciences, 842 38 Bratislava, Czechoslovakia.

considerably lower affinity toward the sugar units at the reducing-end side. This affinity distribution is responsible for transglycosylation and condensation caused by the enzymes[4]. Detailed investigation of the mode of action of endo-glycanohydrolases, such as lysozyme[5–7], alpha amylase[8], and endo-(1 → 3)-α-D-glucanase[9], permitted evaluation of the mutual relationship between hydrolysis and transglycosylation reactions.

Exo-glycanohydrolases and glycosidases differ from endo-glycanohydrolases in the distribution pattern of the binding energy of subsites at the active site. The number of subsites interacting with sugar units at the reducing-end side is usually higher. Because of the higher energy of interaction with the reducing-end portion of the substrate, these enzymes lack the ability to catalyze bimolecular mechanisms[4]. Nevertheless, some exo-glycanohydrolases and glycosidases have been shown to catalyze transglycosylation reactions[10–12]. The mechanism of these reactions has not been defined so far.

D-Galacturonan digalacturonohydrolase [poly-(1 → 4)-α-D-galactosiduronate digalacturonohydrolase, EC 3.2.1.82] catalyzes specific splitting of the second α-(1 → 4) glycosidic bond from the nonreducing end of D-galactosiduronates, releasing di(D-galactosiduronic acid) as the sole product. This action pattern is manifested in a slow decrease in viscosity during degradation of polymeric substrates[13]. The same action pattern was observed also with oligogalactosiduronates having a degree of polymerization (dp) > 3. In the case of tri(D-galactosiduronate), however, specific degradation occurred only at low concentration of the substrate. In contrast, at high concentration, both glycosidic bonds of the molecule were cleaved, indicating the involvement of some bimolecular process. The aim of this work was to elucidate the mechanism of the reaction.

EXPERIMENTAL

Enzyme.—D-Galacturonan digalacturonohydrolase produced by *Selenomonas ruminantium* bacteria, strain No. 777 (isolated from bovine rumen) was purified by a procedure already published[13]. The specific activity of the purified enzyme on sodium pectate at pH 7.0 (pH optimum) and 30° was 0.37 μkat.mg^{-1}. Specificity of the enzyme was evidenced by analyzing the degradation products of polymeric and oligomeric substrates.

Substrates.—Oligogalactosiduronates, dp 2–5, were prepared by enzymic hydrolysis of sodium pectate, followed by separation of the mixture of oligogalactosiduronates on Sephadex G-25 (Fine) and desalting of the products on Sephadex G-10 (ref. 14). The homogeneity of oligogalactosiduronates was monitored by thin-layer chromatography (TLC) on silica gel (Silufol sheets, Kavalier, Czechoslovakia) in 2:3:1 1-butanol–formic acid–water (ref. 15). Indicative were the log $R_F/(1-R_F)$ values which are linearly dependent on the dp of oligogalactosiduronates. D-Galactopyranuronic acid was used as reference. The purity of the substrates was determined on the basis of the ratio of carboxylic to reducing

groups[16]. The [1-^{3}H]-labeled oligogalactosiduronates were prepared at the Institute for Research, Production and Use of Radioisotopes, Prague, Czechoslovakia, by tritiation of the respective galactosiduronates according to Evans et al.[17]. Labeled oligogalactosiduronates were purified by gel chromatography on Sephadex G-25 (Fine)[14] by a procedure described for isolation of unlabeled galactosiduronates. The purity of the labeled products was tested by TLC. After detection with aniline hydrogenphthalate reagent of the standards of oligogalactosiduronates on guide strips, the chromatograms were cut into 0.5-cm segments and the radioactivity measured on a scintillation counter (LKB, Wallace 1214 Rackbeta, Sweden), using SLD-31 scintillation fluid (Spolana, Czechoslovakia). The specific radioactivity of the purified samples was 7.44×10^7, 2.85×10^7, 3.19×10^7, and 2.65×10^7 MBq per 1 mol of di-, tri-, tetra-, and penta-(D-galactosiduronate), respectively.

Enzyme assay.—D-Galacturonan digalacturonohydrolase was routinely assayed at pH 7.0 and 30° by determining the increment of reducing groups by the spectrophotometric method of Somogyi[18]. The enzyme activity was expressed in μmol of reducing groups liberated by 1 mg of enzyme per 1 s and determined by reference to a standard graph for D-galactopyranuronic acid.

Determination of bond-cleavage frequencies.—Various amounts of unlabeled and constant amounts of [1-^{3}H]-labeled substrates (0.05 MBq) dissolved in 50 μl of phosphate buffer (pH 7.0) were incubated with 0.2 mg enzyme at 30°. The sample aliquots withdrawn at preset time-intervals were boiled for 10 min to stop the reaction and then chromatographed on silica gel sheets as already mentioned. The chromatograms were cut and the radioactivity of the products, including substrate, measured. All radioactivities were corrected to a blank background. Fractional radioactivity (the radioactivity of a particular product resulting from hydrolysis of a [1-^{3}H]-labeled oligogalactosiduronate divided by the total radioactivity of the sample, $[^3\mathrm{H}]\mathrm{Gal}pA_i/\Sigma_1^n\,[^3\mathrm{H}]\mathrm{Gal}pA_i$, where $[^3\mathrm{H}]\mathrm{Gal}pA_i$ is the radioactivity in the product of dp i and n denotes the dp of the substrate) for all compounds was determined. The bond-cleavage frequency, expressed as initial molar ratio of labeled products, was determined by plotting fractional radioactivity against the extent of the reaction, $\Sigma_1^{n-1}\,[^3\mathrm{H}]\mathrm{Gal}pA_i/\Sigma_1^n\,[^3\mathrm{H}]\mathrm{Gal}pA_i$ (ref. 19). Bond-cleavage frequencies were determined at the following substrate–enzyme concentrations: tri(D-galactosiduronate): 0.1, 5.0, and 20.0 $\mathrm{mmol.L^{-1}}/2.5 \times 10^{-2}$ $\mathrm{mg.mL^{-1}}$; tetra(D-galactosiduronate): 0.1 and 20.0 $\mathrm{mmol.L^{-1}}/5 \times 10^{-3}$ $\mathrm{mg.mL^{-1}}$; penta(D-galactosiduronate): 0.05 and 25.0 $\mathrm{mmol.L^{-1}}/2.5 \times 10^{-3}$ $\mathrm{mg.mL^{-1}}$. To obtain a comparable degree of degradation in all cases, the concentration of the enzyme was changed in accord with the specific activity on the respective substrates.

Degradation of unlabeled tri(D-galactosiduronate).—Samples of 100 μmol tri(D-galactosiduronate), dissolved in phosphate buffer (pH 7.0) and diluted to the final concentrations of 4.0, 8.0, 25.0, and 50.0 $\mathrm{mmol.L^{-1}}$, respectively, were incubated with 0.4 mg of enzyme at 30° until 30–40% conversion of the substrate was achieved (2–3 h). The course of degradation was monitored in a parallel experi-

ment by measuring at predetermined time-intervals the increment of reducing groups, by the spectrophotometric method of Somogyi[18]. The reaction was stopped by boiling for 10 min. The samples were concentrated to 1.3–2.0 mL and then fractionated on Sephadex G-25 (Fine), with two columns (120 × 2.5 cm), connected in series, using 0.05 M phosphate buffer (pH 9.2) as eluent. The separated products were determined by the method of Somogyi[18]. The precision of the procedure was checked by using a standard mixture containing 40 μmol tri(D-galactosiduronate), 30 μmol di(D-galactosiduronate), and 30 μmol D-galactopyranuronic acid. The yields of the products were not lower than 94.8%.

Localization of the label in [1-^{3}H]di(D-galactosiduronate) produced.—[1-^{3}H]Di(D-galactosiduronate) obtained after resolution of products of the reaction with [1-^{3}H]tri-(D-galactosiduronate) by TLC was eluted from the chromatogram with water and purified by gel chromatography on Sephadex G-25 (Fine). The purified disaccharide was then reduced with $NaBH_4$ according to McCready and Seegmiller[20]. The α-D-galactosyluronic-L-galactonolactone formed was hydrolyzed in 0.2 M CF_3CO_2H for 16 h at 105°. The products were separated by TLC in 17:7:10. EtOAc–AcOH–H_2O. The position of D-galactopyranuronic acid and galactonolactone were found by detecting the former with aniline hydrogenphthalate reagent and the latter by hydroxylamine and ferric chloride[21] on the guide strips. The distribution of radioactivity was then determined by counting 0.5-cm strips.

RESULTS AND DISCUSSION

Degradation of [1-^{3}H]oligogalactosiduronates.—The mode of action of D-galacturonan digalacturonohydrolase was investigated using [1-^{3}H]-labeled oligogalactosiduronates of dp 3–5 prepared from respective galactosiduronates by tritium exchange[17]. The bond-cleavage frequencies were determined as initial product-ratios at low and high concentrations of the substrates. The results are shown in Fig. 1. At low concentrations of the substrates [tri(D-galactosiduronate) and tetra(D-galactosiduronate), both 0.1 mmol.L^{-1}; penta(D-galactosiduronate) 0.05 mmol.L^{-1}] the values of product ratios were in accord with specific unimolecular hydrolysis. The enzyme cleaved specifically the second glycosidic bond from the nonreducing end of the substrates, so that unlabeled di(D-galactosiduronate) was the only reaction product from all three substrates.

Splitting of bond 4 in penta(D-galactosiduronate) was very small at the reaction extent used, obviously attributable to the some 6.5-times lower activity of the enzyme on tri(D-galactosiduronate) (Table I) and lower affinity towards trisaccharide[22]. The initial molar ratio of products of tetra- and penta-(D-galactosiduronate) degradation also remained unchanged at high concentration (25 mmol.L^{-1}), suggesting that unimolecular hydrolysis occurs also under these conditions (Fig. 2). On the other hand, the values obtained with tri(D-galactosiduronate)

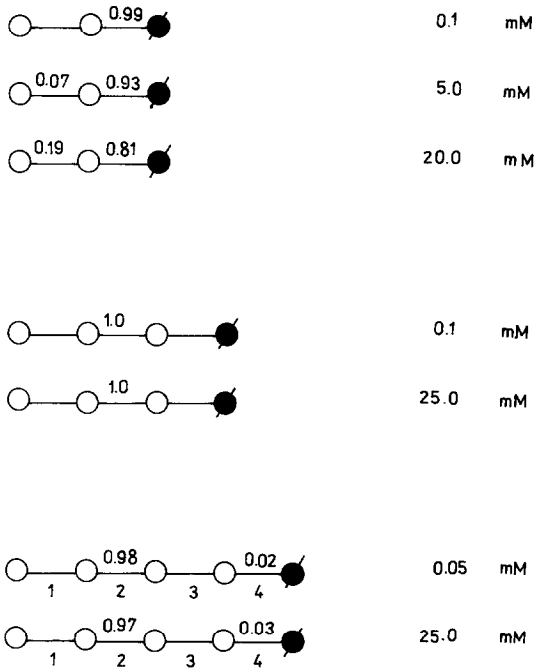

Fig. 1. Schematic illustration of the bond-cleavage frequencies of [1-^{3}H]-labeled tri(D-galactosiduronate), tetra(D-galactosiduronate) and penta(D-galactosiduronate) at different concentrations of the substrates. 1–4 in penta(D-galactosiduronate) is the numbering of glycosidic bonds.

were found to be dependent on substrate concentration. The mode of action on tri(D-galactosiduronate) was therefore investigated in detail.

The initial molar ratios of products at tri(D-galactosiduronate) concentrations of 5 and 20 mmol.L^{-1} (shown in Fig. 1) indicate two alternative modes of degradation of the substrate. The predominant one produces unlabeled di(D-galactosiduronate) and labeled galactopyranuronic acid and the other produces labeled disaccharide and unlabeled monosaccharide. The proportions of radioactivities attributable to

TABLE I

Initial velocity, v_o, of degradation of oligo-D-galactosiduronates by D-galacturonan digalacturonohydrolase [a]

Substrate dp	v_o (μmol.mg^{-1}.s^{-1})	Correlation coefficient
3	0.020 ± 0.009	0.998
4	0.119 ± 0.012	0.997
5	0.133 ± 0.027	0.993
6	0.134 ± 0.030	0.995

[a] The enzyme activity was determined at a substrate concentration of 0.5 μmol.mL^{-1}. The concentrations of the enzyme were the same as mentioned in Experimental for the determination of bond-cleavage frequencies.

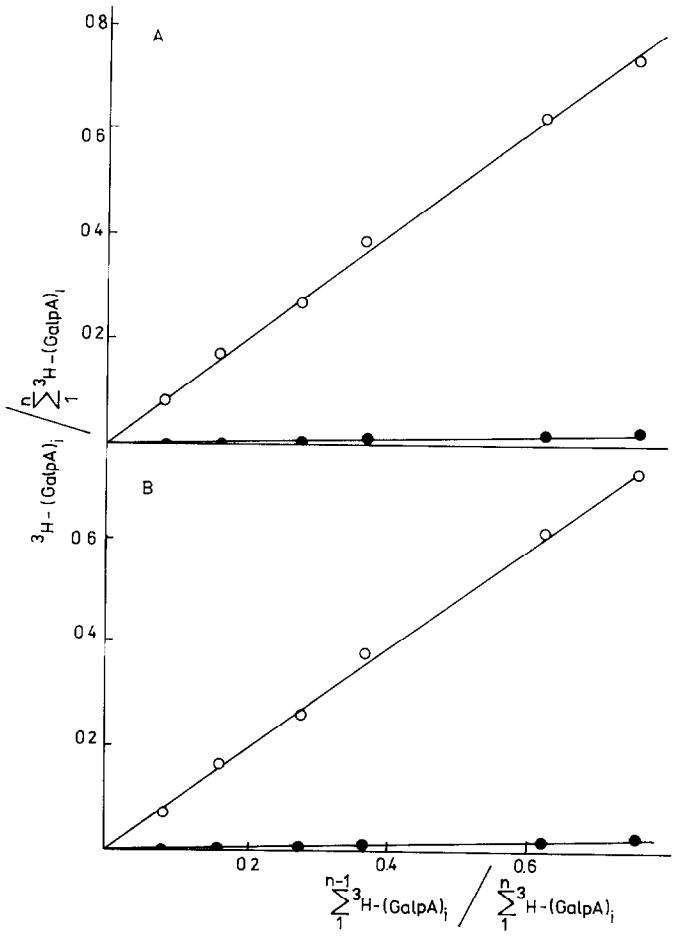

Fig. 2. The fractional radioactivity of products of degradation of [1-^{3}H]penta(D-galactosiduronate) as a function of the extent of the reaction at substrate concentrations of 0.25 mmol.L^{-1} (A), and 50 mmol.L^{-1} (B): (○) ^{3}H-(Gal*p*A)$_3$; (●) ^{3}H-(Gal*p*A)$_1$.

monosaccharide and disaccharide produced at the concentrations of labeled tri(D-galactosiduronate) of 0.1 and 20 mmol.L^{-1} are shown in Fig. 3. The slopes of the straight lines correspond to the initial frequencies of bond-cleavage. The values of ratios of labeled degradation products at 35–40% degradation for the concentration range of the substrate from 5–55 mmol.L^{-1} are plotted in Fig. 4. As shown, the products of the degradation are a function of concentration of the substrate. With increasing concentration of tri(D-galactosiduronate), the ratio of labeled di(D-galactosiduronate) steadily increased at the expense of labeled monomer, thus indicating the participation of more than one molecule in the degradation reaction. Labeled products larger than the starting substrate were not found.

Position of the radioactive label in the di(D-galactosiduronate) produced.—Labeled di(D-galactosiduronate) produced at high concentration of labeled tri(D-galactosid-

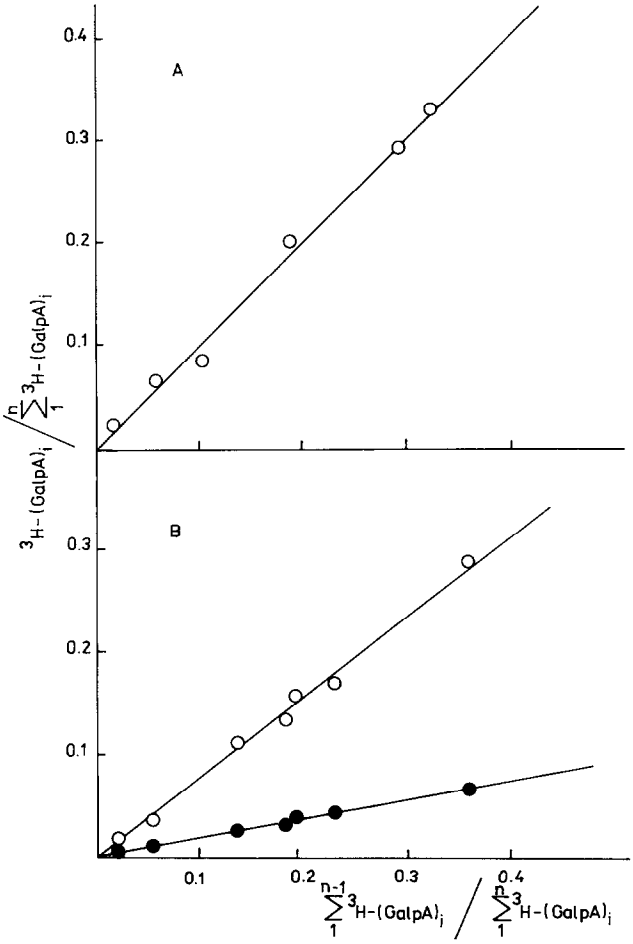

Fig. 3. The fractional radioactivity of products of degradation of [1-^{3}H]tri(D-galactosiduronate) as a function of the extent of the reaction at substrate concentrations of 0.1 mmol.L^{-1} (*A*), and 50 mmol.L^{-1} (B): (○) ^{3}H-(Gal*p*A)$_1$; (●) ^{3}H-(Gal*p*A)$_2$.

uronate) was examined for the position of radioactive label, as described in the Experimental. Galactonolactone was the only radioactive compound found after reduction of di(D-galactosiduronate) and subsequent hydrolysis of D-galacto-pyranuronic acid 3-glycoside of the L-galactonolactone formed. There was no detectable radioactivity present in the second hydrolysis product, namely, D-galactosiduronic acid. This shows that the formation of labeled di(D-galactosid-uronate) was not accompanied by label redistribution.

The degradation of unlabeled tri(D-galactosiduronate).—Valuable information on the action pattern of glycanohydrolases may be obtained from their interaction with uniformly ^{14}C-labeled substrates, which permits determination of the initial molar ratios of all products formed. As we could not prepare ^{14}C-labeled oli-

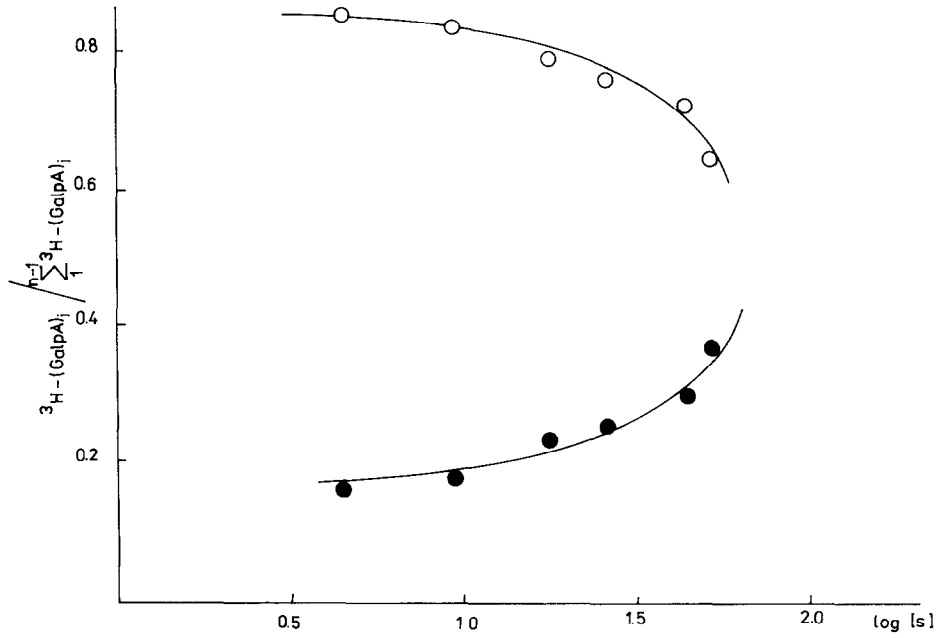

Fig. 4. The ratios of labeled galactopyranuronic acid and labeled di(D-galactosiduronate) produced at 35–40% degradation of [1-^{3}H]tri(D-galactosiduronate) at different concentrations of the substrate: (○) ^{3}H-(Gal$p$A)$_1$; (●) ^{3}H-(Gal$p$A)$_2$.

gogalactosiduronates, the mode of action of D-galacturonan digalacturonohydrolase was examined by using unlabeled tri(D-galactosiduronate) at four concentrations, 4.0, 8.0, 25.0, and 50.0 mmol.L^{-1}. Reaction products obtained at 30–40% degradation of the substrate were determined spectrophotometrically after separation by gel chromatography. The data obtained are summarized in Table II. As shown, the ratio (Gal$p$A)$_2$:(Gal$p$A)$_1$ is dependent on the initial concentration of tri(D-galactosiduronate). At a concentration of 4 mmol.L^{-1} of the substrate, the molar ratio of the products was found to be ~ 1. With increasing concentration of

TABLE II

Products of degradation of tri(D-galactosiduronate) by D-galacturonan digalacturonohydrolase at different concentrations of the substrate

Substrate (mmol.L^{-1})	Separated oligo-D-galactosiduronates [a] (μmol)			Mole ratio (Gal$p$A)$_2$:(Gal$p$A)$_1$
	(Gal$p$A)$_1$	(Gal$p$A)$_2$	(Gal$p$A)$_3$	
4.0	39.6	40.2	60.8	1.00
8.0	30.7	43.7	60.2	1.42
25.0	23.2	38.0	65.7	1.64
50.0	14.9	32.1	71.3	2.15

[a] (Gal$p$A)$_n$ — galactosiduronate having dp n.

the substrate, the mole ratio $(Gal pA)_2:(Gal pA)_1$ increased, reaching 2.2 at a substrate concentration of 50 mmol.L^{-1}. These results are in good agreement with the experimental data obtained with labeled substrate and suggest a bimolecular mechanism in the action of D-galacturonan digalacturonohydrolase.

On the basis of the results here presented it seems that, depending of the concentration of tri(D-galactosiduronate), D-galacturonan digalacturonohydrolase exhibits different modes of action. At the lowest concentration (0.1 mmol.L^{-1}), unimolecular hydrolysis of the substrate occurred. The enzyme formed one productive complex, so that labeled tri-(D-galactosiduronate) was cleaved specifically at the second glycosidic bond from the nonreducing end, giving galactopyranuronic acid as the sole labeled product. The molar ratio of di(D-galactosiduronate) to galactopyranuronic acid was found to be 1. The occurrence of labeled di(D-galactosiduronate) as an alternative reaction product at higher substrate concentration, and its increased production with increasing substrate concentration, is due to the operation of a bimolecular reaction mechanism, that is, a reaction requiring interaction of the enzyme with more than one molecule of the substrate.

The production of labeled di(D-galactosiduronate) under simultaneous maintenace of the molar ratio $(Gal pA)_2:(Gal pA)_1$ close to unity, observed at the concentration 4 mmol.L^{-1}, may be ascribed to the formation of a shifted termolecular enzyme–substrate complex[23], leading to hydrolysis of the first glycosidic bond from the nonreducing end (Fig. 5A). With further increase in concentration of tri(D-galactosiduronate), the molar ratios $(Gal pA)_2:(Gal pA)_1$ attain values > 1; thus the value 2.2 was obtained at a concentration of tri(D-galactosiduronate) of 50 mmol.L^{-1}. For such a case the following two bimolecular processes are commonly considered: (*a*) condensation — a process of coupling of two molecules of substrate to form the product, which then undergoes rapid unimolecular hydrolysis; (*b*) transglycosylation — the transfer of a glycosyl group from one molecule of the substrate to another substrate molecule giving an intermediate product which is then rapidly degraded in unimolecular hydrolysis.

The condensation of two molecules of tri(D-galactosiduronate) and subsequent rapid hydrolysis of the hexa(D-galactosiduronate) formed would lead to equally distributed label from the reducing-end sugar unit of the substrate between the reducing and non reducing galactopyranosyluronic residues of the di(D-galactosiduronate) produced (Fig. 5B). As the test for label redistribution was negative, the condensation mechanism contributing to the degradation of tri(D-galactosiduronate) can be unequivocally rejected.

Transglycosylation is the most frequent bimolecular mechanism in glycanohydrolase action; it has been reported for lysozyme[5,24,25], alpha-amylase[3,19,23], pullulanase[26], and xylanase[27]. In the transglycosylation reaction with tri(D-galactosiduronate) two pathways may be considered: galactosyluronic or digalactosyluronic transfer from the nonreducing end of a donor tri(D-galactosiduronate) to the nonreducing end of the acceptor molecule, resulting in the formation of intermediate tetra- or penta-(D-galactosiduronate) (Fig. 5C). When using [1-^{3}H]tri(D-

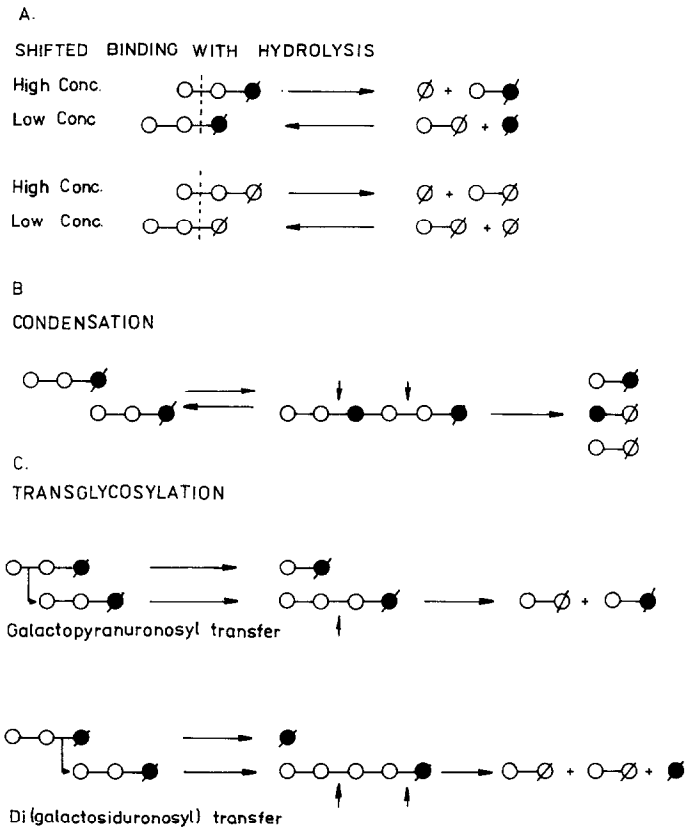

Fig. 5. Reaction pathways considered for D-galacturonan digalacturonohydrolase at high concentrations of tri(D-galactosiduronate): (ϕ) reducing galactopyranuronic acid unit; (●) ^{3}H-labeled reducing galactopyranuronic acid unit.

galactosiduronate) as the substrate, di(D-galactosiduronate) is the labeled reaction product in the former case, whereas labeled D-galactopyranuronic acid would be produced in the latter case.

TABLE III

Distribution of radioactivity in the products of D-galacturonan digalacturonohydrolase action on unlabeled tri(D-galactosiduronate) in the presence of [1-^{3}H]di(D-galactosiduronate)

Incubation time (h)	[1-^{3}H](Gal*p*A)$_3$ [a] (%)	[1-^{3}H](Gal*p*A)$_2$ [a] (%)	[1-^{3}H](Gal*p*A)$_1$ [a] (%)
1	1.6	96.6	1.9
2	2.3	89.4	6.3
3	2.5	90.5	6.2
20	2.7	91.0	6.1

[a] (Gal*p*A)$_n$ — galactosiduronate having dp *n*.

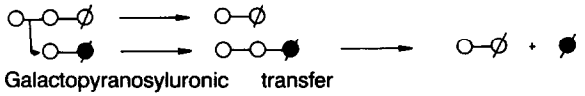

Fig. 6. Reaction pathway considered for the degradation of unlabeled tri(D-galactosiduronate) in the presence of labeled di(D-galactosiduronate). The symbols are as in Fig. 5.

Increasing production of di(D-galactosiduronate) (Table II) suggests the transfer of galactosyluronate groups in the action of D-galacturonan digalacturonohydrolase. The character of the glycosyl transfer was further confirmed in experiments in which the enzyme (0.025 $mg.mL^{-1}$) was incubated with the mixture of unlabeled tri(D-galactosiduronate) (18 $mmol.L^{-1}$) and [1-^{3}H]di(D-galactosiduronate) (9 $mmol.L^{-1}$) at pH 7.0 and 30°. The radioactivity of the products separated by TLC was measured. The data summarized in Table III show 1.6–2.7% of the label to be present in tri(D-galactosiduronate), 1.9–6.1% in D-galactopyranuronic acid in the course of 20 h of reaction. The results well demonstrate the transfer reaction. The formation of [1-^{3}H]tri(D-galactosiduronate) may be envisaged only in the situation when a galactopyranosyluronic group enters the reaction pathways as a glycosyl donor (Fig. 6). Results of transglycosidic reactions of some other glycanohydrolases were interpreted analogously[23,25,27].

On the basis of the results presented we can draw the following conclusions. At low initial concentration of tri(D-galactosiduronate), unimolecular hydrolysis takes place. A termolecular shifted complex in which the nonreducing-end glycosidic bond is cleaved participates in the degradation mechanism at medium concentrations. The shifted termolecular enzyme–substrate complex and subsequent galactosyluronic transfer is the predominant bimolecular mechanism in degradation of tri(D-galactosiduronate) at high concentrations. The mode of action of D-galacturonan digalacturonohydrolase on tri(D-galactosiduronate) seems to be similar to the action of some other glycanohydrolases[25,27]. Unlike these enzymes, however, D-galacturonan digalacturonohydrolase degrades oligogalactosiduronates of dp > 3 by unimolecular hydrolysis also at high concentrations.

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