# THE MODE OF ACTION OF THE ISOLATED FORM OF TOMATO ENDO-D-GALACTURONANASE*

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The mode of action of the isolated form of tomato endo-D-galacturonanase of molecular weight close to 50000 was investigated with oligo-D-galactosiduronic acids of polymerization degree 2—7 and their derivatives the terminal aldehyde group of which was reduced. The rate of hydrolysis, catalysed by this enzyme decreased with the shortening the chain length of oligo-D-galactosiduronates used; di(D-galactosiduronic) acid was not hydrolyzed by this enzyme at all. Tri(D--galactosiduronic) acid was cleaved into monomer and dimer, tetra(D-galactosiduronic) acid was alternatively cleaved into monomer and trimer, as well as into two dimers. The previously proposed conception that the binding site of the tomato endo-D-galacturonanase contains three subsites and that the catalytic groups are localized close to the first bond from the reducing end of the substrate segment bound in the complex were proved. The mode of hydrolysis of the reduced oligomers is in favour of the mentioned conception.

Tomatoes contain, in addition to a relatively great amount of pectinesterase (pectin pectyl--hydrolase, E.C. 3.1.1.11), endo-D-galacturonanase (poly-(1 → 4)-α-D-galactosiduronate glycanohydrolase, E.C. 3.2.1.15), and, with regard to the cleavage of di(D-galactosiduronic) acid[2,3], probably also exo-D-glacturonanase (poly-(1 → 4)-α-D-galactosiduronate glycanohydrolase, E.C. 3.2.1.67).

The original opinion on the action of tomato D-galacturonanase, as one of the depolymerization enzymes of pectic acid ("pectic acid-depolymerase"), have became more precise especially after introduction of chromatographic separations and identification of reaction products formed by cleavage[5,6]. Luh and coworkers[7] examined the action of the crude product of tomato D-galacturonanase on pectic acid and oligo-D-galactosiduronates; the rate of hydrolyses of tetra- tri- and dimer was found to be 7·0, 1·58 and 1·05, respectively, when compared with that of pectic acid = = 100. Patel and Phaff[8] prepared a partly purified tomato D-galacturonanase and obtained the principal information on the mechanism of hydrolysis of oligo-D-galactosiduronates and their reduced and oxidized derivatives[3]; since they found two pH-optima during hydrolysis of trimer, tetramer and a low-molecular pectic acid (polymerization degree 14), they assume that this tomato D-galacturonanase contained at least 2 components, the separation of which failed. Basing upon their results one of the three modes of action of endo-D-galacturonanases was proposed[1]. Pressey and Avants[9] investigated the action of partly purified tomato D-galacturonanase on pectic acid and D-galacturonans of various molecular weights; they ascertained the decrease of the pH-opti-

* Originally called "endopolygalacturonase". The new term corresponding to the name of the substrate has been proposed by Prof. D. Horton[1].

mum of the enzyme with the decrease of the molecular weight of substrates. They consider the presence of an isoenzyme of D-galacturonanase, which is not inhibited by a D-galacturonan of a higher molecular weight. Later, they found the presence of two forms of D-galacturonanases of molecular weight 44000 and 84000, respectively, in extracts of tomatoes. Both forms differed in activation with NaCl and in the rate of cleavage of D-galacturonans of various polymerization degree[10]. The form of molecular weight 44000 caused a rapid decrease of viscosity at a relatively low increment of reducing groups, what indicated a random splitting of the substrate by an endo--action pattern. Takehana and coworkers[11] prepared endo-D-galacturonanase of molecular weight 52000 from tomatoes (var. *Hikari*) electroforetically homogenous, which, they believe, corresponds to D-galacturonanase II described by Pressey and Avants[10].

In the previous paper[12] we described the isolation of one form of tomato endo--D-galacturonanase of molecular weight close to 50000. This paper deals with the use of this endo-D-galacturonanase as a homogeneous enzyme for the study of its action on oligomeric substrates and their reduced derivatives and for the proof of the already suggested dimension of the binding site of the tomato endo-D-galacturonanase[1].

## EXPERIMENTAL

*Enzyme*: The isolated form of tomato endo-D-galacturonanase (var. *Imuna*) was prepared according to a method already described[12].

*Substrates*: Oligo-D-galactosiduronates of polymerization degree (DP) 2—7 were prepared according to[13] from the partial hydrolysate of pectic acid. All prepared oligomers were tested for purity by determination of reducing groups by a modified Nelson–Somogyi[14] method and titration of carboxylic groups. Their homogeneity was tested by chromatography on paper Whatman No 1 in the system ethyl acetate–acetic acid–water (18 : 7 : 8), visualization with aniline--phthalate reagent; calculation employing values $\log [R_F/(1 - R_F)]$, representing the linear functions of polymerization degree of oligo-D-galactosiduronates[15] was used for identification. The reduced oligo-D-galactosiduronates were prepared according to[16] with sodium tetrahydridoborate, whereby the reducing D-galactopyranuronic unit was changed into L-galactonolactone. The products were desalted after reduction on a Sephadex G-10 column and their purity was checked chromatographically on paper in the same system as the original oligo-D-galactosiduronates (detection with hydroxylamine reagent[17]). Pectic acid was obtained from the commercial citrus pectin by a repeated alkaline deesterification with 0·1M-NaOH followed by precipitation with ethanol at pH 2·5; the average molecular weight 33000 was calculated from the limit viscosity number $[\eta] = 158$ ml/g (ref.[18]); the purity of the acid was found to be 95·5% and the esterification degree 0·5%.

*Estimation of the enzyme activity and kinetic parameters*: The enzyme hydrolysis of the employed substrates was estimated by determining the initial rate of reducing group increase in a 0·1M acetate buffer solution of pH 4·5 in 0·15M-NaCl at 30°C. The initial rates were calculated using a CDC 3300 (USA) computer and the Booman and Nieman's program[19]. The initial rate is given in moles of reducing groups liberated by 1 kg of enzyme per 1 s.

The Michaelis constant ($K_m$) and the maximum velocity ($V$) were calculated using a least-squares program[20] at 0·1—1 μmol $ml^{-1}$ substrate concentrations and the enzyme concentration 0·05 mg per 1 ml.

The products of enzymic cleavage of oligo-D-galactosiduronic acids and their reduced derivatives were examined by paper chromatography. Aliquot portions (30 μl) withdrawn in 1, 5, 10, 20, 60 min and 72 h intervals from the original mixture containing 1 μmol of substrate and 0·05 mg of enzyme in 0·1M acetate buffer (1 ml) of pH 4·5 incubated at 30°C were applied on two chromatographic papers (solvent system as mentioned above) and detected alternatively with the aniline--phthalate reagent for reducing groups and hydroxylamine reagent for lactones.

*The protein content* was determined by the method of Lowry and coworkers[21] using the bovine serum-albumin as reference.

## RESULTS

The initial reaction rates and the maximum velocities of degradation of individual oligo-D-galactosiduronic acids catalyzed with the tomato endo-D-galacturonanase decreased with the shortening of the chain length (Table I). Whereas the values of Michaelis constants showed only an unsubstantial increase for the individual oligomers of decreasing degree of polymerization (from $K_m = 1{\cdot}20$ for a heptamer to $K_m = 1{\cdot}38$ for a trimer) the initial rate of cleavage of the heptamer is by 16 times higher than that of a trimer. The greatest difference in the reaction rates of cleavage was found between the trimer and the tetramer (value $v$ for the tetramer is by 3·9 times higher than that for the trimer); the value $v$ was among the next higher oligomers almost constantly by 1·5—1·6 times higher. Differences in maximum rates of cleavage of glycosidic bonds of individual oligomers were similar to those in the initial reaction rates.

TABLE I

Kinetic Parameters of Action of Tomato Endo-D-galacturonanase on Oligo-D-galactosiduronic Acids (GalpUA)

$v$ The initial velocity expressed in mol of cleaved glycosidic bonds per 1 kg of the enzyme within 1 s at the 1 μmol/ml enzyme concentration. $K_m$ the Michaelis constant expressed in mM concentration of the substrate; $V$ the maximum reaction velocity; $h$ the Hill coefficient of interaction determined from the relationship $\log[v/(V - v)]$ *versus* $[S]$.

| Substrate | $v \cdot 10^2$ | $K_m$ | $V \cdot 10^2$ | $h$ |
|---|---|---|---|---|
| $(GalpUA)_2$ | 0·0 | | | |
| $(GalpUA)_3$ | 0·260 | 1·38 | 0·525 | 1·15 |
| $(GalpUA)_4$ | 1·008 | 1·33 | 1·753 | 1·10 |
| $(GalpUA)_5$ | 1·638 | 1·33 | 3·27 | 1·12 |
| $(GalpUA)_6$ | 2·530 | 1·25 | 4·75 | 1·11 |
| $(GalpUA)_7$ | 4·215 | 1·20 | 6·683 | 0·97 |
| Pectic acid (m.w. 33 000) | 21·16 | 0·014 | 24·537 | |

Chromatographic examination of reaction products of oligo-D-galactosiduronic acids showed that the monomer and dimer were formed from tri(D-galactosiduronic) acid; di(D-galactosiduronic) acid did not undergo cleavage nor after 72 h. With tetra--(D-galactosiduronic) acid the trimer and monomer were the first reaction products and after a 10% cleavage of the substrate (monitored by the increase of reducing groups) the content of the dimer became noticealbe what indicated also the alternative cleavage into two dimers. Penta(D-galactosiduronic) acid afforded in the initial stage all lower oligomers with a relatively higher content of the dimer and trimer; this, however, indicates three alternative modes of cleavage. Similarly, hexa- and hepta--(D-galactosiduronic) acids furnished as the first identifiable products all lower oligomers with a negligible amount of a monomer, the content of which substantially raised together with the dimer during the further action of the enzyme. This fact indicates four, or five modes of cleavage to be involved in hexa- or heptamer (Scheme 1). The final products of cleavage of all tested oligomers with tomato endo-D-galacturonanase were the monomer and dimer.

$$(\mathrm{GalpUA})_3 \longrightarrow (\mathrm{GalpUA})_2 + (\mathrm{GalpUA})_1$$

$$(\mathrm{GalpUA})_4 \longrightarrow \begin{cases} (\mathrm{GalpUA})_3 + (\mathrm{GalpUA})_1 \\ (\mathrm{GalpUA})_2 + (\mathrm{GalpUA})_2 \end{cases}$$

$$(\mathrm{GalpUA})_5 \longrightarrow \begin{cases} (\mathrm{GalpUA})_4 + (\mathrm{GalpUA})_1 \\ (\mathrm{GalpUA})_3 + (\mathrm{GalpUA})_2 \\ (\mathrm{GalpUA})_2 + (\mathrm{GalpUA})_3 \end{cases}$$

$$(\mathrm{GalpUA})_6 \longrightarrow \begin{cases} (\mathrm{GalpUA})_5 + (\mathrm{GalpUA})_1 \\ (\mathrm{GalpUA})_4 + (\mathrm{GalpUA})_2 \\ (\mathrm{GalpUA})_3 + (\mathrm{GalpUA})_3 \\ (\mathrm{GalpUA})_2 + (\mathrm{GalpUA})_4 \end{cases}$$

$$(\mathrm{GalpUA})_7 \longrightarrow \begin{cases} (\mathrm{GalpUA})_6 + (\mathrm{GalpUA})_1 \\ (\mathrm{GalpUA})_5 + (\mathrm{GalpUA})_2 \\ (\mathrm{GalpUA})_4 + (\mathrm{GalpUA})_3 \\ (\mathrm{GalpUA})_3 + (\mathrm{GalpUA})_4 \\ (\mathrm{GalpUA})_2 + (\mathrm{GalpUA})_5 \end{cases}$$

SCHEME 1

Cleavage of the reduced derivatives of oligo-D-galactosiduronic acids of DP 3–7 with tomato endo-D-galacturonanase was examined on the basis of reducing group increments[14] and the products analyzed by paper chromatography. The initial reaction rates increased with the polymerization degree of the reduced oligomers (Table II) and their values were very close to those of non-reduced oligomers lower by one grade. The reduced trimer was not cleaved nor after 72 h of action of the enzyme. Products of hydrolysis of reduced oligomers indicated that the reduced tetramer underwent a selective cleavage into a reduced dimer and di(D-galactosiduronic)

acid. The reduced pentamer afforded the reduced dimer and reduced trimer, as well as di- and tri-(D-galactosiduronic) acids, what indicates two alternative splitting pathways. The reduced hexamer was cleaved in three ways, since reduced dimer, trimer and tetramer together with di-, tri- and tetra(D-galactosiduronic) acids were detected at the beginning of the reaction. The final products of hydrolysis catalyzed by endo-D-galacturonanase were the reduced dimer and di(D-galactosiduronic) acid when incubating the reduced tetramer, whilst further higher reduced oligomers yielded the reduced dimer, di(D-galactosiduronic) and D-galactopyranuronic acids.

Hill plots of $\log [v/(V - v)]$ *versus* $\log [S]$ were linear for all substrates (Fig. 1). The corresponding interaction coeficient $h$ was close to 1.

## DISCUSSION

It has been supposed that the active site of enzymes degrading polysaccharides by a random cleavage, including the tomato endo-D-galacturonanase, consists of a certain number of binding subsites, which are geometrically complemental to monomeric units of the polymeric substrate[22,23]. On the basis of mode of action and kinetics of tomato endo-D-galacturonanase with oligo-D-galactosiduronic acids and their reduced derivatives the complex enzyme–substrate could be illustrated as seen in Fig. 2. According to this model the primary binding site is composed of three subsites and the catalytic groups are located between subsites 1 and 2. The tendence of the substrate to fully occupy the binding site and especially the mode of cleavage of tri(D-galactosiduronic) acid and the reduced tetramer together with two alternative splitting pathways of the tetramer and the reduced pentamer backed the proposal of only this type of binding site. According to a semiquantitative evaluation of products

TABLE II

The Initial Velocities of Cleavage of Reduced Oligo-D-galactosiduronic Acids Catalyzed by Tomato Endo-D-galacturonanase

$v$ The initial velocity expressed in moles of glycosidic bonds cleaved by 1 kg of the enzyme per s at 1 μmol of the substrate and 0·05 mg/ml of the enzyme concentrations.

| Substrate | $v \cdot 10^2$ |
|---|---|
| Red $(GalpUA)_3$ | 0·0 |
| Red $(GalpUA)_4$ | 0·23 |
| Red $(GalpUA)_5$ | 0·91 |
| Red $(GalpUA)_6$ | 1·68 |
| Red $(GalpUA)_7$ | 2·83 |

of cleavage of the pentamer (the preponderance of the di- and trimer against tetramer and monomer in the first stage), three alternative modes of splitting could be considered (Scheme 1) under a full occupation of the binding site in such a way that the reducing end of the substrate remained in the binding site, or alternatively, that the reducing end was out of the binding site (similarly as with splitting the tetramer – Fig. 2, $B_1$, $B_2$). The number of productive complexes, identical with that of modes of cleavage, regularly increases in the examined oligo-D-galactosiduronic acids with the increase of polymerization degree. According to[23], the number of productive com-

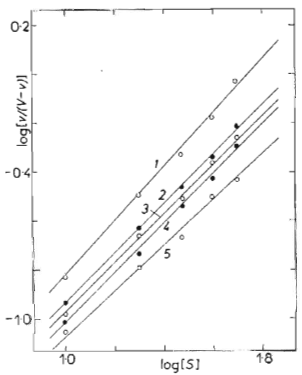


FIG. 1

Hill Plot of log $[v/(V - v)]$ *versus* log $[S]$ for Oligo-D-galactosiduronic Acids

*1* Tri(D-galactosiduronic) acid, *2* tetra(D--galactosiduronic) acid, *3* penta(D-galactosiduronic) acid, *4* hexa(D-galactosiduronic) acid, *5* hepta(D-galactosiduronic) acid.

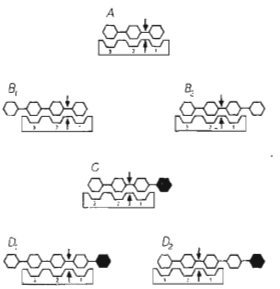


FIG. 2

Plot of the Enzyme-Substrate Complex of Tomato Endo-D-galacturonanase with Oligo--(D-galactosiduronic) Acids and their Reduced Derivatives

*A* Complex of tri(D-galactosiduronic) acid, $B_1$, $B_2$ complexes of tetra(D-galactosiduronic) acid, *C* complex of reduced tetra--(D-galactosiduronic) acid, $D_1$, $D_2$ complexes of reduced penta(D-galactosiduronic) acid; 1—3 binding subsites: — glycosidic α - 1, 4 bonds; open sextagon the D-galactopyranuronic acid unit, full sextagon the reduced D-galactopyranuronic acid unit, arrow catalytic endo-D-galacturonanase groups.

plexes at which all subsites of the enzyme ($m$) are occupied by the substrate of polymerization degree ($n$) equals ($n - m + 1$). When substituting the values of polymerization degree and the final number of modes of cleavage (according to Scheme 1) into the given relationship, the $m = 3$ for all employed substrates, what corresponded to the given model of the binding site of tomato endo-D-galacturonanase (Fig. 2).

The values of Hill interaction coefficient ($h$ close to 1) indicated one catalytic site.

Bearing the presented results in mind, one can conclude that the primary binding site of tomato endo-D-galacturonanase is composed of three subsites and the catalytic site is situated close to the first bond from the reducing end of the substrate segment bound into the complex.

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