MULTI-CHAIN ACTION OF EXO-D-GALACTURONANASE FROM CARROT

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Two possible modes of action of exo-D-galacturonanase from carrot (E.C. 3.2.1.67) were instigated; this enzyme catalyses the sequential hydrolytic cleavage of pectanins and oligogalacturonans by a terminal action from the nonreducing end of the molecule. The experiments indicate that the investigated exo-D-galacturonanase degrades these substrates by a predominantly multi-chain mechanism. Distribution of degradation products of oligomeric substrates (hexa- and pentagalacturonide) under an optimal condition for the action of the enzyme (pH and temperature) indicates that a multi-chain enzyme attack with a prevalent simple collision is involved. Results of the enzyme degradation kinetics are in a good agreement with the above-mentioned presumption.

Two distinct models of action exist for degradation of a polysaccharide by enzymes with terminal or random mechanism of hydrolysis: *1*) the single-chain mechanism, where the enzyme fully degrades one polymeric chain and only afterwards it passes its action to the further macromolecule chain; *2*) multi-chain mechanism, where the enzyme randomly cleaves the individual units from all attainable polymeric terminals. Concurrently, the enzyme can catalyse the hydrolysis of one or more bonds sooner than it has dissociated and formed a new active complex. The single-chain and multi-chain mechanisms are two extreme possibilities of polymer degradation, the multiplicity of the attack is a more general conception involving the multi-chain and single-chain process as specific extreme case.

A single-chain mechanism was reported[1,2] when investigating the action of plant β-amylases on amylose of a high molecular mass (DP = 1 000). On the other hand, other authors[3–5] tended to favourize the multi-chain action of β-amylase. As reported later[6–9], the action of β-amylase on amylose of a lower molecular mass (DP = 44 and 49) and on maltodextrine (DP = 6 and 7) was a degradation process involving transition between the single- and multi-chain action. The reaction conditions (pH and temperature) were found[10] to change the measure of the multi-chain action of β-amylase. These findings stimulated us to seek experimental methods for estimation of the single- and multi-chain action of exo-D-galacturonanase from carrot, which, similarly as plant β-amylases degreades polymeric and oligomeric

substrates by terminal mode of action. The possibility to asses the simple and multiple attacks was examined when degrading the polymeric and oligomeric substrates.

EXPERIMENTAL

Material

Enzyme: Exo-D-galacturonanase was isolated from the carrot (*Daucus carota*) debris which was extracted by a 5%-NaCl at pH 5·0. The isolation process included the salting-out of proteins by ammonium sulfate up to 90% of saturation, separation of the mixture of proteins by chromatography on DEAE cellulose, refiltration on Sephadex G-100, desalting of the enzyme on Sephadex G-25 Medium and freeze-drying[11]. Exo-D-galacturonanase prepared by this process had specific activity $5 . 10^{-2}$ mol s^{-1} kg^{-1} (determined on a polymeric substrate).

Substrates: Sodium pectate was obtained from citrus pectin by a repeated alkaline deesterification with 0·1N-NaOH at pH 10 and 22°C. The deesterified product was precipitated after lowering the pH to 2·5 with hydrochloric acid and neutralized with sodium hydroxide. Sodium pectate contained 82·9% of D-galacturonan, 10% of neutral saccharides and 0·2% of rhamnose. The mean molecular mass 27 000 was determined viscometrically. Oligogalacturonic acids were prepared from the partially or enzymatically hydrolyzed D-galacturonan by gel chromatography on Sephadex G-25 Fine and desalting on Sephadex G-10 by a procedure already described[12,13].

Methods

The substrates were enzyme-hydrolyzed at pH 5·1 and 30°C. The course of degradation was monitored by measurement of the reducing group increases in time intervals. The liberated reducing groups were estimated by the Somogyi method[14]. The starting reaction rate (in mol . $. s^{-1}$ kg^{-1}) was calculated by graphical extrapolation of the experimental data. The limit degradation time was considered that at which no further reducing group increase was observed at a long-lasting hydrolysis. The degree of maximum enzyme degradation of the polymeric substrate determined from the maximum value of reducing groups freed by the enzyme in the limit time of degradation was expressed in per cent of the cleaved glycosidic bonds.

Change of the Molecule Size of the Polymeric Substrate in Correlation with Both the Rate and Degree of Degradation

A) Sodium pectate (100 ml of a 1% solution in an 0·1M-acetate buffer of pH 5·1) was incubated at 30°C with the enzyme (2 mg) dissolved in the same acetate buffer (5 ml) for 158 h. Samples (13 ml) each) withdrawn in time intervals were inactivated by boiling and employed for determination of both the degradation degree of the substrate and degradation products. The concentrated 10 ml-aliquots were transferred on a Sephadex G-50 column (1·8 × 120 cm) and the elution volume of the degraded polymeric molecule was estimated.

B) Sodium pectate (100 ml of a 1%-solution in an 0·1M-acetate buffer of pH 5·1) and hexagalacturonic acid (100 mg, 82 μmol) dissolved in 0·1M-acetate buffer of pH 5·1 (5 ml) were incubated at 30°C with the enzyme (2 mg) dissolved in the same buffer (5 ml) for 168 h. Samples (13 ml) each) were withdrawn in the same time intervals and worked up as under *A*).

C) Sodium pectate (100 ml) of a 1% solution in 0·1M-acetate buffer of pH 5·1) and trigalacturonic acid (100 mg, 150 μmol) were incubated with the enzyme (2 mg) under the same conditions as in the preceding cases.

The elution volumes of polymeric molecules were determined in 10 ml-aliquots of samples A), B) and C) after gel chromatography on Sephadex G-50 equilibrated and eluted with 0·1M-acetate buffer of pH 4·4. Reducing groups in the particular fractions were photometrically estimated after reaction with 3,5-dinitrosalicylic acid at 530 nm; the obtained data were graphically evaluated and the elution volumes of molecules were determined from the curve courses. The degradation products of sodium pectate, hexa- and trigalacturonic acids were chromatographically determined in aliquots of inactivated samples A), B) and C) in the respective phases of hydrolysis. Solvent system for paper chromatography of oligomers was ethyl acetate–acetic acid–water (18 : 7 : 8), for thin-layer chromatography on Silufol sheets 1-butanol–formic acid–water (2 : 3 : 1), *cf.* ref.[15]. The particular oligogalacturonic acids were identified after detection with anilinium hydrogen phthalate reagent according to value $\log [R_F/(1 - R_F)]$, which is the function of polymerization degree. D-Galacturonic acid and standard oligogalacturonic acids were the reference sample.

The Mode of Attack by the Enzyme

A) *Considering the degradation products of hexa- and pentagalacturonic acids*: From the reaction mixture, containing hexa- or pentagalacturonic acid (50 mg) and the enzyme (0·06 mg) in 0·1M-acetate buffer of pH 5·1, samples (0·4 ml each) were withdrawn in 10, 20, 30, 60 and 120 min intervals. Samples of the mixture inactivated by a 20 min-boiling were chromatographically analyzed.

B) *Considering the degradation kinetics of pentagalacturonic acid*: Each sample (2·5 ml), containing pentagalacturonic acid (37·5 μmol) and the enzyme (0·07 mg) was incubated in 0·1M-acetate buffer of pH 5·1. After 30, 60 and 120 min-incubation the increase of reducing groups was estimated in 0·1 ml aliquots. The inactivated reaction mixture (2 ml-samples) were introduced on the Sephadex G-25 column Fine (two tail-to-head coupled columns 1·6 × 120 cm) equilibrated with 0·1M-acetate buffer of pH 4·4 and the degradation products were eluted by the equilibration solution. A standard mixture of oligogalacturonic acids composed of pentagalacturonic acid (12 μmol), tetragalacturonic acid (18 μmol), trigalacturonic acid (10 μmol), and D-galacturonic acid (10 μmol) was worked up in the same way, the fraction volume was 4 ml. Oligogalacturonic acids were identified by thin-layer chromatography; their quantitative representation was determined by the Somogyi method. The per cent yield, with respect to the amounts applied, was: pentagalacturonic acid 80%, tetragalacturonic acid 76%, trigalacturonic acid 74%, and D-galacturonic acid 79%.

RESULTS

To find the proper time for the desired conversion of the substrate, when investigating the single- and multi-chain action pattern, only a little amount of samples was employed. Changes of the substrates were monitored within 144 h, when the maximum degree of polymer degradation was attained, *i.e.* 43·3% of cleaved glycosidic bonds. Upon gel filtration the peak of the polymer substrate lowered with the proceeding degradation of the polymer towards higher elution volumes, this being an indication that the enzyme did not keep associated with the first substrate molecule, but concurrently cleaved various molecules. Table I shows the degradation course upon time of the polymer substrate (sample A), hexagalacturonic acid (sample D)

and trigalacturonic acid (sample E) expressed in μmol of the D-galacturonic acid increase. The substrate in experiment B was 1%-solution of sodium pectate and hexagalacturonic acid (0·1 g, 82 μmol). The interaction of the enzyme with sodium pectate in the presence of trigalacturonic acid (0·1 g, 150 μmol) was investigated in experiment C. Addition of hexagalacturonic acid to the polymeric substrate influences both the rate and degradation degree even after 30 min-incubation (Tables I, II). A different course has the degradation of the polymer in the presence of trigalacturonic acid, where changes due to the action of hexagalacturonic acid on the polymer were observed only after 24 h. The degradation degree of the polymeric

TABLE I

Amount of reducing groups (μmol) liberated by degradation of polymeric and oligomeric substrates by exo-D-galacturonanase

Sample[a]	Incubation, h							
	0·5	1	2	6	24	48	120	144
A	186·3	294·6	449·2	720·6	1 280·4	1 566·8	1 754·4	1 998·6
B	197·8	355·0	491·0	771·0	1 339·2	1 739·2	1 814·3	2 152·3
C	186·2	294·0	449·3	720·0	1 302·0	1 709·2	1 791·0	2 098·1
D	53·9	103·6	130·8	169·9	211·6	248·1	268·2	308·2
E	30·1	50·4	65·3	107·8	174·6	209·0	231·2	247·0

[a] A a 1% solution of sodium pectate, B a 1% solution of sodium pectrate with an addition of hexagalacturonic acid (100 mg), C a 1% solution of sodium pectate with addition of trigalacturonic acid (100 mg), D hexagalacturonic acid (100 mg), E trigalacturonic acid (100 mg).

TABLE II

Degree of degradation (%) of the polymer and oligomer substrates during incubation (h) by carrot exo-D-galacturonanase

Sample[a]	% of degradation after incubation, h							
	0·5	1	2	6	24	48	120	144
A	4·2	6·4	9·2	15·7	28·0	34·3	38·9	43·3
D	12·2	23·5	30·7	38·6	48·1	56·3	62·9	70·1
E	6·8	11·4	14·8	21·5	37·0	47·2	52·5	56·0

[a] A, D, E substrates defined in Table I.

substrate was also reflected in the changes of elution volume of the partially degraded polymer molecules (Table III). Deviations in the rate and degradation degree of sodium pectate due to the effect of hexa- and trigalacturonic acids were also seen in changes of elution volumes of the polymer degraded under modified conditions (Table III).

Degradation products of hexagalacturonic acid during a simultaneous degradation of the polymer substrate (experiment B) are after a 5, 10 and 30 min hydrolysis pentagalacturonic and D-galacturonic acids only. A 6 h hydrolysis afforded also trigalacturonic acid as a degradation product; nonetheless the reaction mixture did not contain, at this stage of degradation, the starting substrate. Digalacturonic acid was found after a 48 h hydrolysis only, when the highest degradable substrate

TABLE III

Time dependence (h) of the elution volume (ml) of the polymer substrate during its degradations by exo-D-galacturonanase in the absence (A) and in the presence of oligogalacturonic acids (B and C)

Sample[a]	Elution volume (ml) after incubation, h						
	0	1	2	6	24	48	120
A	202	205	219	246	290	312	325
B	202	202	214	235	276	290	298
C	202	202	219	245	262	280	290

[a] A, B, C, substrates defined in Table I.

TABLE IV

Kinetics of exo-D-galacturonanase action on pentagalacturonic acid (μmol of acids produced)

Incubation min	D-Galacturonic acid	Tetragalacturonic		Trigalacturonic	
		calculated[a]	found	calculated[a]	found
30	9·80	8·71	8·57	0	0
60	22·50	20·50	17·70	0	2·78
180	30·26	29·50	25·90	25·90	3·84

[a] Calculated on the basis of a total multi-chain process from the gain of D-galacturonic acid and fractionation on a Sephadex G-25 (Fine) column.

in the incubation mixture was tetragalacturonic acid. Exo-D-galacturonanase of carrot degraded pentagalacturonic acid within 30 min at pH 5·1 and 30°C to tetragalacturonic acid and monomer exclusively, within 60 and 180 min the further product was also trigalacturonic acid. Fractionation of degradation products of pentagalacturonic acid by gel chromatography jointly with monitoring the kinetics of the enzyme action showed that after 60 and 180 min degradation a 86% conversion of pentagalacturonic acid to the tetramer was achieved instead of the anticipated 100% one (Table IV, Figs 1–3).

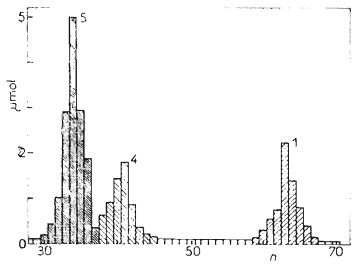


FIG. 1

Quantitative analysis of degradation products of pentagalacturonic acid after a 3 min incubation with exo-D-galacturonanase, after their separation by gel chromatography on Sephadex G-25 (Fine). Symbols *1*, *3*, *4*, and *5* refer to the polymerization degree of oligogalacturonic acids

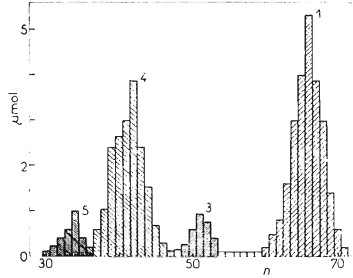


FIG. 2

Quantitative analysis of degradation products of pentagalacturonic acid after a 60 min incubation with exo-D-galacturonanase, after their separation by gel chromatography on Sephadex G-25 (Fine)

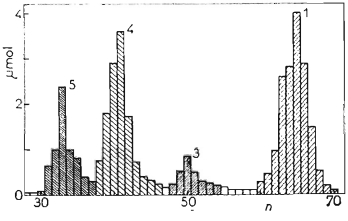
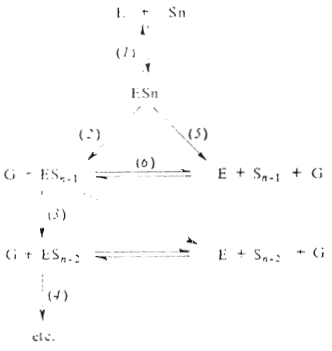


FIG. 3

Quantitative analysis of degradation products of pentagalacturonic acid after a 180 min incubation with exo-D-galacturonanase, after their separation by gel chromatography on Sephadex G-25 (Fine)

DISCUSSION

The action of exo-D-galacturonanase at a single and multi-chain process can be distinguished as follows (Scheme 1):



SCHEME 1

E enzyme, S_n substrate of $DP = n$, G galacturonic acid

The single-chain action is a sum of successive reactions (*1*), (*2*), (*3*), (*4*), where certain enzyme molecule reacts with the same substrate molecule. The multi-chain action proceeds *via* reaction (*1*) and (*5*), or (*1*), (*2*) and (*6*), continuation by reaction (*7*) by a recombination with the same, or another substrate. Both these conception were verified on one type of polymeric substrate of $DP = 131$ and on two low-molecular substrates, namely hexa- and pentagalacturonic acids. An increase of the reducing value at a time-degradation of sodium pectate by exo-D-galacturonanase, and the change of the polymer molecule size reflected in the elution volume of the original and partially degraded molecules were related when using the polymeric substrate. As it follows from the mechanism of exo-D-galacturonanase action on the polymer substrate, it is very difficult in the first phases to distinguish whether the single- or multi-chain process is involved, since the first attack canot, in principle change the nature of the molecule, but changes the chain length to a little e[illegible] Therefore, the degradation was monitored in various time intervals durin[illegible] h-degradation. The single chain action in this case influences less the siz[illegible] mer molecule than does the multi-chain process. A gradual incre[illegible] volume of a a partially degraded polymer molecule indicate[illegible]

of the enzyme. To verify this presumption, the interaction of exo-D-galacturonanase with a polymer substrate was investigated in the presence or in the absence of hexa- and trigalacturonic acids. An addition of hexagalacturonic acid proved the multi-chain process at the polymer substrate. Hexagalacturonic acid was able to compete with the polymer for the enzyme already in the starting phases of the reaction. This was manifested by an enhanced amount of the freed D-galacturonic acid when contrasted with the experiment in which the enzyme degraded the polymer substrate only. At an alternating interaction of the enzyme with the polymer substrate and hexagalacturonic acid an increased amount of freed D-galacturonic acid and particularly, degradation products of hexagalacturonic acid were observed. This was associated with a slowing-down of degradation of polymer chains, and consequently also with a gentle increase of elution volume of the degraded polymer molecules upon time. The products of degradation of hexagalacturonic acid determined after separation of the polymer by gel filtration are also in favour of the multi-chain process. Exlusively pentagalacturonic and D-galacturonic acids were found to be the reaction products after a 5, 10 and 30 min hydrolysis. Only after a 6 h-hydrolysis, when no hexagalacturonic acid was present in the reaction medium, tetramer and trimer were the products of reactions. During a 120 h-hydrolysis none significant amount of the monomer in the excess of hexagalacturonic acid was found in any phase, what could indicate the single-chain process of the exo-D-galacuronanase action. Difference between the simultaneous interaction of the enzyme with the polymer and trigalacturonic acid, and the alternating interaction of the polymer with hexagalacturonic acid was that trigalacturonic acid started to compete for the enzyme only after a 24 h incubation, and even with a lowered effect. This is evidently due to a lowered affinity of exo-D-galacturonanase towards trigalacturonic acid when contrasted with hexagalacturonic acid and sodium pectate; these results are in agreement with those we have already reported[11].

A predominant mode of action of the enzyme process only can be observed during interaction of exo-D-galacturonanase with the polymer substrate in the presence, or in the absence of oligomer substrates. The single- or multi-chain mechanism of action of exo-D-galacturonanase can be distinguished when using the precisely defined oligomeric substrates and monitoring both the kinetics of the enzyme process and the reaction products in the individual phases of the reaction. Four D-galactopyranuronic acid units can be cleaved from each molecule of the pentamer when degrading pentagalacturonic acid by exo-D-galacturonanase. In the case of a single--chain process (under a great excess of the substrate) only D-galacturonic acid should result in addition to the not degraded pentagalacturonic acid molecules. The amount of liberated D-galacturonic acid should be identical with that of the tetragalacturonic acid formed in the exclusive multi-chain process provided a sufficient amount of the original substrate in the medium. Therefore, theoretical values of intermediates for a full multi-chain process were calculated for three time-intervals. The real

amount of intermediates was determined after fractionation of degradation products by gel chromatography. As it follows from results of these experiments only multi--chain process of degradation of pentagalacturonic acid took place at a 30 min lasting hydrolysis. This is in line with findings reported by Balley and Whelan[5] who attained the highest ratio of multi-chain action (98%) with degradation of maltohexose by purified soya-been β-amylase, whereas the ratio for polymeric amylose was much lower. Our experiments showed that the 60 and 180 min hydrolyses of pentagalacturonic acid gave a 14% deviance from the theoretically presumed totally multi-chain process. The fact that digalacturonic acid as intermediate was not observed in the whole phase of minotoring the reaction, and trigalacturonic acid formed only 14% of the amount of the tetramer originated, and only minimal amount of the original substrate was present let us refuse a single-chain mechanism of pentagalacturonic acid degradation. The occurrence of trigalacturonic acid as a product of degradation at a 60 and 180 min hydrolyses, presuming the total multi-chain action, can be explained by two alternative ways. The first is based upon a multi-chain process with a iterative attack (maximum two cleaved D-galacturonic acid units per one effective collision of the enzyme with the substrate). A more probable alternative is based on the presumption of randomness of the attack during the multi-chain process, when this becomes a competitive substrate of the enzyme process in the excess of the product of tetragalacturonic acid. A multi-chain process for the action of exo-D-galacturonanase was also supposed by Pressey and Avants[16], who investigated the degradation course of pectic acid by exo-D-galacturonanase from apricot by gel chromatography on Sephadex G-100. The finding reported by Mill[17], who proposed the multi-chain mechanism on the basis of the course of cleavage of trigalacturonide by exo-D-galacturonanase from *Aspergillus niger* mycellium was in line with our results. The measure for the multi-chain attack showed by any enzyme seems to be close to kinetic constants, governing the vitality of the enzyme–substrate complex and limiting the time of catalytic transformation[18–20]. Following the usual relationships of enzyme kinetics expressed by Michaelis and Menten, the modell of enzyme action depends on the relative rate constants k_1, k_{-1} and k_2 controlling the formation, dissociation and reaction of the inermediate ES complex.

This paper reports the multi-chain mechanism of degradation of polymer substrate by exo-D-galacturonanase from carrot. The employed methods do not, however, exclude involvement of a single-chain process at a gradual degradation of the polymer molecule. This project could be solved on the basis of experimentally determined rate constants responsible for the formation of ES complex, for dissociation of this complex and for the reaction giving the product. The vitality of the enzyme--substrate complex at a simple collision is given by the ratio k_2/k_{-1}. The relationship between the Michaelis constant and rate constants can serve for deducing the multiplicity of the attack. This paper has been aimed to characterize the preferential processes.

REFERENCES

1. Swanson M. A.: J. Biol. Chem. *172*, 805 (1948).
2. Cleveland F. C., Kerr R. W.: Cereal. Chem. *25*, 133 (1948).
3. Hopkins R. H., Jelinek B.: Nature (Lonson) *164*, 955 (1949).
4. Hopkins R. H., Jelinek B.: Biochem. J. *56*, 136 (1954).
5. Bourne E. J., Whelan W. J.: Nature (London) *166*, 258 (1950).
6. Balley J. M., Thomas G. J., Whelan W. J.: Biochem. J. *49*, 141 (1951).
7. Balley J. M., French D.: J. Biol. Chem. *226*, 1 (1957).
8. French D., Knapp D. W., Pazur J. H.: J. Amer. Chem. Soc. *72*, 1866 (1950).
9. French D., Levine M. L., Pazur J. H., Norberg E.: J. Amer. Chem. Soc. *72*, 1746 (1950).
10. Balley J. M., Whelan W. J.: Biochem. J. *67*, 540 (1957).
11. Heinrichová K.: This Journal *42*, 3214 (1977).
12. Rexová-Benková Ľ.: Chem. Zvesti *24*, 59 (1970).
13. Heinrichová K.: Biológia, in press.
14. Somogyi M.: J. Biol. Chem. *195*, 19 (1952).
15. Koller A., Neukom H.: Biochim. Biophys. Acta *83*, 366 (1964).
16. Pressey R., Avants J. K.: Phytochemistry *14*, 941 (1975).
17. Mill P. F.: Biochem. J. *99*, 557 (1966).
18. Robyt J. F., French D.: Arch. Biochem. Biophys. *138*, 662 (1970).
19. Thoma J. A.: Carbohyd. Res. *48*, 85 (1976).
20. Bransk W., Greenwoud C. T., Khan K. M.: Carbohyd. Res. *12*, 79 (1970).

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