ISOLATION, CHARACTERIZATON AND MODE OF ACTION OF EXO-D-GALACTURONANASE FROM CARROT

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D-Galacturonanase (EC 3.2.1 67) was obtained by salting-out from the extract of carrot with ammonium sulphate and separation of the mixture of proteins on DEAE cellulose and Sephadex G-100 columns. Only one form of D-galacturonanase was found in the purified preparation by electrophoresis on polyacrylamide. The maximum activity and stability displayed by the enzyme was at pH 1·5. The enzyme is stable even at high temperatures. D-Galacturonanase from carrot cleaved digalacturonic acid, *p*-nitrophenyl-α-D-galactosiduronate and also substrates having a 4-deoxy-L-*threo*-hexo-4-eno-pyranosyluronate unit at the nonreducing end. The enzyme was characterized by kinetic constants (K_m and V) for polygalacturonic and oligogalacturonic acids with degrees of polymerization 2 to 7 and 10. The enzyme is classified on the basis of the terminal action pattern of the purified enzyme and preference of high-molecular substrates as exo-D-galacturonanase.

Exo-D-galacturonanases are enzymes catalyzing the hydrolytic cleavage of glycosidic α-1,4-bonds of D-galactopyranuronic acid units situated at the end of D-galacturonans and releasing D-galactopyranuronic acid as a sole reaction product. Substrates for these enzymes are polygalacturonic and oligogalacturonic acids and, in contrast to endo-D-galacturonanases, also digalacturonic acid.

Although exo-D- galacturonanases were found to be present in several higher plants, it is little known about their function[1]. Exo-D-galacturonanase from carrot was already partly purified and characterized[2,3]. Exo-D-galacturonanase was isolated also from extracts of peaches[4], bananas[5] and cucumbers[1]. Exo-D-galacturonanases of vegetal origin so far described favourize de-esterified substrates; degradation of substrates proceeds from the nonreducing end of the chain, the particular enzymes differing from each other by the range and rate of the effect on substrate in relation to the chain length. Unlike exo-D-galacturonanases of microbial origin, which favourize oligomers of lower DP[6,7], digalacturonic acid[8], or those in which the substrate chain length is not a factor determining the enzyme effect[9], the most suitable substrates for exo-D-galacturonanases of vegetal origin are D-galacturonan or a partly degraded D-galacturonan of DP 20 (ref.[2,4]). The effect of exo-D-galacturonanases of vegetal origin on substrates containing a 4-deoxy-L-threo-hexo-4-eno-pyranuronate (4,5-unsaturated D-galactopyranuronic acid) unit at the nonreducing end of the

molecule has as yet not been investigated. These substrates were hitherto degraded only by an extracellular[7] exo-D-galacturonanase from *Aspergillus niger* and an enzyme from mycelium[10] of the same microorganism. Exo-D-galacturonanases both of vegetal and microbial origin do not, however, fully degrade pectic acid, the limit degradations being proportionally different with the content of neutral saccharides in the pectin molecule. Further differences concerning the properties of exo-D-galacturonanases (*e.g.* the effect on substrates of various polymerization degree, the effect of mono- and bivalent cations on the enzyme activity, various pH optimum) indicate that also enzymes with terminal effect show a different action pattern similarly, as endo-D-galacturonanases.

To characterize exo-D-galacturonanase from carrot in more detail, we investigated the degradation rate of substrates of various polymerization degrees in an equimolar concentration and ascertained whether differences in the degradation rate reflect the effective concentration of terminal bonds, or result from the mechanism of the enzyme reaction. Examination of the effect of exo-D-galacturonanase from carrot on modified substrates threw light on the role of some functional groups of the employed substrate. In this paper we verified the presumption of Hatanaka and coworkers[2] concerning the presence of isoenzymes of exo-D-galacturonanase in carrot.

EXPERIMENTAL

Material

Sodium pectate (polygalactosiduronate content 75·5%, molecular weight 27000) used as substrate for exo-D-galacturonanase activity determination was prepared from apple pectin by a repeated alkaline deesterification with 0·1M-NaOH at pH 10 and 22°C. The deesterified product was precipitated with hydrochloric acid added to pH 2·5.

Pectic acid (polymerization degree 10 ± 1) was obtained from acid hydrolysis of pectic acid[11]. A product of a great distribution of the polymerization degree was obtained and therefore, it was separated by a multiple gel chromatography over Sephadex G-25 Fine[12] and desalted on Sephadex G-10. The purity criterion was the ratio of carboxyl groups determined titrimetrically to the content of reducing groups estimated according to Somogyi[13].

Oligogalacturonic acids of polymerization degree 2 to 7 were prepared from a partial acid hydrolyzate of pectic acid, separation by gel chromatography on Sephadex G-25 Fine (*cf.*[12]) and desalting on Sephadex G-10. Purity of the individual oligogalacturonic acids was monitored by paper chromatography in a solvent system ethyl acetate–acetic acid–water (18 : 7 : 8), or by TLC on silica gel (Silufol, Kavalier) in a solvent system n-butanol–formic acid–water (2 : 3 : 1) according to Koller and Neukom[14]. Oligouronic acids were detected with aniline–phthalate reagent and identified on the basis of the linear dependence of log $[RF/(1 - RF)]$ values on degree of polymerization of oligogalacturonic acids[15] the reference substance being D-galacturonic acid. The second purity criterion of oligogalacturonic acids was the ratio of the carboxyl to reducing groups content. The 4,5-dehydrogalacturonosyl-D-oligogalactosiduronates were prepared from a highly esterified pectin (degree of esterification 95%) by heating in a boiling water bath at pH 6·8.

p-Nitrophenyl-α-D-galactopyranuronide was kindly supplied by Dr K. Schwabe, Central Institute for Cancer Research, Academy of Sciences, Berlin-Buch, GDR.

Methods

The activity of exo-D-galacturonase was estimated by measuring the increment of reducing groups during the enzymic reaction by the Somogyi method[13], sodium pectate (0·5% solution in 1M pH 5·1 acetate buffer) being the substrate. Solutions of the substrate and the enzyme (0·5 ml each) were mixed together and incubated at different time intervals at 30°C. The activity was expressed in μmol of reducing groups liberated within one min per one mg of the protein. Degradation products were monitored by TLC on silica gel in a solvent system n-butanol–formic acid–water (2 : 3 : 1) (ref.[14]) after visualization with aniline phthalate reagent.

Michaelis constants and maximum reaction rates of exo-D-galacturonase were estimated according to Lineweaver and Burk[16] employing sodium pectate (degree of polymerization 153) in a $1{\cdot}3 \cdot 10^{-4}$ mol . l^{-1} to $9{\cdot}0 \cdot 10^{-4}$ mol . l^{-1} concentration range, galacturonic acid (degree of polymerization 10) in a 0·1 to 2·0 μmol concentration range in 1 ml of reaction mixture. Concentration of the enzyme for all substrates used amounted 0·25 mg/1 ml of the reaction mixture. The incubation time for sodium pectate and galacturonic acid of degree of polymerization 10 was 10 min, for hepta, hexa and pentagalacturonic acids 15 min and for tetra, tri and digalacturonic acids as substrates 20 min at 30°C. The K_m value was calculated by regress analysis always from 18 experimental v and s values. Michaelis constant is given in mol . l^{-1}, maximum rate V in μmol of reducing groups freed during 1 min per 1 mg of protein. A 95% interval of reliability of these values and a correlation coefficient of linear dependence $1/v$ on $1/s$ are reported in this case. Discontinuous electrophoresis on polyacrylamide gel was performed according to Ornstein[17] and Davis[18]. Proteins were stained with amido black 10 B, the activity of D-galacturonanase was detected after incubation of the gel in a 1·25% solution of sodium pectate by dyeing with ruthenium red[19], proteins were estimated according to[20].

RESULTS AND DISCUSSION

The enzyme was isolated from fresh ground carrot (*Daucus carota* L.) from which the liquid was removed by pressing and the pulp was extracted for 24 h with a NaCl solution of pH 5 in a 1 : 1 (w/w) ratio under occassional stirring at 4°C, so as the final NaCl concentration reached 5%. The supernatant, obtained by pressing and centrifuging (at 6000 *g*), was precipitated with ammonium sulphate up to 90% saturation. The precipitate was dialyzed against water for 24 h and then freeze-dried. This partially desalted product was completely desalted with Sephadex G-25 Medium and the protein-nonhomogeneous preparation thus obtained was chromatographically separated on DEAE-cellulose equilibrated with a 0·005M phosphate buffer of pH 7. The separation was tested by means of absorbance recorded at 280 nm. A gradual elution with phosphate buffers afforded four fractions (Fig. 1).

The individual fractions collected from this chromatography were desalted on a Sephadex G-25 Medium column and subjected to an enzyme activity determination.

The activities of D-galacturonase M_1 and M_2 eluted with 0·05M and 0·1M with 0·005M NaCl phosphate buffers, respectively, were determined. Both fractions contained an enzyme which, on the base of a sole degradation product – D-galacturonic acid (using sodium pectate as a substrate) – and the ability to cleave digalacturonic acid,

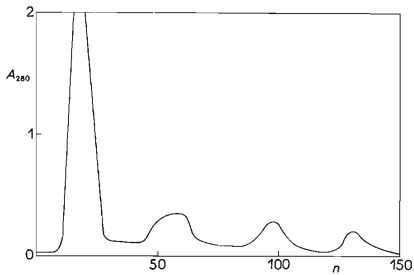

FIG. 1

Chromatography of the Raw Preparation M_0 on a DEAE-Cellulose

The column (3·8 . 24 cm) was eluted with phosphate buffers: (A) 0·005M, (B) 0·05M, (C) 0·1M at pH 7·0, (D) 0·2M at pH 6·0. (Buffers B, C and D contained 0·05M-NaCl). Elution volume 17 ml/30 min, n — the number of fractions.

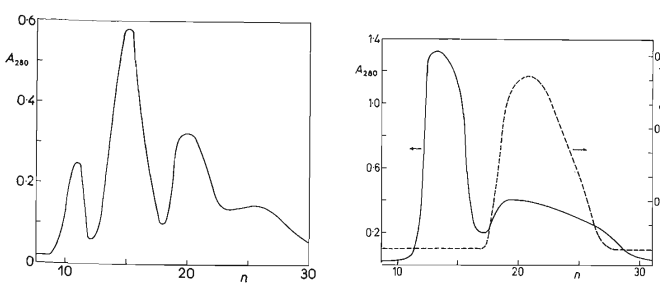

FIG. 2

Chromatography of the Purified Product on Sephadex G-100

The column (1·4 . 90 cm) was eluted with 0·1M acetate buffer in 0·5M-NaCl, pH 5·1. a — separation of the combined product M_1 and M_2; flow-rate 6 ml/30 min (1 fraction); n — the number of fractions; b — separation of product M_3; flow-rate 5 ml/30 min (1 fraction); full line — absorbance at 280 nm, dashed line — the specific activity μmol/mg, n — the number of fractions.

was characterized as exo-D-galacturonase. The fact that this activity was found in two fractions could indicate the presence of several forms of exo-D-galacturonanase in carrot. The freeze-dried products M_1 and M_2 with the same content of protein differed in the specific activity: the first product revealed 0·44 μmol of cleaved glycosidic bonds, the latter 0·56 μmol when using sodium pectate as a substrate at pH 5·0 and 30°C. Therefore, the fractions M_1 and M_2 were subjected to an electrophoresis on polyacrylamide gel. Staining with amido black 10 B showed four protein zones in both fractions, visualization with ruthenium red only one zone at the same place of the gel column with polygalacturonanase activity. These arguments and the estimated pH optimum (both fractions displayed the identical pH 5·1 value) allow us to assume the exo-D-galacturonase to be present in one form only in the extract from carrot.

Fractions M_1 and M_2 were collected and the enzyme further purified by separation on Sephadex G-100 equilibrated with an acetate pH 5·1 buffer containing 0·5M-NaCl. Of three fractions thus obtained the activity of exo-D-galacturonanase was found in the second one (Fig. 2*a*). Desalting of this product on a Sephadex G-25 Medium column and freeze-drying afforded the product M_3 in which 3 protein zones were detected after discontinuous electrophoresis on polyacrylamide gel, but only one of them showed an exo-D-galacturonanase activity. Aiming to obtain a protein-homogeneous enzyme this fraction was rechromatographed under the afore-mentioned conditions on a Sephadex G-100 column (Fig. 2*b*). Two fractions were obtained the latter of

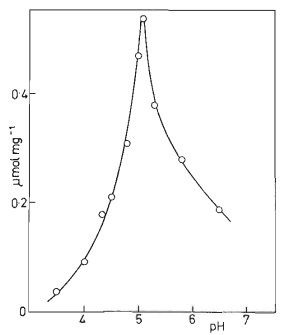

FIG. 3

Effect of pH on the Reaction Rate Catalysed by Exo-D-galacturonanase

Substrate — 0·5% solution of sodium pectate in 0·1M acetate buffer for pH 3 to 6; for pH 6·5 0·1M phosphate buffer.

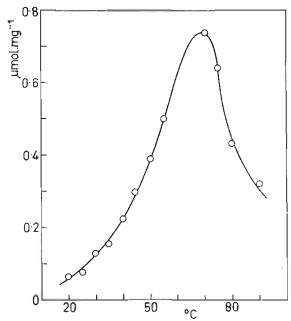

FIG. 4

Dependence of Exo-D-galacturonanase Activity on Temperature

Substrate — 0·5% solution of sodium pectate in 0·1M acetate buffer at pH 5·1.

which displayed an exo-D-galacturonase activity and was electrophoretically homogeneous. The investigation of degradation products of each fraction collected in the three-step chromatography of the raw product excluded the presence of endo-D-galacturononanase in carrot. The raw extract from carrot was shown to contain pectinesterase[21] when the concentration and pH of the extraction solvent (0·2M-NaCl, pH 7·8) were changed.

This purification procedure afforded a homogeneous enzyme M_4 with a 70 times higher activity than that of the raw product with a 82·9% protein content. The relationship between the exo-D-galacturononanase activity and pH is plotted in Fig. 3. The enzyme has a narrow pH activity range with optimum at 5·1, it is relatively stable in acid, neutral and unstable in alkaline media (Fig. 4). The optimum temperature for action of this enzyme lies between 60 and 70°C at which it is highly stable.

Experiments examining the effect of the enzyme on modified substrates clarified the mode of action of exo-D-galacturononanase. It has thus been shown that exo-D-galacturononanase from carrot cleaves 4,5-unsaturated tetragalacturonic acid to form trigalacturonic acid as a primary saturated product the degradation rate of the unsaturated substrate being equal to that of its saturated analogue. It means that the group at $C_{(4)}$ of the D-galactopyranuronic acid unit at the nonreducing end is not essential either for the enzyme-substrate complex formation, or the catalytic reaction.

The effect of NaCl on the activity of polygalacturononanases of vegetal origin was investigated by several authors. The enhancement of the activity by NaCl was reported with endo-D-galacturononanase from tomato[22,23] and banana[5]; a partially inhibitory effect of NaCl was observed with exo-D-galacturononanase from carrot[3]. We have also observed an inhibitory effect of NaCl with exo-D-galacturononanase from carrot, the inhibition degree being dependent on the concentration of NaCl in the reaction medium (Table I).

Exo-D-galacturononanase from carrot was further characterized by the effect of polymerization degree of the substrate on the degradation rate of glycosidic bonds.

TABLE I

Effect of NaCl on the Activity of Exo-D-galacturononanase

Specific activity was determined using sodium pectate as substrate at 30°C and pH 5·1.

Addition of NaCl mol/l	0	0·025	0·050	0·075	0·100	0·150	0·250
Specific activity μmol/mg . min	0·72	0·68	0·65	0·57	0·48	0·38	0·20

Values K_m and V for the series of homologous oligomeric substrates and for two polymeric substrates (DP = 153 and 10) were determined at optimum enzyme conditions (Table II). The slowest degradation rate was found for digalacturonic, the highest for polygalacturonic (DP = 10) acids. The K_m values decreased with extension of the substrate chain length. Degradation of the polymeric substrate did not proceed completely. The substrate of DP = 153 was hydrolyzed to 29%. In addition to the major product D-galactopyranuronic acid, even di and trigalacturonic acids were found after a 36 h hydrolysis. Cumulation of these products can be rationalized by a low affinity of exo-D-galacturonanase to those substrates. Our results concerning exo-D-galacturonanase from carrot indicate, in accordance with those published, that even for enzymes with terminal action pattern a finding verified for endo-D-galacturonanase, that enzymes isolated from the same biological individual can differ in some properties, holds. Our exo-D-galacturonanase has identical pH optimum with the enzyme from carrot isolated by Hatanaka and Ozawa[2] and differ from that described by Pressey and Avants[3] in the pH optimum. As we have evidenced by electrophoresis on a polyacrylamide gel, exo-D-galacturonanase is not present in form of isoenzymes in carrot in contrast to presumption of Hatanaka ans Ozawa[2]. On the other hand, in accordance with these authors, no endo-D-galacturonanase was found in carrot.

TABLE II

Kinetic Constants of Exo-D-galacturonanase form Carrot
(GalpUA) n oligogalacturonic acid of degree of polymerization n.

Substrate	K_m mol/l	V μmol/mg . min	Correlation coefficient
Sodium pectate	$(4{\cdot}23 \pm 0{\cdot}13) \cdot 10^{-5}$	$0{\cdot}522 \pm 0{\cdot}016$	0·9970
(GalpUA) 10	$(2{\cdot}45 \pm 0{\cdot}09) \cdot 10^{-4}$	$0{\cdot}579 \pm 0{\cdot}017$	0·9951
(GalpUA) 7	$(2{\cdot}09 \pm 0{\cdot}08) \cdot 10^{-4}$	$0{\cdot}489 \pm 0{\cdot}015$	0·9959
(GalpUA) 6	$(2{\cdot}70 \pm 0{\cdot}09) \cdot 10^{-4}$	$0{\cdot}452 \pm 0{\cdot}016$	0·9923
(GalpUA) 5	$(3{\cdot}18 \pm 0{\cdot}10) \cdot 10^{-4}$	$0{\cdot}400 \pm 0{\cdot}013$	0·9956
(GalpUA) 4	$(4{\cdot}93 \pm 0{\cdot}14) \cdot 10^{-4}$	$0{\cdot}219 \pm 0{\cdot}007$	0·9991
(GalpUA) 3	$(4{\cdot}50 \pm 0{\cdot}14) \cdot 10^{-4}$	$0{\cdot}146 \pm 0{\cdot}005$	0·9972
(GalpUA) 2	$(4{\cdot}55 \pm 0{\cdot}14) \cdot 10^{-3}$	$0{\cdot}131 \pm 0{\cdot}004$	0·9981

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