ISOLATION AND CHARACTERIZATION OF ONE FORM OF TOMATO *endo*-D-GALACTURONANASE

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One form of *endo*-D-galacturonanase of molecular weight close to 50000 was isolated by chromatography on a CM-Sephadex C-50 column from a purified preparation of tomato *endo*-D-galacturonanase which had been obtained by gel chromatography on Sephadex G-75. The homogeneity of this enzyme form was demonstrated by gel electrophoresis and by ultracentrifugation analysis. The sedimentation constant ($s^0_{20,w} = 3{\cdot}7$ S) and the amino acid composition of the enzyme were determined; the cleavage of low molecular weight substrates by the enzyme, its pH-optimum and kinetic constants have also been characterized.

Studies on plant D-galacturonanases* (poly-α-1,4-galacturonide glycanohydrolase, EC 3.2.1.15) have concentrated so far mostly on tomato *endo*-D-galacturonanases. These studies have been done on crude products[1-3] or on partly purified preparations[4-7].

Some time ago McColloch[8] used electrophoresis to demonstrate the presence of two to three components showing D-galacturonanase activity in tomato extracts. In our preceding study, dealing with the isolation of pectin esterase from tomatoes[9], we reported on the presence of *endo*-D-galacturonanase in peak I and II obtained by gel chromatography on Sephadex G-75. Later using starch-gel electrophoresis we were able to show[10] the presence of three zones with D-galacturonanase activity in tomato extracts. Pressey and Avants[11] recently described the separation of two forms of tomato D-galacturonanase by chromatography on DEAE-Sephadex A-50; these forms differed both in molecular weight (44000 and 84000) as well as in the mode of action. None of the forms described was obtained in homogeneous state by the authors.

The isolation of one form of tomato *endo*-D-galacturonanase from a preparation obtained by gel chromatography on Sephadex G-75 and the partial characterization of this form are described in the present study.

* Referred to so far as "polygalacturonases". The new name has been proposed by Prof. D. Horton (private communication) because it corresponds to the name of the substrate preferred.

EXPERIMENTAL

Preparation of enzyme. Ripe tomatoes (*Lycopersicum esculentum*, var. *Immuna*) were used to start with. The tomatoes were homogenized, the homogenate was pressed and the juice was extracted with 5% NaCl solution at pH 7·5. The extract was treated with ammonium sulfate (20—90% of saturation) and the product obtained was dialyzed, freeze-dried, and desalted on Sephadex G-25; colored contaminants and inactive proteins of acidic character were removed by chromatography on DEAE-Sephadex A-50 (ref.[9]). The fraction showing D-galacturonanase activity was concentrated by ultrafiltration through an Amicon (Holland) UM 10 membrane, desalted on a Sephadex G-25 column, and subjected to gel chromatography on Sephadex G-75 as described elsewhere[9]. The second peak obtained which contained the majority of *endo*-D-galacturonanase was desalted on Sephadex G-25 and chromatographed on a CM-Sephadex C-50 column equilibrated with 0·01M phosphate buffer at pH 6·0 containing 0·01M-NaCl. A stepwise gradient of phosphate and NaCl concentration increasing up to 0·2M in both cases was used (Fig. 1). The material emerging in individual peaks from the column was concentrated by ultrafiltration (Amicon, UM 10 membrane) and desalted on a Sephadex G-25 column (Table I).

The activity of endo-D-*galacturonanase* was determined by measurement of the increase of reducing groups according to Somogyi[12] with pectic acid (average molecular weight 33000, esterification degree 0·5%, purity 95·5%) as substrate. The reaction mixture contained 0·5 ml of 0·5% solution of sodium pectate in 0·1M sodium acetate buffer, pH 4·5, containing 0·3M-NaCl and 0·5 ml of the enzyme solution. The mixture was incubated at 30°C and the determination of reducing groups was effected at various time intervals (1—20 min). The activity was expressed in μmol of reducing groups liberated in 1 min at 30°C.

The products of the reaction in which oligogalacturonic acids were used as substrates was effected by chromatography on Whatman No 1 paper in the solvent system ethyl acetate–acetic acid–water (18 : 7 : 8) as well as on thin layers of silica gel (Silufol sheets, Cavalier, Czechoslovakia) in the system n-butanol–formic acid–water (2 : 3 : 1); the aniline phthalate reagent was used for detection. Oligogalacturonic acids of polymerization degree 2—7 were prepared according to Rexová-Benková[13].

The activity of pectinesterase was determined by a modification of the continuous titration method[9] in TTT 11 Autotitrator (Radiometer, Copenhagen, Denmark) with purified citrus pectin (esterification degree 65·1%) as substrate. One unit of pectinesterase activity was defined as 1 μmol of ester hydrolyzed in 1 min at 30°C and pH 7·5.

Molecular weight determinations were made by chromatography on thin layers of Sephadex G-150 Superfine equilibrated with 0·9% NaCl. The apparatus manufactured by Pharmacia Uppsala, Sweden was used and ribonuclease A, chymotrypsinogen, ovalbumin, and human serum albumin served as standards. The proteins were stained with bromophenol blue on a Whatman No 3 MM paper replica of the chromatogram. The D-galacturonanase activity was detected on a replica impregnated with 0·5% solution of sodium pectate in 0·1M sodium acetate buffer which contained 0·15M-NaCl. After 20-min incubation the paper was stained with 0·05% aqueous solution of ruthenium red. The spots with *endo*-D-galacturonanase activity appeared white on a red background.

Ultracentrifugation analysis. The sedimentation analysis was carried out in Model E Beckman Spinco Ultracentrifuge at 56100 revolutions per min. The enzyme samples were dissolved in 0·1M phosphate buffer, pH 7·8, containing 0·1M-NaCl, to 0·4, 0·65, and 0·75% concentration. The value of the sedimentation constant was determined by extrapolation to zero concentration of values obtained at the concentrations given. The sedimentation equilibrium was investigated with 0·03% enzyme solution in the same buffer at 40000 rev./min.

Protein content was determined by the method of Lowry and coworkers[14].

Amino acid analysis was carried out in an automatic amino acid analyzer (type 6020, Instrument Development Workshops, Czechoslovak Academy of Sciences, Prague) according to Spackmann and coworkers[15]. Tryptophan was determined after hydrolysis of the sample in the presence of thioglycolic acid according to Matsubara[16].

Electrophoresis. Discontinuous electrophoresis in polyacrylamide gel was performed according to Ornstein[17] and Davis[18] using the arrangement for basic proteins[19]. Proteins were stained with amido black 10 B and D-galacturonanase activity was detected according to Lisker and Retig[20] by incubation of the gel in sodium pectate and staining with ruthenium red.

Electrophoresis in hydrolyzed, cross-linked starch gel[21] was carried out in 0·3M Tris-HCl buffer at pH 7·5; proteins were stained with nigrosine and D-galacturonanase activity was detected on the paper replica as described for the molecular weight determination by thin-layer chromatography on Sephadex.

RESULTS AND DISCUSSION

The fraction showing maximum *endo*-D-galacturonanase activity which had been obtained by separation of the purified tomato extract on a Sephadex G-75 column and designated as M3/II in the preceding study[9], was subjected to chromatography on a CM-Sephadex C-50 column; a stepwise concentration gradient of phosphate buffer and NaCl was used for elution.

Six fractions were obtained (Fig. 1); the main portion of *endo*-D-galacturonanase was contained in the sixth peak and maximum pectinesterase activity showed the

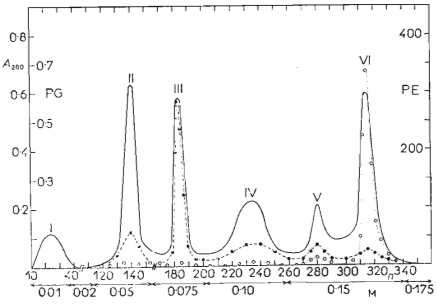

FIG. 1

Chromatography of Purified Tomato *endo*-D-Galacturonanase on Column of CM-Sephadex C-50

Full line A_{280}; broken line, activity of pectinesterase in μmol of hydrolyzed ester. min^{-1} . . ml^{-} (PG); dotted line, activity of *endo*-D-galacturonanase in μmol of reducing groups. min^{-1} . ml^{-1} (PE); I–VI peaks.

third peak. The *endo*-D-galacturonanase thus obtained was still contamined with a small amount of pectin esterase (Table I). The separation of residual pectinesterase was achieved by repeated chromatography of peak VI (Fig. 1) on a column of CM-Sephadex C-50; a continuous elution gradient of sodium phosphate buffer, pH 6·5, containing NaCl was used in the range 0·05M phosphate/0·05M-NaCl-0·2M phosphate / / 0·2M-NaCl. After the main peak containing exclusively *endo*-D-galacturonanase activity had been concentrated and desalted on Sephadex G-25, a product was obtained which on discontinuous electrophoresis in polyacrylamide gel at acidic or basic pH as well as on starch-gel electrophoresis gave one single zone. This zone was positively stained for proteins and showed D-galacturonanase activity. The yield of this form of *endo*-D-galacturonanase calculated in terms of total activity of the parent tomato extract was 4·2%.

The molecular weight examination of the isolated product by thin-layer chromatography on Sephadex G-150 Superfine gave a value close to 48000. A value of 51400 ± ± 2500 was obtained by the sedimentation equilibrium method[22] assuming a partial specific volume of 0·72 ml/g.

Pressey and Avants[11] analyzed the molecular weight of their polygalacturonase II by gel filtration on Sephadex G-100 and obtained a value of 44000. Their product, however, represents only one fraction obtained by resolution of the crude tomato extract on a DEAE-Sephadex A-50 column and obviously contains other contaminants which may have affected the molecular weight determination. The similarity

TABLE I

Characterization of Products of Separation of Tomato *endo*-D-Galacturonanase on CM-Sephadex C-50 Column

Product	Total activity	
	endo-D-galacturonanase %	pectinesterase %
Starting product[a]	100·00	100·00
Ist peak[b]	0·00	0·00
IInd peak[b]	0·65	7·12
IIIrd peak[b]	0·82	52·85
IVth peak[b]	0·38	1·08
Vth peak[b]	1·48	2·15
VIth peak[b]	40·60	0·29
Final product[c]	25·02	0·00

[a] IInd peak from Sephadex G-75 column; [b] peaks from CM-Sephadex C-50 column; [c] after rechromatography on CM-Sephadex C 50 column.

of molecular weight as well as of the pH-optimum and activation by NaCl seems to suggest that the form of *endo*-D-galacturonanase isolated is identical with polygalacturonase II described[11].

The pH-optimum of the *endo*-D-galacturonanase form isolated examined with pectic acid (mol. wt. 33000) as substrate was close to 4·5 in 0·1M sodium acetate buffer not containing NaCl (Fig. 2). The addition of NaCl made the pH-optimum shift to the acidic pH-range. The enzyme activity increased with the increasing NaCl concentration up to 0·25M; and activity decrease was observed at higher NaCl concentrations.

Patel and Phaff[5] investigated the action of purified tomato D-galacturonanase on oligomeric substrates, namely on di-, tri-, and tetragalacturonic acids. They were able to show that their preparation cleaved all the three substrates examined at different reaction rates; this seemed to point to the presence of a mixture of *endo*- and *exo*-D-galacturonanase. By contrast, the isolated form of *endo*-D-galacturonanase exclusively cleaved oligomeric substrates higher than trigalacturonic acid; digalacturonic acid was not cleaved at all.

The value of the Michaelis constant of this form of *endo*-D-galacturonanase examined with sodium pectate of mol.wt. 33000 was $1{\cdot}4 \cdot 10^{-5}$M and the V_{max}-value was $14{\cdot}7 \cdot 10^{-5}\text{M} \cdot \text{min}^{-1}$.

The isolated form of *endo*-D-galacturonanase behaved on ultracentrifugation at 56100 rev/min as a homogeneous peak showing a sedimentation constant of $s^0_{20,w} = 3{\cdot}7$ S.

The amino acid composition of the enzyme was determined (Table II). The relatively high content of amide nitrogen indicates the presence of asparagine and glutamine

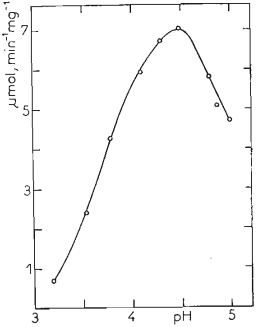

FIG. 2

pH-Optimum of Isolated Form of Tomato *endo*-D-Galacturonanase

Ordinate, activity of *endo*-D-galacturonanase in μmol of reducing groups. $\text{min}^{-1} \cdot \text{ml}^{-1}$; abscissa, pH of substrate (0·5% sodium pectate of molecular weight 33 000 in 0·1M sodium acetate buffer).

TABLE II

Amino Acid Composition of Form of Tomato *endo*-D-Galacturonanase Isolated

Amino acid	Content %	Molar ratio (for mol.wt. = = 50 000)	Nearest integer
Lysine	9·85	38·70	39
Histidine	2·22	8·29	8
Arginine	2·79	9·08	9
Aspartic acid	13·56	59·20	59
Threonine	5·46	27·28	27
Serine	8·21	47·07	47
Glutamic acid	10·95	42·98	43
Proline	4·23	22·35	22
Glycine	4·25	37·52	38
Alanine	3·71	26·55	27
Half-cystine[a]	1·11	5·60	6
Valine	8·13	41·64	42
Methionine	0·78	3·12	3
Isoleucine	9·42	42·08	42
Leucine	4·75	21·34	21
Tyrosine	3·20	10·06	10
Phenylalanine	4·48	15·64	16
Tryptophan[b]	1·42	3·90	4
Amide nitrogen	1·48		

[a] After performic acid oxidation; [b] determined after hydrolysis in the presence of thioglycolic acid[16].

and this may explain the basic character of this enzyme which moved toward the cathode on gel electrophoresis. The isoelectric point determined in orienting experiments on cellulose acetate strips in various buffer solutions was 8·1 – 8·3.

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