PURIFICATION AND CHARACTERIZATION OF ANOTHER D-GALACTURONANASE FROM *Aspergillus niger*

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Pectinase, a commercial preparation of Koch-Light Ltd. (Colnbrook, England) is a mixture of pectolytic enzymes: it contains endo-D-galacturonanase, exo-D-galacturonanase, and pectin esterase. We have been able to isolate from this mixture of enzymes, which is produced by *Aspergillus niger* cultures, by repeated chromatography on a DEAE-cellulose column and subsequent chromatography on Sephadex G-100 a preparation of specific, sixty-three times enriched endo-D-galacturonanase of molecular weight about 46000. The final preparation of purified endo-D-galacturonanase contained a small quantity of enzymatically inactive protein. The enzyme showed maximum activity at pH 5·0 and 40–45°C. Endo-D-galacturonanase was characterized by cleavage of high and low molecular weight substrates, kinetic constants K_m and V for oligogalacturonic acids and pectic acid. The enzyme has been classified on the basis of kinetic constants and products of cleavage and its mode of action is discussed.

Endo-D-galacturonanases (poly-1→4-α-D-galacturonide glycanohydrolase, EC 3.2.1.15) catalyze hydrolytic cleavage of glycosidic α-1,4-bonds of nonesterified units of D-galactopyranuronate in molecules of pectic substances effected by an endo type action pattern. The extent and mode of action of various endo-D-galacturonanases on pectic substrates is different. Differences have been observed not only with endo-D-galacturonanases of different origin but also with enzymes produced by the same biological individual. Endo-D-galacturonanases produced by *Aspergillus niger*[1], *Coniothyrium diplodiella*[2–4], endo-D-galacturonanases found in tomato extracts[5], *etc.* may be adduced by way of example. An atypical mode of degradation of pectic acid has been observed with D-galacturonanases produced by *Colletotrichum lindemuthianum*[6] and *Botrytis cinerea*[7]. The differences are most marked as regards the effect on oligomer substrates where the ability, mode, and rate of cleavage of these substrates are factors which permit the individual endo-D-galacturonanases to be distinguished and classified.

This study was designed to purify an endo-D-galacturonanase from *Aspergillus niger*, to characterize its mode of action on polymer and oligomer substrates, and to compare the mechanism of its action with that described for other *Aspergillus niger* endo-D-galacturonanases. The commercial preparation of Koch-Light Ltd., Coln-

brook, England was chosen as a source of the enzyme because of its relatively high D-galacturonanase activity.

EXPERIMENTAL

Substrates

Sodium pectate (polyuronide content 75·5%, molecular weight 27000), used as substrate for determination of endo-D-galacturonanase activity, was prepared from apple pectin by repeated alkaline deesterification in 0·1M-NaOH at pH 10 and 22°C. The deesterified product precipitated after the pH of the reaction mixture had been decreased to pH 2·5 by hydrochloric acid.

Oligogalacturonic acids with a polymerization degree 2 to 5 were prepared from a partial acid hydrolysate of pectic acid by gel chromatography on Sephadex G-25 Fine, and desalting on Sephadex G-10 as described elsewhere[8]. The purity of the individual oligogalacturonic acids was tested by paper chromatography in the system ethyl acetate–acetic acid–water (18 : 7 : 8) or by thin layer chromatography on silica gel (Silufol sheets, Kavalier) in the system 1-butanol–formic acid–water (2 : 3 : 1) according to Koller and Neukon[9]. Oligogalacturonic acids were detected in both cases by the aniline–phthalate reagent and identified by their log $R_F/(1 - R_F)$ values which linearly depend on the degree of polymerization of oligogalacturonic acids[10]. D-Galactopyranuronic acid served as a reference sample. Another criterion of purity of the oligogalacturonic acids was the ratio of content of carboxyl groups, determined titrimetrically, to the content of reducing groups determined by the carbazole method[11].

Determination of Enzymatic Activity

The activity of endo-D-galacturonanase was determined in terms of increase of reducing groups during the enzymatic reaction by the method of Somogyi[12]; sodium pectate served as substrate (0·5% solution of sodium pectate in 0·1M acetate buffer at pH 5·0). In a typical experiment 0·5 ml samples of substrate were mixed with 0·5 ml of the enzyme solution and were incubated at 30°C for various time intervals. Activity was expressed in micromol of reducing groups liberated in one minute per milligram of protein.

The products of cleavage (sodium pectate and oligogalacturonic acids were used as substrates) were identified by thin-layer chromatography on silica gel in the system 1-butanol–formic acid–water (2 : 3 : 1) (ref.[9]) and detected by the aniline phthalate reagent.

The Michaelis constants and the maximum rates of the endo-D-galacturonanase effect were carried out according to Lineweaver and Burk[13] with sodium pectate (polymerization degree 153, concentration range 0·0025—0·5%), pentagalacturonic, tetragalacturonic, and trigalacturonic acid (concentration range 0·1—2 micromol) in 1 ml of reaction mixture. The enzyme concentration was 0·0025 mg ml^{-1} with sodium pectate as substrate, and 0·02 mg ml^{-1} with the individual oligogalacturonic acids. The time of incubation at 30°C was 3 min with sodium pectate, 5 min with pentagalacturonic and tetragalacturonic acid, and 30 min with trigalacturonic acid. For the determination of K_m, 24 experimental values of v and s were subjected to regression analysis to give an equation of a straight line; the data were treated statistically in a Cellatron computer. The Michaelis constant is given in mol l^{-1}, the maximum rate V is expressed by the number of micromoles of reducing groups per mg of protein in one minute.

The molecular weight of endo-D-galacturonanase was determined by thin-layer chromatography on Sephadex G-150 Superfine equilibrated with 0·9% NaCl in the apparatus of Pharmacia, Uppsala (Sweden); ribonuclease A, chymotrypsinogen, ovalbumin, and human serum albumin

served as standards. The proteins were stained with bromophenol blue on a Whatman 3MM paper replica. The activity of D-galacturonanase was detected on a Whatman 3MM paper replica impregnated with 0·5% sodium pectate in 0·1M sodium acetate buffer. After a 20 min incubation the paper was stained with 0·5% aqueous solution of ruthenium red; sites with endo-D-galacturonanase activity appeared as white spots on a red background.

Discontinuous electrophoresis in polyacrylamide gel was carried out according to Ornstein and Davis[14,15]. Proteins were stained with amido black 10B and the activity of D-galacturonanase was detected according to Lisker and Rettig[16] by incubation of the gel in 1·25% solution of sodium pectate and by staining with ruthenium red. Proteins were determined according to Lowry and coworkers[17].

RESULTS AND DISCUSSION

For the purification of endo-D-galacturonanase Pectinase, a commercial product of Koch-Light (England), was used as starting material. Pectinase (100 g) was dissolved in 2000 ml H_2O with stirring. The insoluble portion was centrifuged off at 6000*g*. A crude enzyme preparation was obtained by salting out the protein fraction of the supernatant with ammonium sulfate (saturation 0·9), ethanol precipitation (four parts of ethanol per one part of supernatant), dialysis, lyophilization of the dialyzed product, and desalting of the lyophilisate on Sephadex G-25 Medium. The individual pectolytic enzymes were separated by chromatography on DEAE-cellulose eluted by a concentration and pH-gradient of phosphate buffers. In this manner six fractions were obtained. The fraction eluted by 0·005M phosphate buffer at pH 7·0 had no pectolytic activity. The fraction eluted by 0·05M phosphate buffer at pH 7·0 showed endo-D-galacturonanase activity, *i.e.* catalyzed the cleavage of glycosidic bonds of sodium pectate and of pectinate esterified to a low degree at random giving rise to oligogalacturonides. The fraction emerging in 0·1M phosphate buffer at pH 7·0 showed a low endo-D-galacturonanase activity. The fraction emerging in 0·15M phosphate buffer at pH 7·0 had predominantly exo-D-galacturonanase activity; the products of cleavage contained mostly D-galactopyranuronic acid and the enzyme readily cleaved digalacturonic acid. The fraction eluted by 0·2M phosphate buffer at pH 7·0 showed no pectolytic activity whereas the fraction eluted by 0·4M phosphate buffer at pH 6·0 was (according to its substrate specificity) pectin esterase.

Fraction N_2 (eluted by 0·05M phosphate buffer and desalted on Sephadex G-25 medium) was rechromatographed on a DEAE-cellulose column eluted by a concentration gradient of phosphate buffers in the molarity range 0·005 – 0·2M, pH 7·0. Endo-D-galacturonanase activity was found in the second fraction. This fraction was desalted on Sephadex G-25 Medium, freeze-dried, and designated N_3. Even though this procedure afforded an endo-D-galacturonanase preparation rich in protein content and with a higher specific activity, it showed the presence of three zones when subjected to polyacrylamide gel electrophoresis. The enzyme was therefore further purified on Sephadex G-100 equilibrated with 0·1M acetate buffer at pH 5·0 containing 0·5M-NaCl. Endo-D-galacturonanase activity was found in the second

TABLE I

Purification of endo-D-galacturonanase from Commercial Pectinase

The yield represents average values obtained with a 300-g batch of crude pectinase. The specific activity was determined with sodium pectate at 30°C and pH 4·5.

Product	Yield mg	Protein content %	Activity		
			specific μmol mg^{-1} . . min^{-1}	relative	% of original
N 1	700	44·3	1·2	1·0	100
N 2	51·6	76·2	8·4	7·0	87·2
N 3	16·3	60·3	18·2	15·1	62·0
N 4	3·1	82·2	75·4	62·8	54·0

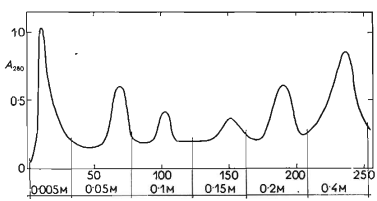

FIG. 1

Chromatography of Crude Preparation N_1 on DEAE-Cellulose

Column (3·8 × 24 cm) eluted by phosphate buffers at the concentration given. The pH of the eluting solutions was 7·0 (up to 0·2M concentration); the pH of the 0·6M solution was 6·0. Flow rate 18 ml per 30 min *n*, number of fractions.

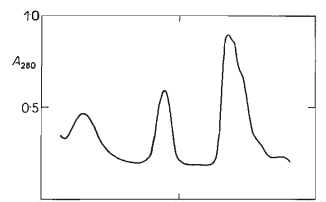

FIG. 2

Chromatography of Purified Product N_2 on DEAE-Cellulose

Column (2·5 × 60 cm) eluted by a concentration gradient of phosphate buffers in the range 0·005—0·2M, pH 7·0. Flow rate 12 ml per 30 min. *n*, number of fractions.

fraction. This product was desalted on Sephadex G-25 Medium and yielded the final product N_4 after lyophilization.

By using the purification procedure described in Fig. 1–3, and in Table I we obtained 20·6 mg of endo-D-galacturonanase of total activity 1554 units from 2000 g of the commercial pectinase preparation. The final product of purification, N_4, was a 63-times purified specific enzyme with 82·6% of protein, contaminated (according to electrophoresis) with a small amount of protein lacking pectolytic activity. The approximate molecular weight of endo-D-galacturonanase determined by thin-layer chromatography on Sephadex G-150 Superfine is 46000. The pH-dependence of endo-D-galacturonanase activity is shown in Fig. 4. The enzyme has a narrow activity range with a pH-optimum at pH 5·0, is relatively stable in acid and neutral media and instable in alkaline media (Fig. 5). The optimum temperature of its action lies between 40 and 45°C.

To the elucidation of the mode of action of endo-D-galacturonanase contributed experiments in which the effect of these enzymes on defined oligomer substrates was examined[18]. It has been observed that the individual endo-D-galacturonanases differ in the extent of their effect and in the manner in which they degrade these substrates. Whereas one type of endo-D-galacturonanase (from *Aspergillus niger*[1], *Acrocylindrium*[19], *Aspergillus japonicus*[20], *etc.*) can degrade tetragalacturonic acid as the smallest substrate, another type of endo-D-galacturonanase can split even trigalacturonic acid (endo-D-galacturonanase from *Erwinia carotovora*[21], *Aspergillus saitoi*[22], *Coniothyrium diplodiella*[23], the extracellular endo-D-galacturonanase from *Aspergillus niger*[18], tomato endo-D-galacturonanase[23], *etc.*); these enzymes cleaved trigalacturonic acid at different rates.

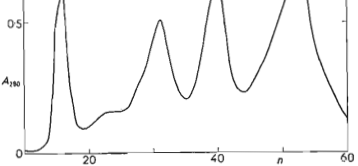

FIG. 3

Chromatography of Fraction N_3 on Sephadex G-100

Column (1·4 × 90 cm) eluted by 0·1M acetate buffer in 0·5M-NaCl, pH 5·0. Flow rate 6 ml per 30 min. *n*, number of fractions.

TABLE II

Kinetic Constants of Endo-D-galacturonanase Isolated from Commercial Pectinase

Substrate	K_m mol l^{-1}	V μmol mg^{-1} min^{-1}
Sodium pectate	$(1{\cdot}96 \pm 0{\cdot}06) \cdot 10^{-5}$	224
$(GalpUA)_5$[a]	$(1{\cdot}85 \pm 0{\cdot}06) \cdot 10^{-3}$	21·0
$(GalpUA)_4$	$(3{\cdot}31 \pm 0{\cdot}09) \cdot 10^{-3}$	11·7
$(GalpUA)_3$	$(1{\cdot}25 \pm 0{\cdot}12) \cdot 10^{-2}$	3·0

[a] $(GalpUA)_n$ oligogalacturonic acid, n degree of polymerization.

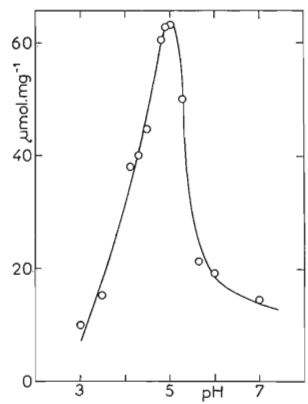

FIG. 4

Effect of pH on Rate of Reaction Catalyzed by Endo-D-galacturonanase

The substrate was 0·5% solution of sodium pectate in 0·1M acetate buffer for pH 3—6 and in 0·1M phosphate buffer for pH 6·5 and 7.

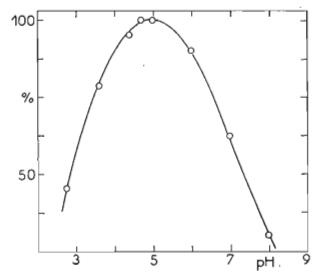

FIG. 5

Effect of pH on Stability of Endo-D-galacturonanase

The enzyme was incubated in 0·1M acetate buffer of the given pH for 2 h. After the adjustment of constant volume and of pH to 5·0 the activity was determined with sodium pectate as substrate. The results are given in % of original activity.

Endo-D-galacturonanases have been observed to degrade oligomer substrates[24] in three different manners. The first one is a typical specific cleavage of tetrasaccharide to trisaccharide and D-galactopyranurate and an extremely slow cleavage or even absence of cleavage of trigalacturonic acid. The second type of degradation is characterized by alternative cleavage of tetragalacturonic acid and by a relatively fast degradation of trisaccharide. The third type of cleavage differs from the first two types in specific hydrolysis of pentasaccharide to trisaccharide and disaccharide and by only two alternative cleavages of hexagalacturonic acid. Both bonds of trigalacturonic acid are cleaved. These differences in the mode of cleavage of oligomer substrates as well as the differences in the cleavage rates suggest that the properties of the enzyme, *i.e.* the location of its active center are factors determining the enzyme substrate reaction.

As follows from what has been stated above, the investigation of degradation of defined substrates may provide information on the character of the active center and thus contribute to the elucidation of the mode of action of the enzyme. We have therefore examined the effect of endo-D-galacturonanase isolated in this study on the degradation of sodium pectate where all oligogalacturonides were obtained; this shows that the degradation of high molecular weight substrates proceeds via cleavage of glycosidic bonds at various sites of the D-galacturonan chain. The 235 nm absorbance of the reaction mixture does not change during the action of the enzyme; therefore the cleavage of glycosidic bonds follows the mechanism of acid hydrolysis.

When the enzyme was allowed to act on the individual oligogalacturonic acids at its pH-optimum and 30°C (20 min incubation with tetra- to hexasaccharide, 60-min incubation with trisaccharide and disaccharide), the following products of cleavage were found by thin-layer chromatography on silica gel:

Substrate	Products of cleavage
$(GalpUA)_6$	$(GalpUA)_4 + (GalpUA)_3 + (GalpUA)_2$
$(GalpUA)_5$	$(GalpUA)_3 + (GalpUA)_2$
$(GalpUA)_4$	$(GalpUA)_3 + (GalpUA)_1$
$(GalpUA)_3$	$(GalpUA)_2 + (GalpUA)_1$
$(GalpUA)_2$	–

The nature of the products of cleavage shows that of all the substrates investigated hexagalacturonic acid only is cleaved in two different manners: (*a*) to tetrasaccharide and disaccharide and (*b*) to two molecules of trisaccharide.

The values of the Michaelis constants with sodium pectate and tri-to pentagalacturonic acids as substrates as well as the values of maximum rates are given in Table II.

* $(GalpUA)_n$ oligogalacturonic acid, *n* degree of polymerization.

The character of the products of cleavage of the individual oligogalacturonic acids shows that the endo-D-galacturonanase prepared by us differs in its mode of action from the extracellular endo-D-galacturonanase from *Aspergillus niger*[18] and from the endo-D-galacturonanase obtained by Koller[1] even though it has the same pH-optimum as the latter. The main difference rests in the specific cleavage of pentagalacturonic acid and in the mode of cleavage of hexagalacturonic acid. With regard to the effect on these two oligomer substrates and to the rate of cleavage of trisaccharide our endo-D-galacturonanase can be classified as falling in the group of endo-D-galacturonanases degrading substrates in the latter manner. An identical type of endo-D-galacturonanase has been found so far only in the filtrate of a *Erwinia carotovora*[21].

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