Biochimica et Biophysica Acta, 422 (1976) 349—356

BBA 67701

PURIFICATION AND CHARACTERIZATION OF AN EXTRACELLULAR EXO-D-GALACTURONANASE OF *ASPERGILLUS NIGER*

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(Received August 12th, 1975)

Summary

A D-galacturonanase (EC 3.2.1.67) catalyzing the degradation of D-galacturonans by terminal action pattern was purified from a culture filtrate of *Aspergillus niger* by a procedure including the salting-out with ammonium sulfate, precipitation by ethanol, chromatography on DEAE-cellulose, and gel chromatography on Sephadex G-100 The obtained preparation was slightly contaminated by an enzymically inactive protein fraction Maximum activity and stability of the enzyme was observed at pH 5 2. The enzyme degrades digalacturonic acid, *p*-nitrophenyl-α-D-galactopyranuronide, as well as oligogalacturonides containing at the nonreducing end 4-deoxy-L-threo-hexa-4-enopyranosyluronate It differs from all *A niger* enzymes so far described which degrade D-galacturonans by the terminal action pattern, in not clearly preferring low-molecular substrates It is therefore classified as an exo-D-galacturonanase

Introduction

Exo-D-galacturonanases*, (poly-1,4-α-D-galacturonide-galacturonohydrolase, EC 3 2.1.67) catalyze the hydrolytic cleavage of terminal glycosidic α-1,4 linkages of D-galacturonans, releasing D-galacopyranuronic acid as the sole reaction product The degradation catalyzed by microbial and plant exo-D-galacturonanases proceeds from the nonreducing end of the D-galacturonan chain

The differences in some properties, especially in the preference of substrates with respect to their degree of polymerization, metal ion requirement, and pH optimum, observed with some of the exo-D-galacturonanases so far described, indicate that in spite of the same action pattern, the mechanism of action of

* So far called exopolygalacturonase The new name, proposed by Professor Derek Horton (private communication) is in agreement with the name of the preferred substrate

these enzymes may not be identical These differences are displayed not only by enzymes of different origin, but also by enzymes produced by the same biological species Mill [1,2] purified two exo-D galacturonanases from a culture extract of *Aspergillus niger*, of which one, activated by mercury ions, preferentially degraded low-molecular substrates The ratio of degradation rates of high-molecular D-galacturonans to digalacturonic acid was 1 85 The other enzyme, not activated by metal ions, degraded digalacturonic acid 11 times faster than polymeric substrates Hatanaka and Ozawa [3] described an *A niger* exo-D-galacturonanase which, unlike many other exo-D-galacturonanases, also degraded the substrates containing 4-deoxy-L-threo-hexa-4-eno-pyranosyluronate at the nonreducing end This enzyme degraded digalacturonic acid 6 3 times faster than a polymeric D-galacturonan For exo-D-galacturonanases purified from a culture extract of *Acrocylindrium* [4] and *Penicillium digitatum* [5], the chain length of a substrate is not a factor determining the degradation rate In none of the mentioned papers were substrates with different degree of polymerization used at equimolar concentrations, so that it is not clear, in some cases, to what extent the differences in the rate of cleavage reflect the influence of the effective concentration of the cleavable terminal bonds, and to what extent they reflect the differences in the mechanism of enzyme action. It was proposed to differentiate between the enzymes operating by terminal action pattern according to preferred substrates and to classify those clearly preferring low-molecular substrates as oligogalacturonide hydrolases [6,7]

In the course of the purification of an extracellular endo-D-galacturonanase from a culture filtrate of *A niger* [8] it was found, unlike a previous finding, [9] that the starting material has a low activity of a D-galacturonanase which acts by the terminal action pattern It was the purpose of our experiments to purify the enzyme and to compare its properties with those of other exo-D-galacturonanases of *A niger* described so far

Materials and Methods

Substrates

Pectic acid (polygalacturonide content 75 5%, molecular weight 27 000) was prepared from a commercial apple pectin by repeated alkaline de-esterification with 0 1 M NaOH, followed by the precipitation at pH 2 5

Homologous oligogalacturonic acids, degree of polymerization 2—4, were prepared from a partial acid hydrolyzate of pectic acid by gel chromatography on Sephadex G-25 Fine as described elsewhere [10] The purity of oligogalacturonic acids was tested by thin-layer chromatography on silica-gel according to Koller and Neukom [11], as well as by the determination of reducing groups by the Nelson-Somogyi method [12] and by titration of carboxylic groups

Acid soluble pectic acid (degree of polymerization 10 ± 1) was prepared by treating apple pectin in 0 5 M H_2SO_4 at 100°C for 1 h [13], followed by repeated chromatography on Sephadex G-25 Fine [10] and desalting on Sephadex G-10 The content of polyuronide and the degree of polymerization were characterized on the basis of carboxylic group and reducing group content, determined as in the case of oligogalacturonic acids

4,5-Dehydrogalacturonosyl-α-D-trigalacturonic acid (4,5-unsaturated tetragalacturonic acid) was prepared by the β-eliminative degradation of highly esterified pectin (degree of esterification 95%) carried out at pH 6.8 and 100° C The degradation products were fractionated by chromatography on Dowex 1X8, followed by chromatography on Sephadex G-25 Fine [10] and desalting on Sephadex G-10

p-Nitrophenyl-α-D-galactopyranuronide was a preparation obtained from Dr K. Schwabe, Central Institute for Cancer Research, Berlin-Buch (Germany)

Enzyme assay

Exo-D-galacturonanase activity was determined by measuring the initial rate of reducing group increase by a spectrophotometric method using Nelson-Somogyi reagent [12] In a typical experiment, a series of 0 5-ml aliquots of the substrate solution and 0 5 ml of enzyme solution were mixed and incubated for different time intervals at 30° C. An 0.5% solution of sodium pectate, pH 5.2 (0 1 M acetate buffer) or digalacturonic acid (1 μmol per ml) in the same buffer were used as substrates The reducing groups were determined by means of a standard graph for D-galactopyranuronic acid The initial velocity is defined in μmol of reducing groups liberated by 1 mg of protein per min The protein content was determined by the method of Lowry et al [14]

For the determination of the pH optimum, digalacturonic acid in 0 1 M acetate buffers of pH 3—6 and in 0 1 M phosphate buffers, pH 6 5 and 7 0, respectively, was used as a substrate. The effect of pH on the stability of the enzyme was examined by incubating the enzyme in the above-mentioned buffers for 2 h The enzyme solution was then adjusted to pH 5 2 and a constant volume, and the activity on digalacturonic acid was determined

The activity of exo-D-galacturonanase on *p*-nitrophenyl-α-D-galactopyranuronide was determined by measuring the increase in absorbance at 405 nm, after adjusting pH of the reaction mixture to 10, as well as by the analysis of liberated D-galactopyranuronic acid by thin-layer chromatography

Specificity of exo-D-galacturonanase was examined on the basis of the analysis of reaction products by thin-layer chromatography using sodium pectate as a substrate

Purification of exo-D-galacturonanase

Exo-D-galacturonanase was purified from a culture filtrate of *A niger* growing 10 days on Czapek-Dox nutrient medium containing 1 5% citrus pectin as carbon source A crude preparation of pectic enzymes was prepared by a procedure including the salting-out of proteins with ammonium sulfate (0 9 saturation), precipitation with ethanol and desalting by gel filtration on Sephadex G-25 Medium, as described previously [14]

1 5 g of the crude preparation containing 30 units of exo-D-galacturonanase were chromatographed on a column of DEAE-cellulose, 3 8 cm × 22 cm, equilibrated with 0 005 M phosphate buffer, pH 7 0 The column was eluted using phosphate buffers of gradually increasing concentration. The course of the fractionation was followed by recording the absorbance at 280 nm The fractions eluted with 0.15 M phosphate were collected, freeze-dried, desalted by gel filtration on Sephadex G-25 and again freeze-dried

The obtained preparation (37 mg containing 21 units of enzyme activity) was then chromatographed on a column of Sephadex G-100, 1 4 cm × 90 cm, equilibrated with 0.5 M solution of sodium chloride in 0 1 M acetate buffer, pH 5 2 In addition to absorbance at 280 nm, D-galacturonanase activity was determined in individual fractions, using sodium pectate as a substrate The fractions containing exo-D-galacturonanase were desalted on Sephadex G-25 and freeze-dried

The purity of the preparation was tested by electrophoresis on polyacrylamide gel performed in Tris/glycin buffer, pH 8 3, at 4°C using a potential of 3 V/cm [16,17]

Results and Discussion

The course of the purification of exo-D-galacturonanase from the crude enzyme preparation is shown in Figs. 1 and 2 and in Table I The mixture of proteins present in the crude preparation (see disc-electrophoretic pattern, Table I) was separated by chromatography on DEAE-cellulose into six fractions The fractions eluted with 0 1 M phosphate (3), 0 2 M phosphate (5), and 0 4 M phosphate (6) showed respectively, endo-D-galacturonanase, pectinesterase, and polymethylgalacturonase activity The fraction eluted with 0 15 M phosphate (marked by the arrow), which has been overlooked in the previous experiments [15], contained a D-galacturonanase which degraded digalacturonic acid and liberated D-galactopyranuronic acid from a polymeric D-galacturonan as the main product Traces of oligogalacturonic acids found besides D-galacopyranuronic acid as the product of pectic acid degradation, indicated a slight contamination of the enzyme by endo-D-galacturonanase, the main portion of which was eluted from the column with the preceding buffer By polyacrylamide gel electrophoresis three protein fractions were found The removal of the endo-D-galacturonanase was achieved by gel chromatography on Sephadex G-100 By this procedure two peaks were obtained (Fig 2) The first

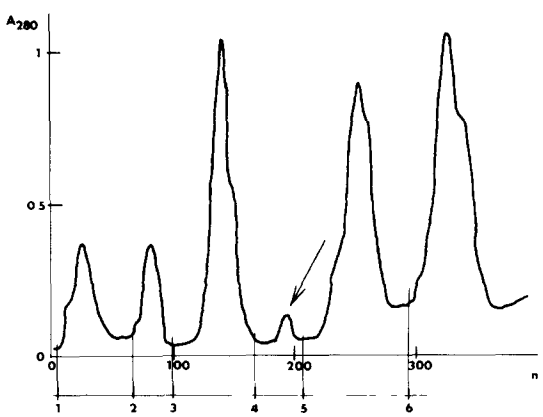


Fig 1 Chromatography of the crude enzyme preparation on DEAE-cellulose The column (3 8 cm × 22 cm) was eluted with sodium phosphate buffers of (1) 0 005 M, (2) 0 05 M, (3) 0 1 M, (4) 0 15 M, (5) 0 2 M (all pH 7 0), (6) 0 4 M, pH 6 0 18-ml fractions were collected at 30 min intervals The course of the separation was followed by recording the absorbance at 280 nm n, fraction number

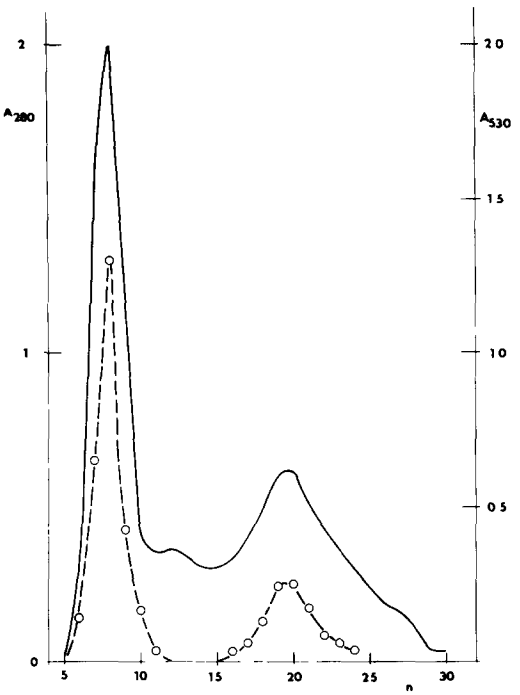


Fig 2 Chromatography on Sephadex G-100 of the fraction eluted from the DEAE-cellulose column with 0 15 M phosphate buffer. The column (1 4 cm × 90 cm) was eluted with 0 5 M solution of NaCl in 0 1 M acetate buffer, pH 5 2, and 5-ml fractions were collected at 30 min intervals Full line, absorbance at 280 nm, dashed line, D-galacturonanase activity determined by Nelson-Somogyi method and expressed in absorbance at 530 nm n, fraction number

TABLE I

SUMMARY OF EXO-D-GALACTURONANASE PURIFICATION

Specific activity is expressed in μmol of reducing groups liberated by 1 mg of protein min^{-1} using di-galacturonic acid as substrate N_0, crude enzyme preparation, N_1, fraction eluted from DEAE-cellulose column with 0 15 M phosphate buffer, N_2, final exo-D-galacturonanase preparation Polyacrylamide gel electrophoresis was performed in Tris/glycin buffer, pH 8 3 The proteins were stained by Amido Black 10 B

Product	Specific activity (μmol mg^{-1})	Disc electrophoresis
N_0	0 034	
N_1	0 70	
N_2	1 44	

peak contained the purified enzyme, endo-D-galacturonanase was present in the second peak

As shown in Table I, the purification procedure led to the removal of the majority of contaminating proteins The activity of the final preparation was 42 times higher than that of the crude preparation However, the final product was still slightly contaminated by an unknown, inactive protein fraction, which could not be separated, even by further chromatography on Sephadex G-150

The enzyme operates by the terminal action pattern liberating D-galacto-pyranuronic acid as the sole reaction product It is active on digalacturonic acid, as well as on *p*-nitrophenyl-α-D-galactopyranuronide Its pH optimum of activity and stability lies at pH 5 2 (Figs 3 and 4) In both cases the shift of pH to acidic or alkaline side results in a sharp decrease in specific activity The effect of temperature on the activity and stability of the enzyme is illustrated in Figs 5 and 6 The temperature stability was investigated by incubating the enzyme solution in 0 1 M acetate buffer, pH 5 2, for 4 h at various temperatures The activity of the enzyme was then determined at 30° C

With respect to the pH optimum, the enzyme is similar to the exo-D-galacturonanase isolated from a mycellium extract of *A niger* [1] which was activated by Hg^{2+} ions Therefore the effect of $HgCl_2$ on the activity was examined It was found that neither Hg^{2+} nor other divalent cations (Ca^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+}) affected the activity of the enzyme

For the comparison of the enzyme with exo-D-galacturonanase described by Hatanaka and Ozawa [3], the activity on 4,5-dehydrogalacturonosyl-D-trigalacturonic acid was determined It was found that the substrate is degraded at the same velocity as the corresponding saturated oligogalacturonic acid Trigalac-

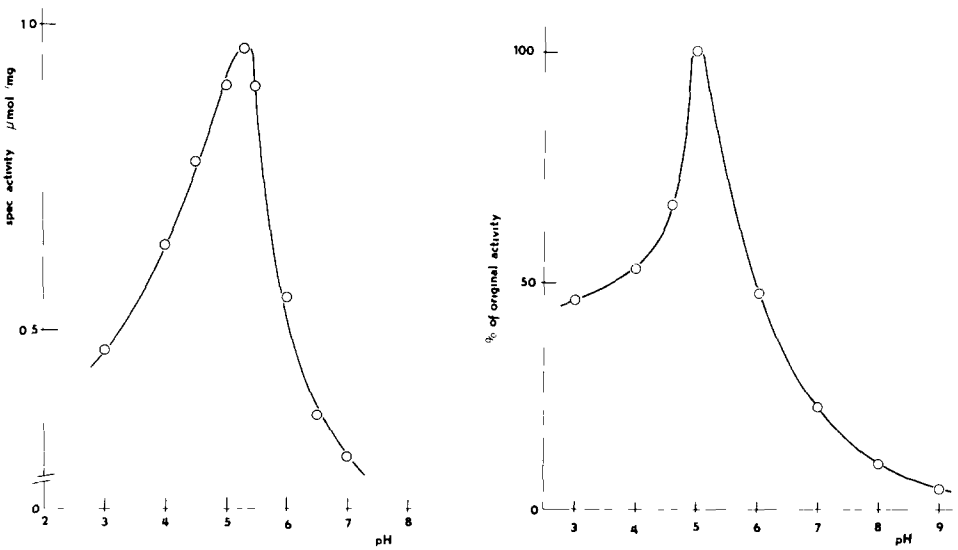
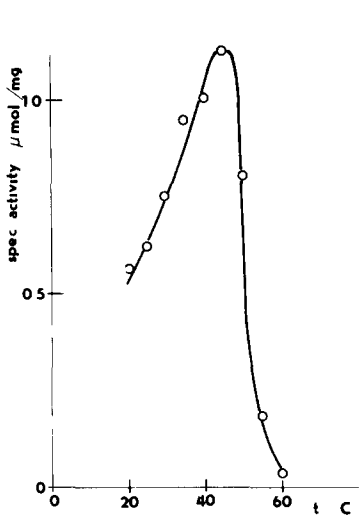


Fig 3 Effect of pH on the activity of exo-D-galacturonanase Digalacturonic acid in 0 1 M acetate buffers of pH 3—6 and in phosphate buffers, pH 6 5 and 7 0, was used as substrate

Fig 4 Effect of pH on the stability of exo-D-galacturonanase The enzyme solutions in buffers of varying pH were incubated at 30° C for 2 h The solutions were then adjusted to pH 5 2 and to a constant volume, and the initial velocity of digalacturonic acid degradation was determined



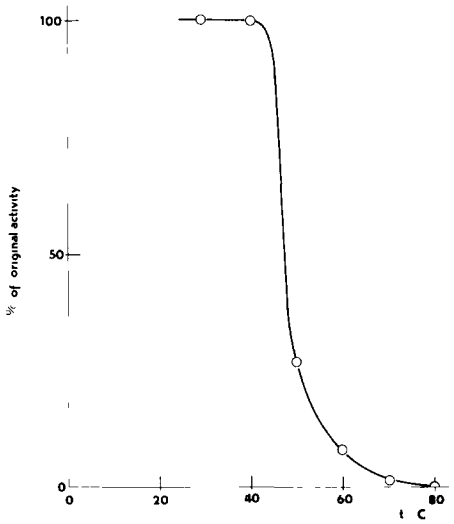


Fig 5 Effect of temperature on the activity of exo-D-galacturonanase An 0 5% solution of sodium pectate in 0 1 M acetate buffer, pH 5 2, was used as substrate

Fig 6 Effect of temperature on the stability of exo-D-galacturonanase Enzyme solutions in 0 1 M acetate buffer, pH 5 2, were incubated at the respective temperatures for 4 h The initial velocity of sodium pectate degradation was then determined at 30° C

turonic acid was the primary saturated product of the reaction. This indicates that the -OH group at C-4 of the sugar unit at the nonreducing end of the substrate is not essential for the catalytic reaction

For the characterization and classification of the enzyme, the effect of the degree of substrate polymerization on the activity was investigated. In order to exclude the effect of the unequal concentration of the terminal cleavable bonds, which could lead to an inadequate interpretation of the results, the activity was determined on the substrates used at equimolar concentrations. For comparison with the data presented in other papers, measurements were

TABLE II

ACTIVITY OF EXO-D-GALACTURONANASE ON SUBSTRATES OF DIFFERENT DEGREE OF POLYMERIZATION

$(GalpUA)_n$, oligogalacturonic acid, degree of polymerization n, I, substrates used in the concentration of 1 µmol ml^{-1}, II, substrates used in the concentration of 0 05% Specific activity is expressed in µmol of reducing groups mg protein^{-1} min^{-1}

Substrate	Specific activity	
	I	II
$(GalpUA)_2$	1 66	1 99
$(GalpUA)_3$	4 30	3 61
$(GalpUA)_4$	6 80	
$(GalpUA)_{10}$	4 00	2 42
Sodium pectate av DP = 153	1 90	0 52

made in which the substrates with a degree of polymerization 2, 3, and 10, as well as sodium pectate, were all used at the same percentage concentration

As is seen in Table II, among the substrates used, tetragalacturonic acid is the substrate of preference A shortening, or elongation of the substrate chain length resulted in a decrease in the rate of glycosidic bond splitting The ratio of the rates of tetragalacturonic acid and digalacturonic acid degradation was found to be 4.1 1 The degradation rates of digalacturonic acid and the polymeric substrate, used at equimolar concentrations, are approximately equal, while at a concentration of 0 05% of both substrates, digalacturonic acid was degraded 3.8 times faster than the polymeric substrate From this point of view, the extracellular enzyme of *A niger* differs from all mycellial D-galacturonanases so far described which operate by terminal action pattern, by not preferring low-molecular substrates, therefore it may be classified as exo-D-galacturonanase

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