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SEZIONE III

(Botanica, zoologia, fisiologia e patologia)

Botanica. — *Polygalacturonase from culture filtrates of* Aspergillus flavus *Link. ex Fr.* Nota di ANNA ADELE FABBRI, CORRADO FANELLI, MAURO SERAFINI e LAURA SPANÒ (*), presentata (**) dal Corrisp. G. B. MARINI-BETTÒLO.

RIASSUNTO. — Nel presente lavoro è descritta la purificazione mediante cromatografia su CM-cellulosa ed isoelectrofocusing (pH 3.5-5) di una poligalatturonasi isolata da filtrati colturali di *Aspergillus flavus*.

Analisi eseguita mediante cromatografia su carta dei prodotti di idrolisi suggerisce che l'enzima attacca il substrato mediante un meccanismo casuale e produce come composti finali l'acido di- e trigalatturonico. Gli spettri di assorbimento dei gruppi cromogeni formati dai prodotti dell'idrolisi e l'acido tiobarbiturico confermano che l'enzima è classificabile come endopoligalatturonasi (poli (1.4-α-D-galacturonide) glycanohydrolase E.C.3.2.1.15). L'enzima è una glicoproteina con un contenuto di carboidrati dello 0.017% e si focalizza a un pH 4.35±0.04.

INTRODUCTION

In a previous report it was shown that some of the different fungi isolated from damaged plant seeds produced pectolytic activity [1]. Many strains of the genus *Aspergillus* were isolated from wheat and sunflower seeds and the isolates of *Aspergillus flavus* Link. ex Fr. were the most active in producing pectolytic activity. Considering the role of *Aspergillus flavus* Link. ex Fr. in producing aflatoxins and the ability of the pectolytic enzymes to macerate and attack plant tissue we decided to purify these enzymes and analyze some of their properties.

MATERIALS AND METHODS

Reagents. – Pure citrus pectin was supplied by N. B. C. Corp. (Cleveland, Ohio), sodium polypectate and D-galacturonic acid by K and K laboratories, dinitrosalicylic acid, thiobarbituric acid and bromophenol blue by Merck Chemicals, 3 MM chromatographic paper by Whatman, anpholine carriers by LKB Sweden. All other chemicals were reagent grade.

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(**) Nella seduta del 15 dicembre 1979.

Fungal cultures. – All the strains were grown for 7 days at 23 °C in medium previously described [2] $8 \cdot 10^6$ conidia were then inoculated in the same liquid medium with a) 12 g/l pectin or 12 g/l glucose. The mycelium was then grown in a 200 ml Erlenmeyer flask containing 40 ml of liquid medium culture at 23 °C. The mycelium was then removed by filtration through a Ha Millipore filter (0.45 μ) and the culture filtrate was centrifuged at 3000 g at 4 °C for 20 min and then dialysed for 18 h against several changes of distilled water at 0–4 °C.

Polygalacturonase assay. – Pectolytic activity was determined with a Cannon–Fenske viscometer (size 300) in 30 mM citrate phosphate buffer, pH 5, by measuring the decrease in relative viscosity of a 0.6 % solution of sodium polypectate. Enzyme activity is reported in relative viscosimetric units; one unit is the reciprocal of the time in minutes required to reduce to one half the viscosity of 6 ml of the reaction mixture. The hydrolysis of glycosidic bonds was followed by reducing end-group analysis using the dinitrosalicylic acid procedure [3]. As the complete hydrolysis of the substrate produced reducing groups corresponding to the monomer concentration, this latter value was assumed to be 100 % of the substrate degradation.

Paper chromatography. – The products formed by the action of polygalacturonase on sodium polygalacturonate were examined by descending paper chromatography [4]. The reaction mixture, containing 0.6 % sodium polypectate, 20 mM citratephosphate buffer, pH 5.0, and 10 viscosimetric units/ml enzyme was incubated at 30 °C.

Thiobarbituric acid (TBA) test. – The products formed by the action of polygalacturonase on sodium polypectate were examined using the thiobarbituric acid procedure of Neukomb as modified by Ayers *et al.* [4].

Isoelectric focusing. – Isoelectric focusing was performed in an LKB 8100 column, cooled to 4 °C at pH 3.5 to 6 in the stabilizing linear sucrose gradient (0–40 %). Focusing was obtained in 36 h by applying a constant voltage of 1000 volts, 2.0 ml fractions were collected from the column for pH and activity determinations.

Carbohydrate assay. – Carbohydrates in homogeneous enzymes were determined by the anthrone method using glucose as the standard [6].

Protein determination. – Protein determination was done according to the method of Lowry *et al.* [7].

Results and discussion

The pectolytic activity produced by different isolates of the genus Aspergillus is reported in Table I. Among these all three strains of *Aspergillus flavus* were peculiar in producing pectolytic activity when grown on medium

with glucose. One isolate from sunflower seeds presents higher pectolytic activity than the other strains. The dry weight does not correspond to the enzymatic production. The strain of *Aspergillus flavus* from sunflower seeds was used for the purification of pectolytic enzymes. The isolate was surface cultured on the synthetic medium with glucose. The fungus was allowed to grow at 23 °C for 15 days and then removed by filtration through a Ha Millipore filter (0.45 μ). Then the culture filtrate was lyophylized and redissolved in 10 mM acetate buffer, pH 3.6. Then it was dialysed overnight against 10 mM acetate buffer pH 3.8 and loaded into a CM-cellulose column (1.6 × 18 cm) equilibrated with the same buffer. The column was washed with 100 ml of the buffer and then eluted with a linear 0–0.7 M NaCl gradient. The peak (Fig. 1) fractions with the highest specific activity were pooled and subjected to extensive dialysis against 1 % glycine.

TABLE I

Extracellular pectolytic activity of the strains of Aspergillus. *Dry weight values are indicated.*

(a) = fungi isolated from sunflower seeds; (b) = fungi isolated from wheat seeds.

FUNGI	Pectolytic activity (units/ml)		Dry weight of mycelium (mg)	
	with glucose	with pectin	with glucose	with pectin
Aspergillus awamori (a)	0	1.00	67.8	106.5
Aspergillus awamori (b)	0	0.90	65.7	108.8
Aspergillus candidus (a)	0	0	12.8	18.2
Aspergillus flavus (a)	0.7	2.55	70.2	78.6
Aspergillus flavus (b)	0.4	0.50	79.4	69.1
Aspergillus flavus (b)	0.6	0.76	75.0	71.1
Aspergillus niger (a)	0	0.62	84.3	91.4
Aspergillus niger (b)	0	0.54	79.8	98.8
Aspergillus ochraceus (a)	0	0	181.4	20.7
Aspergillus versicolor (a)	0	0.02	33.7	30.3
Aspergillus versicolor (b)	0	0.06	37.9	48.2

Upon isoelectric focusing in a pH range of 3.5 to 5 one distinct peak was obtained (Fig. 2). The isoelectric point of the enzyme was 4.35 ± 0.04. All the operations described were repeated using the pectolytic enzyme produced

in medium containing pectin. Table 2 *a*, *b* shows the purification procedure for 150 ml (enzyme produced in medium with pectin) and 100 ml (enzyme produced in medium with glucose). The purity of the two enzyme preparations

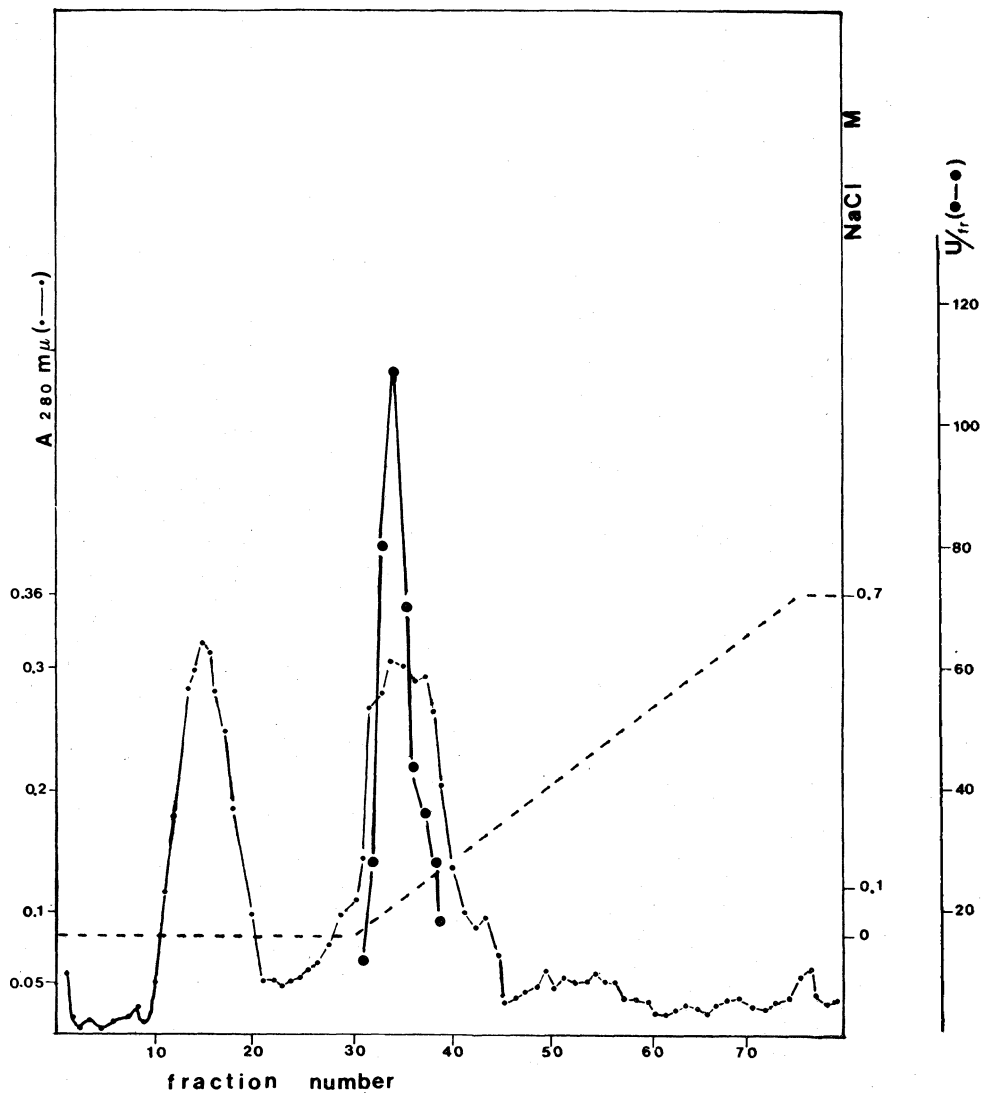

Fig. 1. – Elution profile of polygalacturonase activity from CM-cellulose columm. 3 ml fractions were collected and assayed for activity. ●——● polygalacturonase activity; ——— absorbance at 280 nm; - - - - - - NaCl concentration.

was demostrated by refocusing polyacrilamide gel electrophoresis and gel filtration. Only one peak was obtained when each enzyme was separately refocused and there was no shift in the pH value at which each enzyme banded was observed.

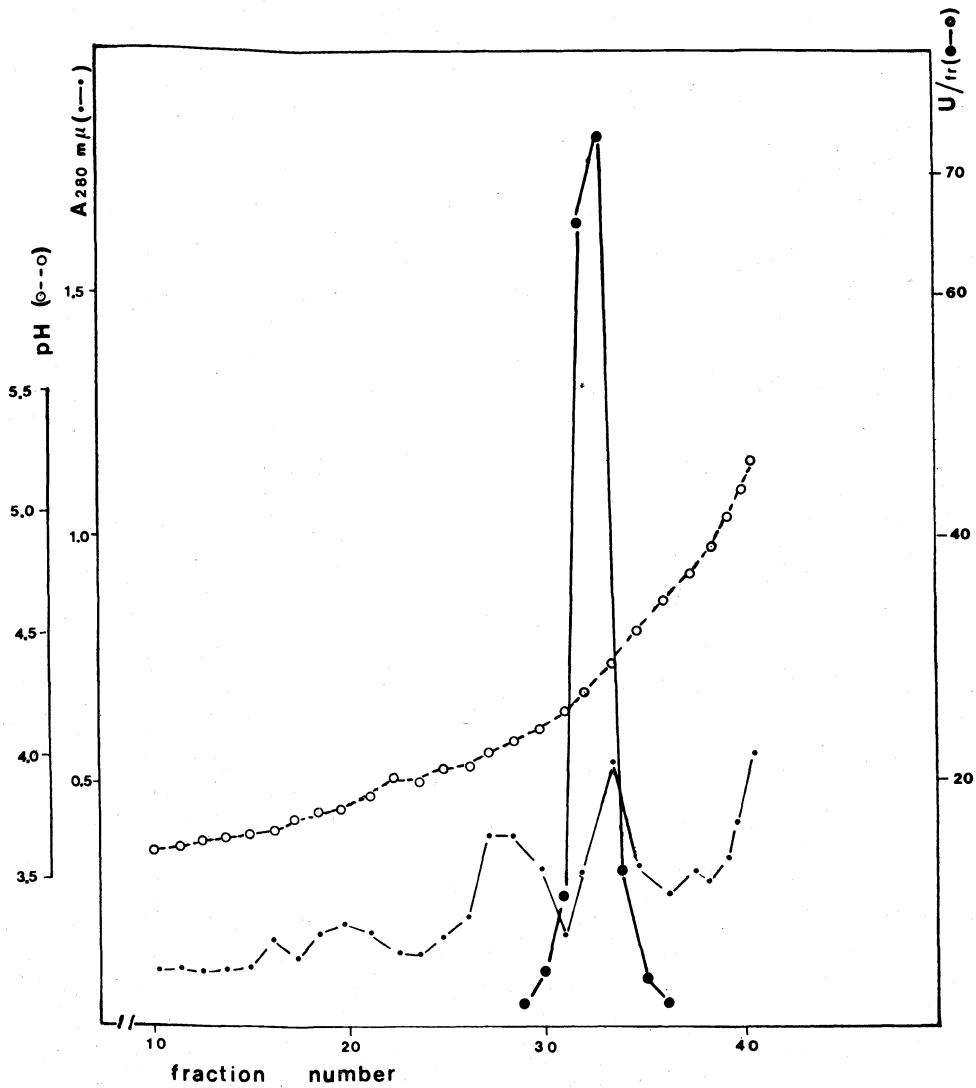

Fig. 2. – Isoelectric focusing of polygalacturonase produced in glucose medium. 15 ml from the CM-cellulose column were mixed with the lighter component of the sucrose gradient. 2.0 ml fractions were collected for pH and enzymatic activity determinations. ●——● polygalacturonase activity; ——— absorbance at 280 nm; - - - - pH. Other experimental details are given in the text.

The properties of both enzymes are very similar to those observed for polygalacturonase from phytopathogenic fungi [8] and saprophytic fungi [9]. Both enzymes showed optimal activity at pH 5 and the high rate of viscosity decrease compared with the low rate of reducing group increase during the enzymatic breakdown of sodium polypectate shows that the enzymes hydrolase the glycosidic bonds of the substrate in an " endo " manner (Fig. 3). This is confirmed by paper chromatography analysis of the products of hydrolysis

TABLE II *a*

Purification of polygalacturonase from Aspergillus flavus *produced in pectin medium.*

Fraction	Volume (ml)	Total activity	Total proteins	Specific activity	Yield %	Purification (fold)
Filtrate	150	800	80	10	100	—
Lyophilized	10	761	6.9	108	95	10.8
CM-cellulose	8	240	0.92	260	30	26
Electrofocusing	4	120	0.20	300	15	40

TABLE II *b*

Purification of polygalacturonase from Aspergillus flavus *produced in glucose medium.*

Fraction	Volume (ml)	Total activity	Total proteins	Specific Activity	Yield %	Purification (fold)
Filtrate	100	500	97.3	5.1	100	—
Lyophilized	10	410	9.4	43.6	82	8.5
CM-cellulose	8	225	2.8	80.3	45	15.7
Electrofocusing	4	180	0.97	185.5	36	36.3

The absorption spectra of the chromogens formed by hydrolysis products of sodium polypectate with thiobarbituric acid exhibited a maximum at 510 nm which was considered good evidence for polygalacturonase activity [10], while the absence of a peak at 550 nm indicated that lyase activity was not present in culture filtrates of *Aspergillus flavus*. Paper chromatography shows that the enzymes do not attack the three galacturonic acids like the results obtained with the saprophytic fungus *Trichoderma koningii* [9]. The endopolygalacturonase of some pathogenic fungi attacks the three galacturonic acid and shows mono- and digalacturonic acid as products of hydrolysis [8, 11].

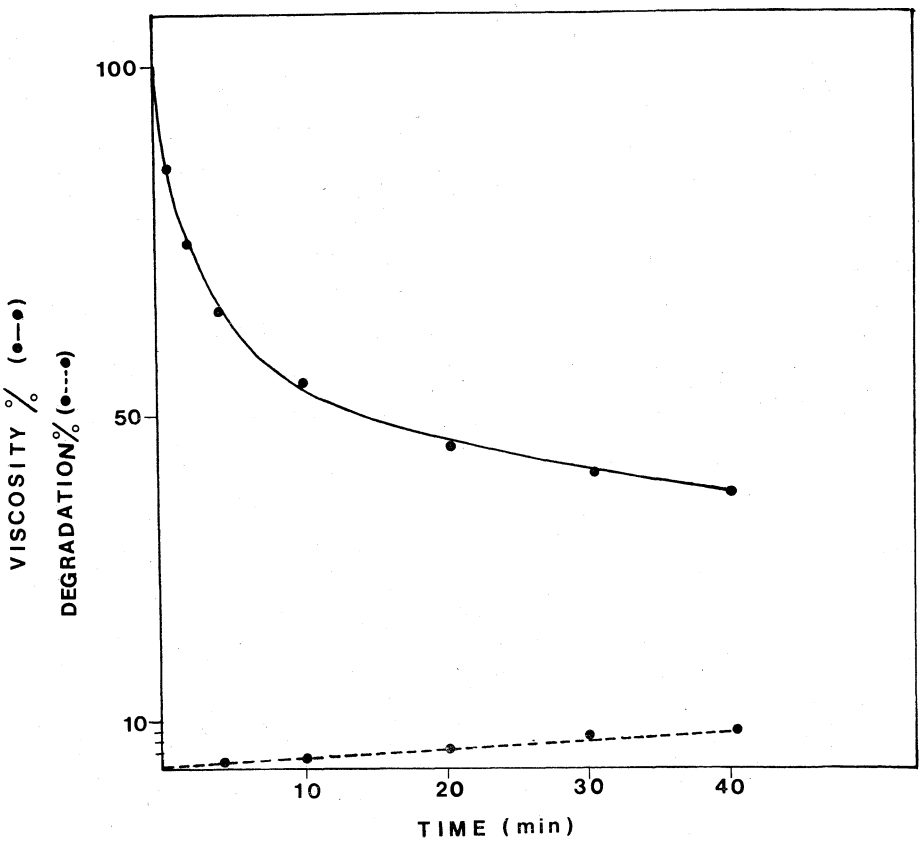

Fig. 3. – Viscosity change and hydrolysis of sodium polypectate by polygalacturonase as a function of time. ●——● relative viscosity (%); ●----● hydrolysis of glycosidic bonds (% monomer concentration).

The enzymes of *Aspergillus flavus* are glycoproteins with a carbohydrate content of 0.017 mg of sugar/mg of protein and can be classified as endopolygalacturonases (poly-(1.4-α-D-galacturonide)glycanohydrolase E.C.3.2.1.15).

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