ISOLATION AND CHARACTERIZATION OF A POLYGALACTURONASE ACTIVITY IN FIVE LOCAL STRAINS OF MICROSCOPIC FUNGI

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Abstract

Five strains of microscopic fungi belonging to genus : *Mucor, Aspergillus, Penicillium, Alternaria* and *Nigrospora* were isolated from a soil of El Kala region (north east Algeria).

The study of the pectolytic activity showed that the five strains were active on the pectic substrat used as the only carbon source and they have secrete polygalacturonases in the liquid medium.

These enzymes are inducible, have an acting pH varying between 4 and 5.5 and are activated by monovalent ions (NaCl) situated between 5 and 10mM.

Polygalacturonases act on the pectic acid as a preferential substrat and galacturonic acid is the final product of degradation.

Moreover, the detailed study of *Mucor sp.* strain has permitted to isolate and to partially purify an endopolygalacturonase EC (3.2.1.15).

Résumé

A partir d'un sol de la région d'El Kala (Nord Est de l'Algérie), cinq souches de champignons microscopiques appartenant aux genres : *Aspergillus, Penicillium, Alternaria, Mucor* et *Nigrospora* ont été isolées.

L'étude de l'activité pectinolytique a montré que les cinq souches sont actives sur leur substrat pectique utilisé comme seule source de carbone. Elles sécrètent des polygalacturonases dans le milieu liquide.

Ces enzymes sont inductibles, ont un pH d'action variant entre 4 et 5.5 et sont activés par des ions monovalents (NaCl).

Ces polygalacturonases agissent sur l'acide pectique comme substrat préférentiel et le produit final de dégradation est l'acide galacturonique.

Par ailleurs, l'étude détaillée de la souche *Mucor sp.* a permis d'isoler une endopolygalacturonase EC (3.2.1.15) et de la purifier partiellement.

Mots clés : polygalacturonases, acide pectique, champignons.

ملخص

خمس سلالات من الفطريات المجهرية المنتمية للأجناس الآتية: *Penicillium, Aspergillus, Mucor* . *Nigrospora* و *Alternaria*, قد تم عزلها من تربة بمنطقة القالة (شمال شرق الجزائر)

لقد بينت دراسة النشاط البكتيني أن السلالات الخمس تبدي نشاطا محللا للبكتين و المستعمل كمصدر وحيد للكربون. هاته السلالات تفرز في الوسط الخارجي إنزيم (Polygalacturonases). تحليل متعدد الغلوكورونيك

تعمل هاته (4 إلى 5.5) وهي تحتاج لنشاطها إلى أيونات أحادية التكافؤ (NaCl). كما الأنزيمات في وسط حامضي الإنزيم نشاطا تفاضليا للجوهر (حمض متعدد الغلوكورونيك) اد أن يبدي آخرجزئ للتحليل هو حمض الغلوكورونيك.

على غرار ما تقدم سمحت الدراسة من عزل إنزيم *Mucor sp.* محلل لمادة البكتين دو النمط المفصلة لسلالة

التأثير الداخلي EC (3.2.1.15) و من تنقيته جزئيا.

INTRODUCTION

Pectinases are a group of enzymes that act on pectic substances by a mechanism of desesterification and hydrolysis or by β elimination [1].

Various phytopathogens or saprophyte microorganisms produce polygalacturonases such as bacteria (Erwinia chrysanthemi), yeasts (Kluyveromyces fragilis) *and microscopic* fungi (Aspergillus niger) [2][3][4].

Plants (tomato, banana) [5][6] and animals (Helix pomatia) [7] have also been reported to produce these enzymes which are often associated to esterases [4].

Pectic enzymes ensure the transformation of plant biomass to liberate oligomers easily assimilated by microorganisms and can be used in industry for the clarification of fruit juices rich in pectin.

In this work, however, five strains of saprophyte fungi obtained from El Kala region have been isolated, and the pectolytic activity of each strain was investigated.

MATERIALS AND METHODS

1. Microorganisms isolation and identification

Five strains of microscopic fungi : Mucor sp., *Aspergillus sp., Penicillium sp., Nigrospora sp. and Alternaria sp.* were isolated from a soil of El Kala region.

The isolation of different strains was achieved according to the method of Marcelou Kinti et al [8] and identification of the five strains was made on the basis of their cultivation and morphological characters [8][12].

2. Crude enzyme preparation

The five strains of fungi were cultivated at 25°C on a solid then liquid Czapek medium [9] containing pectin as the only carbon source, slightly modified (use of KH_2PO_4 instead of K_2HPO_4) and adjusted to pH 5. It contained (gl^{-1}) : $NaNO_3$, 2 ; KH_2PO_4, 1.2 ; $Mg\ SO_4\ 7\ H_2O$, 0.5 ; $FeSO_4\ 7\ H_2O$, 0.01.

Citrus pectin (Sigma P9135) was added to the medium at a rate of 5 g per liter. Cultures were grown in non-agitated conditions at 25°C. For solid medium agar was added (15 g per liter).

After 7 days of growth, cultures were filtered and total proteins were precipitated with ammonium sulfate (80% of saturation). The whole was centrifuged at 4°C at 3000 × g during 30mn. The resulting precipitate was dissolved in a minimum volume of water, then dialysed for 24 hours at 4°C against distilled water. The obtained solution constituted the crude enzyme preparation.

3. Analytical methods

3.1. Assay for polygalacturonase activity.

After incubation of the crude extract with 0.2% polygalacturonic acid in 0.1M acetate buffer at pH 5 for 16h at 30°C, pectolytic activity was detected by Nelson [10] and Somogyi [11] test by measuring of reducing power at 650nm.

3.2. Acid thiobarbituric specific test.

Polygalacturonase and/or lyase activity is put in evidence by measuring the absorbance between 450nm and 600nm using TBA test [12]. However, maximum of absorption has been given for polygalacturonases at 515nm and for lyases at 550nm.

3.3. Reaction products analysis by thin layer chromatography.

The degradation products of polygalacturonate by the crude enzyme preparation were analyzed by thin layer chromatography on silicagel plates (Schleicher and Schüll F1500) in butanol-1 – formic acid – water (3 : 2 : 1)V/V. Chromatograms were developed for 3h.
After drying, plates were sprayed with a solution of 5% sulphuric acid in ethanol and heated at 100°C for 5 to 10mn.

4. Enzyme properties

4.1. optimum pH.

The variation of enzyme activity according to the pH was studied by incubating the crude enzyme extract at 30°C overnight on 0.2% polygalacturonic acid in 0.1 M acetate buffer ajusted to different pH ranging from 3.5 to 6. Pectolytic activity was detected by the Nelson-Somogyi test.

4.2. Preferential substrate.

The variation of depolymerising activity according to the degree of etherification of the pectic substrate was studied after incubating the crude enzymes extract for : 5, 10 and 15mn with two different substrates: polygalacturonic acid (DE = 0) and partially esterified pectin (DE = 52%).
Enzyme activity was measured by the Nelson-Somogyi test.

4.3. Effect of sodium chloride (NaCl).

Concentration of ionic strength on enzyme activity was achieved after incubation at 40°C for 3 hours, the crude enzyme extract with 0.2% polygalacturonic acid with different concentrations of NaCl varying from 0 to 15 mM. Enzyme activity was measured at 650nm by the Nelson-Somogyi test.

5. Determination of the specific galacturonase activity of the five strains.

The specific activity of the five strains was measured from the rate of reducing sugars released during hydrolysis of polygalacturonate in UI (µM/ min/ mg of protein) as determined by the Bradford method.

Comparison of specific activity of the five strains allowed us to select one strain for further purification.

6. Enzyme purification

Ion exchange chromatography was achieved on a column (15 x 3 cm) of CM trisacryl previously equilibrated with 0.05M acetate buffer at pH 4.7. The enzyme was eluted by a gradient of 0.1M to 0.5M NaCl in the same buffer at a flow rate of 60 ml/h.

The active enzymic fractions collected from CM trisacryl were combined, dialysed against 0.05M, NaCl 0.1M acetate buffer at pH 5 and applied for the second chromatography on sephadex G-75 column (25 x 1 cm) equilibrated with 0.05M NaCl 0.1M acetate buffer at pH 5.5 with a flow rate of 15 ml/h.

Proteins were detected at 280 nm and the enzyme activity was measured by the Nelson-Somogyi test.

7. Enzyme mode of action

The products formed from depolymerization of polygalacturonic acid by the crude enzyme extract and by the fraction collected on CM trisacryl in function of time were detected by thin layer chromatography. The study was done after incubating at 40°C, the two samples on 0.2% polygalacturonic acid buffered with acetate at pH 5 during 5, 15, 30, 60mn, 2, 3, 4, 24 and 48 hours.

RESULTS AND DISCUSSION

After 7 days of growth on solid then liquid medium containing pectin as the only carbon source, pectolytic activity was detected by measuring the reducing power of released sugars. The analysis of results (table1) showed that all strains were active on their pectic substrate. *Aspergillus sp.*, *Mucor sp.* and *Penicillium sp.* strains seemed to be more active than the others. These strains secreted in the liquid medium depolymerases polygalacturonases or lyases.

Table 1. Research of depolymerases in the crude enzyme preparations by measuring the reducing power at 650nm (Nelson-Somogyi test).

Crude enzyme from :	Enzymatic activity µM of reducing sugar/ml
Mucor sp.	13.44
Aspergillus sp.	12.64
Penicillium sp.	12.00
Nigrospora sp.	9.08
Alternaria sp.	4.96

Culture medium is at pH 5 which favourites the synthesis of polygalacturonases. These enzymes act at acidic pH and attack pectic acid preferentially [13].

Then as, the source of carbon used here is highly esterified pectin, a mixture of esterases and polygalacturonases is certainly produced by these strains. Various microorganisms are known by their synthesis of these enzymes as *Clostridium thermosulfurogenes* [15] and *Aspergillus niger* [4].

Polygalacturonases release saturated oligogalacturonates which react with thiobarbituric acid at a maximum of absorption of 515nm. It is apparent from figure (1) that maximum of absorption was at 515nm. This technique confirms the presence of polygalacturonases in the culture medium.

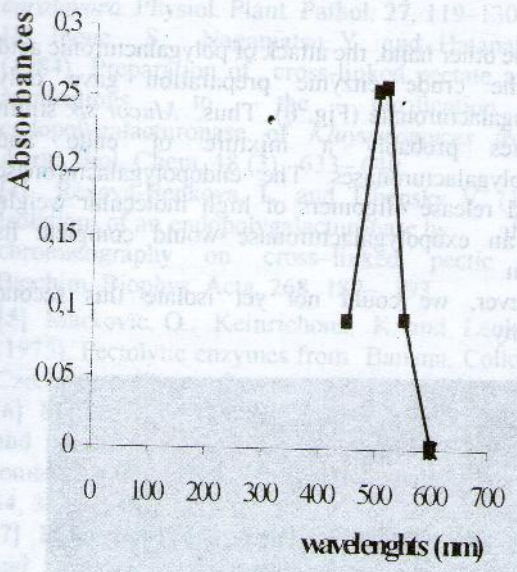

Fig. 1 : Detection of a polygalacturonase activity in the filtrate culture of *Mucor sp.* strain (TBA Test).

Ascending thin layer chromatograms of the resulting reaction mixture of crude enzyme preparations of all strains on polygalacturonate have shown that the nature end product is monogalacturonate (Fig.2). The final product is therefore galacturonic acid used directly by the microorganisms. This is the result of action of endo and exopolygalacturonase enzymes as shown for other pectolytics extracts [1]. Various microorganisms such as *Streptomyces sp., Erwinia carotovora, Aspergillus niger, Penicillium expansum, Penicillium frequentens* are known to produce these enzymes [4][16].

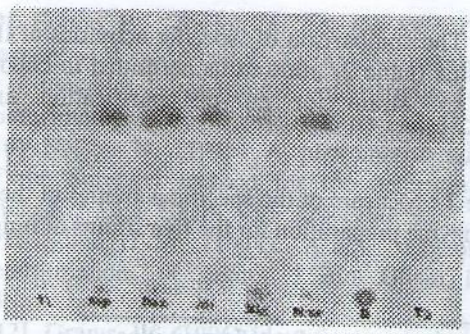

Fig. 2 : TLC of products formed from depolymerization of polygalacturonic acid by crude enzyme preparations of the five strains.
B: sample heated to 100°C to inactive enzyme.
T_1 and T_2 : standards two standards obtained from an industrial polygalacturonase incubated with polygalacturonic acid at pH 5 during 1h30mn for T_1 and 25h for T_2.
Asp: *Aspergillus sp.*, Pen: *Penicillium sp*
Alt : *Alternaria sp.*, Nig : *Niigrospora sp.*,
Muc : *Mucor sp*

The optimum pH for our pectic enzymes was found to be situated between 4.5 and 5.5 for all active strains. Some other similar results have been observed for several strains like *Aspergillus sojae, Aspergillus niger* and *colletotrichum lindemuthianum* [1][17]. These enzymes are also activated bysodium chloride (NaCl) at a rate of 5mM to 10mM.

The polygalacturonase activity on pectic substrate with different degrees of esterification (Fig.3) has shown that the reaction rate on polygalacturonate was higher than on partially esterified pectin (52%). The same result was found with other polygalacturonases secreted by *Aspergillus niger* and *Trichoderma koningii* [1][18].

Fig. 3 : Determination of the preferential substrate of the polygalacturonase isolated from *Mucor sp.* strain.

The comparison of specific activities of the five strains (table 2) allowed us permitted to choose *Mucor sp.* strain for enzyme purification and characterization.

Table 2 : Measure of the specific activities of the crude enzyme preparation for the five strains of fungi.

Strains	Specific activities (UI / mg)
Mucor sp.	19.80
Aspergillus sp.	16.74
Penicillium sp.	07.26
Nigrospora sp.	02.32
Alternaria sp.	00.68

By ion exchange chromatography on CM trisacryl and molecular filtration on sephadex G-75, an endopolygalacturonase activity was partially purified (Fig. 4).

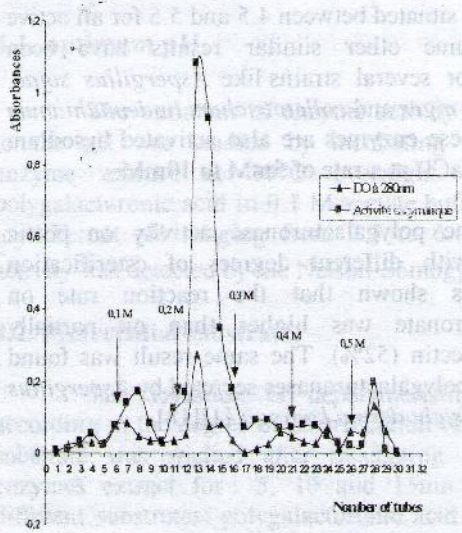

Fig. 4 : Profil of elution of *Mucor sp.* crude enzyme preparation on column of CM trisacryl by adding NaCl.

The " endo " character is demonstrated by thin layer chromatography. However figure (5) has shown that at the beginning of the reaction, are released oligomers of high molecular weight. After 48 hours of incubation, three oligomers are seen corresponding to monoer, dimer and trimer of galacturonic acid. This mode of attack is known at the endopolygalacturonases and the enzyme could be classified (EC : 3.2.1.15). This type of enzyme has been described in other microorganisms as *Aspergillus niger* where an endopolygalacturonase belonging to the same classification has been isolated [19].

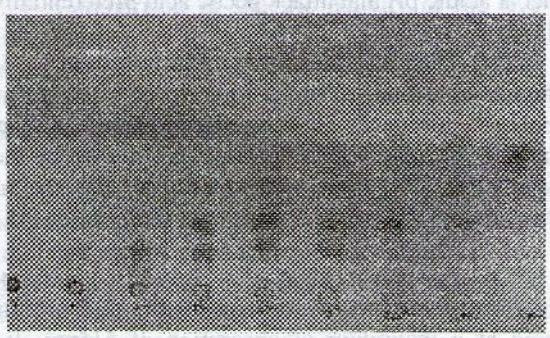

Fig. 5 : Mode of action of the endopolygalacturonase isolated by CM trisacryl from *Mucor sp.* strain on polygalacturonic acid.

On the other hand, the attack of polygalacturonic acid by the crude enzyme preparation gave only monogalacturonate (Fig. 6). Thus, *Mucor sp.* strain secretes probably a mixture of endo and exopolygalacturonases. The endopolygalacturonase would release oligomers of high molecular weight and an exopolygalacturonase would complete its action.

However, we could not yet isolate this second activity.

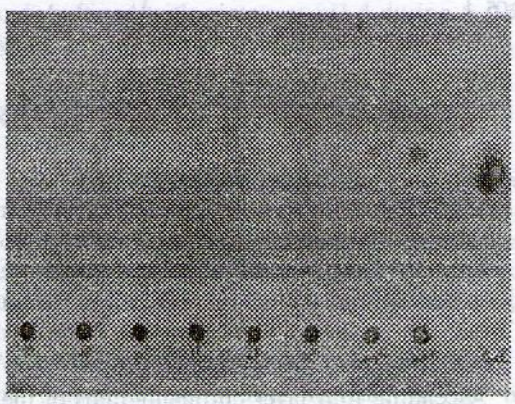

Fig.6 : Mode of action of the crude enzyme of *Mucor sp.* strain on polygalacturonic acid.

CONCLUSION

Polygalacturonases secreted by the strains studied here attak polygalacturonic acid as a preferential substrate. The culture medium used here contained pectin. The five strains of fungi degrade pectin by, a secretion of a mixture of esterase and polygalacturonase. An endopolygalacturonase

activity has been isolated and identified by thin layer chromatography.

The isolated enzyme would be an endopolygalacturonase classified (EC : 3.2.1.15) that attacks pectic substrate randomly inside the chain to release oligogalacturonates of small size easily assimilated by the microorganisms.

Another activity of exo type might be present in the enzymatic mixture but has not be yet isolated.

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