Electrophoretic Characterization of a Cutinase Released by *Ascochyta pisi*

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Abstract

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Cutinase activity was released from *Ascochyta pisi* grown in culture medium containing cutin + cutin hydrolysate as sole source of carbon. No cutinase activity was released when mineral culture medium, supplemented or not with glucose as carbon source, was used. Cutinase was traced by its esterase activity, using ∝ -naphythyl acetate as substrate. This method allowed the revelation of cutinase by its esterase activity, after polyacrylamide gel electrophoresis (PAGE). Upon SDS-PAGE, *A. pisi* cutinase showed a single peptide with an estimated molecular weight of 21, 200 daltons.

Key words: Cutinase, *Ascochyta pisi,* Electrophoresis.

Introduction:

The role of cutinolytic enzymes in the penetration of fungi in host plants has been studied by many researchers (5, 6, 7, 11, 16, 17). The fungal cutinases purified and characterized so far (4, 8, 10, 14) had a molecular weight ranging between 20,000 and 25,000 daltons, and showed similarities in their amino acid composition.

In our previous work (12), we have shown that *Ascochyta pisi* and *A. pinodes* release cutinase activity in nutrient medium containing cutin as the sole source of carbon. In the present paper, we analyze the electrophoretic properties of *A.pisi* cutinase.

Materials and methods

Experimental conditions. Cultures of *A.pisi* were maintained as described previously (12). To induce cutinase, the mineral solution of Dickman and Patil (3) was neutralized to pH 7 with phosphoric acid, and was supplemented with 0.2% (w/v) of cutin + cutin hydrolysate. Cutin was prepared and purified from peelings of apples cv. «Golden Delicious», as described by Baker and Bateman (1). Cutin hydrolysate was obtained by incubating cutin for 24 hr in a solution of 10% KOH (w/v), followed by neutralization to pH 7 with phosphoric acid. The mineral solution, alone or supplemented with 0.2% (w/v) of glucose was used as cutinless control culture medium.

All media were autoclaved for 20 min at 120°C. Spore suspensions of *A.pisi* were inoculated to the culture, medium in Erlenmeyer flasks, to reach a final volume of 50 ml containing 10^6 spores/ml. The vials were incubated during 15 days on a rotary shaker at 26°C and the cultures were then filtrated.

In our previous experiments, the cutinase of *A.pisi* was evaluated by measuring its esterase activity, using paranitrophenyl acetate (PNPA) as substrate (12). In the present work, in order to allow *in situ* revelation of esterase after gel electrophoresis, we used ∝ -naphthyl acetate (∝ NA) as substrate with the reaction medium described by Wheeler *et al.* (18). The effect of the cutinase inhibitor diisopropyl fluorophosphate (DFP) was tested in some experiments.

Assay medium (1 ml) contained 50 mM tris-maleate pH 6.8, 2mM ∝ NA (obtained from a solution of 100 mM ∝ NA in methanol), and *A.pisi* culture filtrate. Reaction started upon addition of the substrate and was stopped after 15 min by addition of 0.2 ml $HClO_4$ (5M). The liberated ∝ -naphthol was extracted by 5 ml chloroform and the phases were separated by centrifugation at 8,400 g for 20 min. Four ml were removed from the upper chloroform phase and were supplemented with 0.1 ml of Fast Blue RR (5 mg/ml in chloroform/methanol; 1:1 v/v); several drops of $NaHCO_3$ were then added to neutralize the residual $HClO_4$.

After addition of 3 ml methanol, the mixture was shaken and centrifuged for 20 min at 8,400 g to remove the precipitated bicarbonate. Finally, the absorbance at 535 nm was read against a reagent blank.

Polyacrylamide gel electrophoresis. Culture filtrates were dialysed for 48 hr and were then lyophilized. The solid material thus obtained was dissolved in 50 mM tris-maleate pH 6.8, with a final volume 30 times less than the initial volume. The total protein content of the culture filtrates was estimated by the method of Bradford (2).

Polyacrylamide gel electrophoresis (PAGE) was performed using a combination of techniques described by Laemmli (9) and Pasteur *et al.* (13). The stacking gel contained 5% (w/v) of polyacrylamide and 0.14% (w/v) of bis-acrylamide. The separation gel contained 15% (w/v) of acrylamide and 0.42% (w/v) of bis-acrylamide. Both gels were polymerized chemically by addition of 0.07% (w /v) of ammonium persulfate and 0.1% (v/v) of TEMED.

Buffers were used as described by Shannon *et al.* (15). Electrode buffer consisted of borate buffer pH 8.5, containing 85 mM sodium hydroxyde and 299 mM boric acid. Gel buffers were obtained by diluting the electrode buffer 1/10 (v/v) in distilled water.

Samples were mixed with 1/10 of a solution of the dye bromophenol blue (0.1%, w/v) and sucrose (60%,w/v). ß -lactoglobulin (molecular weight of 18,400 daltons) used as marker was prepared in the same way.

Electrophoresis was carried out for about 3 hr at 4°C, with a current intensity of 20 mA, until bromophenol blue reached the bottom of the gel.

Revelation of esterase and marker. Cutinase was revealed by its esterase activity, using ∝ NA as substrate. Phosphate buffer Na/K (100 mM, pH 6.5) was prepared as described by Shannon *et al.* (15), and was supplemented or not with DFP (2 mM). After addition of acetone (20%, v/v) containing 4% of ∝ NA (w/v), the gels were submerged in the mixture for 15 min at room temperature. Fast Blue RR (0.2%, w/v) was then added with continuous shaking. When bands appeared, gels were placed in the fixator (methanol/distilled water/acetic acid, 4:5:1, v/v/v).

Revelation of the marker protein was performed as described by Pasteur *et al.* (13), by submerging the gel in a solution of 1% (w/v) Coomassie Brillant Blue R-250 in methanol/distilled water/acetic acid (4.5: 4.5:1, v/v/v). Gels were incubated for 2 hr and then rinsed three times in the fixator, until bands formed.

SDS-polycarylamide gel electrophoresis. For SDS-PAGE, electrode and gel buffers were supplemented with 0.1% SDS (w/v). All samples and markers (lysozyme: 14,300 daltons, ß -lactoglobulin: 18,400 daltons and trypsinogen: 24,000 daltons) were prepared as for PAGE and were supplemented with 1% SDS (w/v). They were then shaken and incubated for 3 min at 100°C. SDS-PAGE was performed at room temperature, under a constant current intensity of 20 mA. Revelation of the proteins was as for PAGE.

Results

Esterase activity. Cutinase, as evaluated by measuring esterase activity of the culture filtrate using ∝NA as substrate (table 1), was present only when the culture medium contained cutin + cutin hydrolysate; its activity was inhibited by DFP.

Table 1: Esterase activity⋆ of *A.pisi* culture filtrate, measured by using PNPA or ∝ NA as substrate

Culture medium	Absorbance/15min	
	PNPA sustrate	NA substrate
mineral solution (MS)	0.007	0.003
MS + glucose	0.009	0.007
MS +cutin + cutin hysrolysate	0.303	0.438

⋆ Results are the mean of three replicates

Polyacrylamide gel electrophoresis. Cutinase was revealed by its esterase activity, using ∝ NA as substrate. Upon PAGE, filtrate from the culture medium containing cutin + cutin hydrolysate gave one band with sole source of carbon, no band was observed. Upon addition of DFP (2 mM) during esterase revelation, no band was formed. Figure 1 shows that the molecular weight of the esterase band was close to that of the ß -lactoglobulin marker protein.

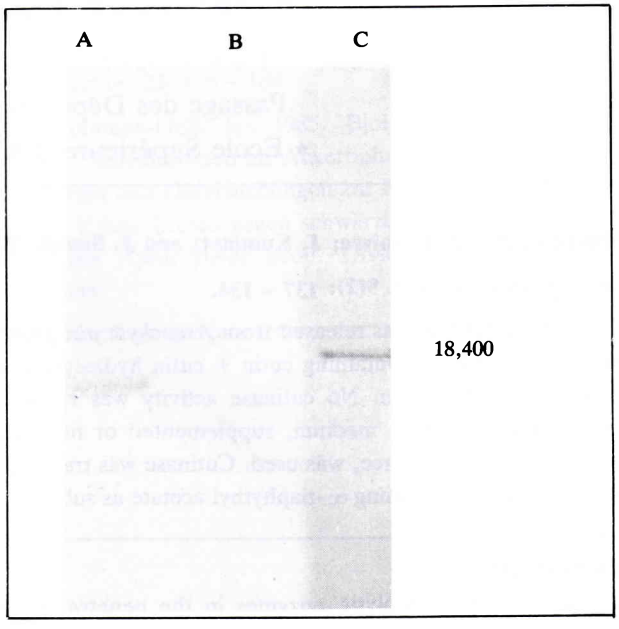

Figure 1: PAGE analysis of (A) culture filtrate of *A. pisi* grown on cutin + cutin hydrolysate as sole source of carbon (esterase band revealed by staining with Fast Blue RR); (B) culture filtrate of *A. pisi* grown on glucose as sole source of carbon (stained with Fast blue RR); (C) -lactoglobulin (18,400) used as molecular weight marker (revealed by staining with Coomassie Brillant Blue).

SDS-Polyacrylamide gel electrophoresis. SDS-PAGE showed that *A.pisi* esterase activity is linked to a single peptide which formed only when the culture medium contained cutin + cutin hydrolysate as sole source of carbon (figure

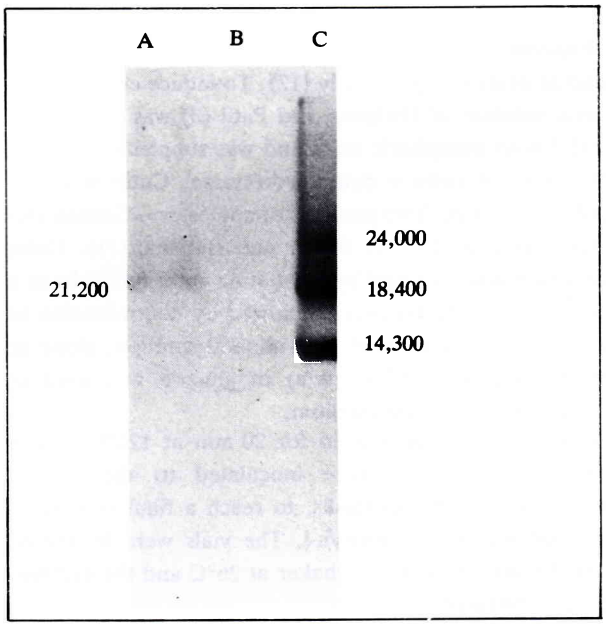

Figure 2: SDS-PAGE analysis, followed by staining with Coomassie Brillant Blue, of (A) culture filtrate of *A.pisi* grown on cutin + cutin hydrolysate as sole source of carbon; (B) culture filtrate of *A.pisi* grown on glucose as sole source of carbon; (C) lysozyme (14,300), ß -lactoglobulin (18,400), and trypsinogen (24,000) used as molecular weight markers.

2). As compared to our markers, Rf of the cutinase band, calculated from three replicated, corresponds to an estimated molecular weight of 21,200 ± 1,600 daltons, (95% of limits of confidence).

Discussion

Cutinase activity has been ususally evaluated by measuring its esterase activity, using para-nitrophenyl esters as substrate (6). We have shown previously that when *A. pisi* was grown in nutrient medium containing cutin as sole source of carbon, cutinase was released in the medium, as measured by microtitration of fatty acids liberated from cutin, or by esterase activity using PNPA as substrate (12).

In the present paper, we show that esterase activity can be revealed also using ∝ NA as substrate, thus allowing the revelation of cutinase band upon PAGE, by precipitating with Fast Blue RR the ∝ -naphthol product liberated from NA. Such revelation is not feasable when para-nitrophenyl esters are used as substrate.

A single band of esterase activity was always found upon PAGE of the culture filtrate of *A. pisi,* when cutin + cutin hydrolysate were used as sole carbon source. DFP, a serine active inhibitor, inhibited both esterase activity in *A. pisi* culture filtrate (as measured using NA as substrate) and the formation of the cutinase band in the gel upon PAGE (as revealed by Fast Blue RR staining). When glucose was used as crabon source, no esterase activity was released in the culture filtrate and no cutinase band was observed in the gel.

The results of analysis of *A. pisi* cutinase by revelation upon PAGE, were confirmed by SDS-PAGE, yielding a single peptide with an estimated molecular weight of 21,200 ± 1,600 daltons. Our results are consistent with those obtained so far with other fungal cutinases.

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الملخص

نصراوي، ب وف. وبوافر وج. كومار وج. صومال. 1991 . توصيف الإنزيم الحالّ للكيوتين الذي يفرزه الفطر *Ascochyta pisi* بوساطة الرحلان الكهربائي. مجلة وقاية النبات العربية 9 (2) : 134 - 137.

يُفرز الفطر *Ascochyta pisi* ، النامي على مستنبت يحوي الكيوتين أو الكيوتين المحلمة كمصدر وحيد للكربون، إنزيماً حالاً للكيوتين (كيوتيناز). ولم يفرز الفطر الانزيم نفسه عند نموه على مستنبت معدني، سواء دعّم أو لم يدعّم بالغلوكوز كمصدر للكربون. وقد اقتفي أثر «الكيوتيناز» بوساطة نشاطه المؤستر باستخدام مادة ألفا ـ نفتيل أسيتات، وذلك بتقنية الرحلان الكهربائي على هلام من البولي أكـرميد. وعنـد استخدام كبريتـات دوسوديـل الصوديـوم، تبين أن الإنزيم المدروس يتكون من ببتيد وحيد يقدر وزنه الجزيئي بـ 21000 دالتون.

كلمات مفتاحيـة: كيوتينـاز، *Ascochyta pisi* ، الرحـلان الكهربائي.

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