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Studies on the Zymogram Method for the Detection of Pectinolytic Activities Using CTAB

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Abstract Zymogram analysis is a useful tool for the identification of several enzymes. The present study was undertaken to investigate the efficiency gains from the characterization of pectic enzymes on zymograms by staining of pectin–agarose overlays using cetyl trimethyl ammonium bromide also known as cetrimide or CTAB. The method is based on the fact that the enzymatic hydrolysis of the pectic substrates included in the agarose matrix gel inhibited their precipitation by CTAB, leading to the appearance of cleared zones in front of the pectin hydrolases and lyases. Conversely, esterases led to the increase of pectin precipitation. Fungal pectinolytic enzymes were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to the zymogram detection technique, using two pectin substances, namely citrus pectin and polygalacturonic acid. Overall, the findings presented in the current study indicate that several elements (ions, salts, pH, temperature, chelators, and reducing agents) may significantly affect the results of zymogram analysis and can, therefore, be employed to enhance the discriminatory and operational potential of the analysis in terms of accurate discrimination between several pectinolytic activities involved and effective implementation of the purification procedures required in the process.

Keywords Zymogram · Pectinase activities · Cetyl trimethyl ammonium bromide (CTAB) · CT1 mutant: *Penicillium occitanis*

Introduction

The hydrolysis of pectin backbones can be achieved through the synergistic action of several enzymes, including depolymerases (polymethylgalacturonases, polygalacturonases, pectate lyases, and pectin lyases) and pectin methylesterases [1]. Several organisms are able to produce pectin-degrading enzymes, which are, in fact, widely used in a variety of food

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industries as a means to improve the yield and clarification of fruit juices [2]. The fungus *Aspergillus niger* is, for instance, one of the most extensively investigated fungi whose industrial application for the production of pectinolytic enzymes is well established [3]. This impressive and steady flow of studies on organisms suitable for the industrial production of pectin degrading enzymes is, however, mired by a relative scarcity of work on potential pectinase producer strains from the genus *Penicillium* [3].

A previous study by the authors reported on the isolation of the mutant CT1 from *Penicillium occitanis*. This strain was observed to possess a promising ability for the production of pectin-degrading enzymes [4]. Besides and when cultivated on media containing a cheap by-product from the wheat manufactory (gruel), this mutant was noted to produce high titers in pectinases. In fact, such enzymes were successfully applied to increase the oil yield extracted from olive pumice [5].

The dosage of the pectinolytic activities is generally carried out in liquid media. For most enzymes, the type of cleavage is random (endo) or terminal (exo). These activities were routinely determined by spectrophotometer assays using dinitrosalicylic acid (DNS) (for exo-activity) [6] and thiobarbituric acid (for pectin and pectate lyase activities) [7] or by viscosimetry (for endo-activity) [8]. These techniques are not specific for a single enzyme but concern all the pectinases that can release reducing sugars from the pectic structure (the DNS method), liberate unsaturated pectin oligomers (the thiobarbituric method), or induce a decrease in the viscosity due to the random degradation inside the pectic chain (the viscosimeter method).

Several techniques have been proposed for the detection of pectic enzymes in electrophoretic gels. Some studies tended to detect pectic activity through the introduction of pectic polymers into the gel [9] or after electrophoresis [10, 11]. Other studies detected pectinolytic activities using an overlay technique based on the contact between the electrophoresis gel and the pectin–agarose gel, and other studies identified pectinases using ruthenium red [10, 12, 13]. The present work was undertaken to investigate the optimal conditions for the analysis and potential gain effects from the identification of pectinolytic activities in solid media through the staining of pectin–agarose overlays with the insoluble acidic precipitant polysaccharide cetyl trimethyl ammonium bromide (CTAB).

Materials and Methods

Substrates

The polygalacturonic acid (PGA) from orange and the citrus pectin (CP) with different degrees of esterification (28%, 67%, and 90%) were purchased from Sigma. The apple pectin (AP) with the degree of esterification of 70% was from Fluka.

Microorganisms

The CT1 mutant was selected from CL100 wild-type strain after a single round of mutagenesis by nitrous acid HNO_2 [4]. This strain was propagated on potato dextrose agar and spores were maintained in 20% glycerol at −80 °C.

Fermentation Conditions

The basal medium used was a slightly modified Mandels medium buffered at pH 5.5 [14]. It contained (in grams per liter) $(NH_4)_2SO_4$, 1.4; $MgSO_4$, 0.3; KH_2PO_4, 2; $CaCl_2$, 0.3;

$NaNO_3$, 5; Tween 80, 1 ml; and 1 ml l^{-1} of trace element solution (in grams per liter) $CoCl_2$, 2; $MnSO_4.H_2O$, 1.6; $ZnSO_4.H_2O$, 1.4; and $FeSO_4.7H_2O$, 0.5. The carbon sources (pectin, glucose, and gruel) were set at a concentration of 1% unless otherwise indicated. In the presence of glucose, the composition of the nitrogen source was as follows: urea 0.64 g/l, ammonium sulfate 1.4 g/l, and yeast extract 1 g/l.

Enzyme production was carried out in 500-ml Erlenmeyer flasks where 100 ml of basal medium was inoculated with spores (10^6 spores/ml). The cultures were incubated at 30 °C on a rotary shaker set at 150 rpm for 5 days. The culture broths were filtered on 0.45-μm Millipore membrane filters and used for various analyses, namely of pectinolytic activities, reducing sugars, and proteins. The filtrate was concentrated by ethanol ($2v/1v$ of enzyme solution) before loaded on the electrophoresis gel.

Determination of Reducing Sugars and Proteins

Reducing sugars were determined using the 3.5 DNS method of Miller [15] and expressed as galacturonic acid equivalents. Protein content was estimated by the method of Bradford [16], using bovine serum albumin as a standard.

Pectinolytic Activity Assays

Exo-pectinolytic activities were determined by measuring the amount of reducing sugars released [15] after 1 h of incubation at 50 °C with 0.9% citrus pectin or 1% polygalacturonic acid (for polymethylgalacturonase and polygalacturonase activities, respectively) as described by Aguilar and Huitron [6]. One unit (IU) of exo-pectinolytic activities was defined as the amount of enzyme that liberated 1 μmol of galacturonic acid per minute.

Endo-pectinolytic activities were determined through the estimation of the relative change in viscosity measured by an Ostwald viscosimeter [8]. This activity was assayed by measuring the viscosity reduction of the citrus pectin solution (0.25%). Volumes of 6 ml of those substrates were dissolved in citrate buffer, pH 4.8, and pre-incubated at 37 °C for 3 min. A 1-ml enzyme solution was then added and the mixture was incubated at 37 °C for 5 min. One unit of endo-pectinolytic activity was defined as the amount of enzyme necessary to reduce the viscosity of the substrate solution by 50% in 5 min. The rate of viscosity reduction (A) is calculated using the following equation:

$$\mathrm{A} = (\mathrm{Ta} - \mathrm{T}/\mathrm{Ta} - \mathrm{To}) \times 100$$

Where T is the flow time (seconds) of the reaction mixture, Ta is the flow time (seconds) of polygalacturonic acid solution or citrus pectin solution added to the heat-inactivated enzyme, and To is the flow time (seconds) of water added to the heat-inactivated enzyme.

Pectin lyase activity was determined according to the method described by Pitt [7] in which 5 ml of citrus pectin solution (1% w/v in 20 mM Tris–HCl buffer, pH 8.5), 1 ml of CaCl2 (0.01 M), and 1 ml of diluted enzyme were used. The total volume was adjusted to 10 ml with water and incubated for 30 min at 60 °C. The reaction was then stopped by the addition of 0.6 ml ZnSO4·7H2O (9% w/v) followed by 0.6 ml NaOH (0.5 M). The precipitate was centrifuged at $3{,}000 \times g$ for 10 min. Three milliliters of thiobarbituric acid (0.04 M) was added to 5 ml of the supernatant, followed by 1.5 ml HCl (0.1 M) and 0.5 ml water, and the enzyme assay mixture was incubated in a boiling water bath for 30 min and cooled. Any precipitate was removed by centrifugation. Absorbance was measured at 550 nm. In the control tubes, the enzyme was added after the addition of ZnSO4 and

NaOH. One unit of pectin lyase was defined as the amount of enzyme causing a change in absorbance of 0.01 under standard assay protocol.

Pectin esterase activity (PE) activity was measured by using a pH stat according to the method described by Schejter and Marcus [17] in which apple pectin solution (30 ml, 0.5% *w*/*v* in 10 mM acetate buffer, pH 6.5) was incubated at 30 °C. The reaction is initiated by the addition of enzyme. One unit of PE activity (U) was taken as the amount of NaOH (micromoles) consumed per minute to keep constant pH value under the above-mentioned reaction conditions.

Denaturing Polyacrylamide Gel Electrophoresis

Electrophoresis under denaturing conditions was carried out according to the method of Laemmli [18]. The stacking and separating gels contained 5% and 10% acrylamide, respectively. The running buffer was Tris-glycine (pH 8.9) containing 0.1% SDS. Samples were either heated at 100 °C for 1 min or not heated in loading buffer (1% SDS, 10% glycerol, 0.01% bromophenol, and 5% 2-mercaptoethanol) in 0.06 M Tris, pH 6.8. The gels were stained with Coomassie brillant blue G-250. The molecular weights of the proteins were determined using molecular weight markers (Amersham).

Activity Stain Overlay Technique for Detecting Pectic Enzymes

After electrophoresis, the gel was incubated for 2 h in Tris–HCl 20 mM to get rid of SDS from the gel and thus allowing the renaturation of proteins. The pectinase gel was then applied against an overlay of 1% agarose containing 0.2% of citrus pectin or 0.1% polygalacturonic acid dissolved in appropriate buffer solutions. The sandwiched gels were incubated at 37 °C for 2 to 4 h; the overlays were stained with 1% CTAB for 30 min. Pectinase-degrading activities were detected as clear zones and pectin esterase activity as white zones.

Results

Effect of Heat Treatment

In order to detect pectinolytic activities in solid media, the present work involved a comparative study wherein the activities obtained by the samples that were heat-treated for 1 min at 100 °C (before loading on SDS-PAGE) were compared to those obtained by nonheated ones. Prior to this analysis, the samples were obtained from cultures realized on different carbon sources, such as glucose, citrus pectin, and gruel (obtained from the by-product of a local wheat manufactory).

After SDS-PAGE analysis, proteins were renatured and the overlay technique was applied on an agarose gel containing citrus pectin (DE=67%) at pH 4.8 (Fig. 1). The findings revealed a net difference in the electrophoretic protein profile between the heated and unheated samples (see the Coomassie-stained gels in Fig. 1). On zymogram, a net difference of activity was also noted according to the treatment. In other words, while the heat-treated samples presented a single clear band (depolymerizing activities) corresponding to the size of 25 kDa for all loaded filtrates, the heat-untreated samples presented diverse bands on the agarose gel (clear and white zones). With regards to the CP filtrate, two clear zones of proteins whose size was lower than 25 kDa were detected

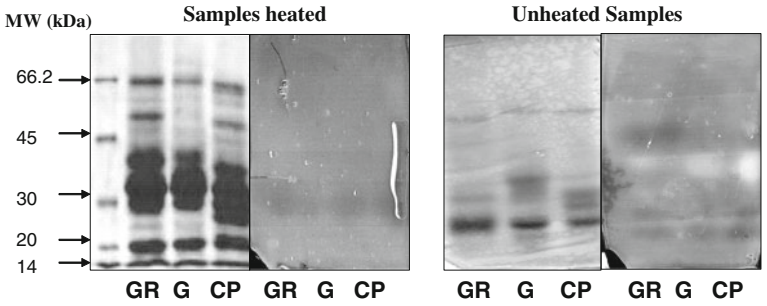

Fig. 1 Polyacrylamide gel SDS-PAGE and zymogram analysis of various enzymatic extracts on different carbon sources. *G* glucose, *GR* gruel, *CP* citrus pectin. Samples heated (**a**); unheated samples (**b**)

together with a white zone (at 35 kDa) that corresponded to pectin esterase activity. This esterase is present in all filtrate extracts of different cultures. The filtrate obtained from the culture grown on gruel presented a hydrolytic activity at the level of proteins having the size of 39 kDa, almost absent in the other filtrates, which should correspond to pectin and pectate lyases [19] (Ben Rhomdhane et al., in preparation).

The findings from the dosage of pectinolytic activities (exo–endo-pectinases) in the enzymatic extract revealed that the exo-pectinolytic activities were significantly higher in gruel (GR) than in CP or glucose. This result could be reflected by the strong presence of 39 kDa band in the GR zymogram. Pectin esterase was present in all filtrate dosages (Table 1), confirming the results of the zymogram analysis but the order is not the same. This could indicate that the differences detected in pectinolytic activities assayed in various liquid enzymatic extracts were not exactly those detected in solid media. This is very obvious since the assay methods in liquid media allow to get a global idea on enzymes while the zymogram analysis reveals a set of enzymes, depending on the overlay conditions. Such statement will be strengthened throughout the following examples.

Effect of Substrate Nature and Esterification Degree

In order to investigate the effect of the substrate nature and the esterification degree of pectin on pectinolytic activity detection, different types of pectic were assayed (Fig. 2). In fact, three pectin sources (orange PGA, citrus pectin CP, and apple pectin AP were used in the overlay gel with CP being present at three percentages of esterification (28%, 67%, and 90%). The findings revealed a difference of detection in terms of pectinolytic activity that correlated with the pectin sources. An interesting finding distinguishes the methylated pectin (either CP or AP) from PGA: a clear zone corresponding to a 25-kDa protein is very intense in AP and

Table 1 Pectinase production by the CT1 mutant using different carbon sources

Pectinolytic activities	CP (1%)	GR (1%)	G (1%)
Exo-pectinases (U/ml)	220	1100	380
Endo-pectinases (U/ml)	265	275	400
Pectin lyase (U/ml)	20	40	8
Pectin esterase (U/ml)	17	50	20

G glucose, *GR* gruel, *CP* citrus pectin

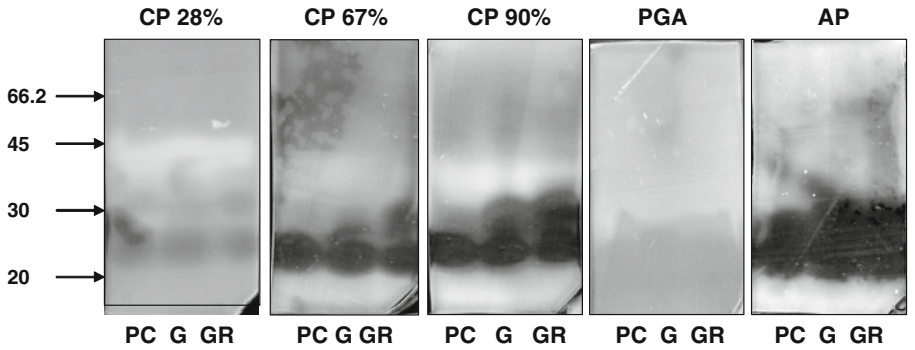

Fig. 2 Zymogram analysis on citrus pectin (*CP*) at various esterification degrees and apple pectin (*AP*); polygalacturonic acid (*PGA*) at pH 4.8

in CP with the degree of esterification of 90% and 67%. This enzyme could be a pectin lyase since it is very weak on CP with degree of esterification of 28. In PGA, such activity seems absent and is replaced by a smaller one, which could be either a pectate lyase or a polygalacturonase. Concerning the white zones, corresponding to pectin esterase with molecular weights of the first at about 20 kDa and the second at more than 30 kDa, are also more pronounced in methylated pectin CP or AP and almost absent in PGA. This result is very obvious with regard to the absence of the substrate of the esterase in PGA, namely the methoxy groups.

Effect of the pH and the Presence of β-Mercaptoethanol in the Zymogram Method

The latest results using β-mercaptoethanol in sample buffer improved the presence of pectinolytic activities under the operating conditions.

The present study deemed it essential to investigate the activity in the presence and absence of β-mercaptoethanol at two pH values, 3 and 4.8, with citrus pectin 67% as substrate (Fig. 3). The findings revealed firstly small differences on the Coomassie-stained SDS-PAGE gels. Secondly, the revelations by CTAB gave different responses according to the presence or absence of the reducing agent for both pHs (mainly at pH 4.8). Indeed, in the absence of β-mercaptoethanol and in all filtrates, a strong white zone (pectin esterase) is observed at pH 3 (mainly for glucose filtrate) and a strong clear zone is observed at pH 4.8.

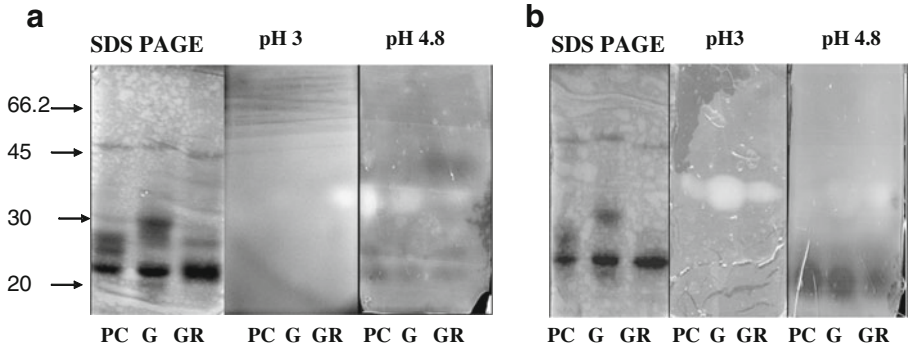

Fig. 3 Polyacrylamide gel SDS-PAGE and zymogram analysis of enzymatic extracts in the presence (**a**) and absence (**b**) of β-mercaptoethanol using citrus pectin 67% at two pH buffer values, 3 and 4.8

Effect of Divalent Cations and EDTA in the Zymogram Method

Regarding the importance of ions (and mainly calcium ions) on pectinase activities, we aimed to investigate the influence of Ca^{++} and Mg^{++} ions on enzymatic activity by performing a series of assays at various $CaCl_2$ and $MgSO_4$ concentrations at pH 4.8. Figure 4 shows the disappearance of the band corresponding to the molecular weight of 25 kDa for a concentration of 25 mM of the divalent cations Ca^{++} or Mg^{++}. A concentration of 5 mM of Ca^{++} or Mg^{++} in overlay gels allowed for a better detection of two pectinolytic activities (depolymerizing and pectin esterase). Inversely, when 5 mM EDTA was added to the overlay gels, no more pectin esterase activity was detected (no white zone for the three filtrates).

Discussion

The present study deals with a pectinolytic activity-staining procedure system that worked in conjunction with electrophoretic techniques, a system that allowed for the easy detection and preliminary characterization of pectic enzymes. The detection technique was based on the ability of the enzymes diffusing from bands in a polyacrylamide gel to degrade the polymers of pectin in agarose overlay as well as on the ability of CTAB to precipitate and stain the surroundings of the degraded substrate.

The detection system provides a rapid means to visualize the molecular weights, pH activities, and substrate preferences of pectin-degrading enzymes The revelation by CTAB yielded into a change in agarose gel as well as into an observable clear zone, corresponding to depolymerizing activities, and a noticeable white zone (precipitate), corresponding to pectin esterase activity. This protein was purified and its N terminal sequence confirmed its identity as pectin esterase. With such activity, the different enzymatic extracts exhibited a preference towards citrus pectin as a substrate, particularly at high degrees of pectin esterification (Tounsi et al., in preparation).

The pH of the agarose overlay gel allows for the differential identification of certain pectinolytic activities. Particularly, the pH has a great influence on the revelation of esterase activity; pH 3 was shown to be the best one since it allows at the same time the disappearance of the depolymerizing activities. Moreover, the presence of β-mercaptoethanol in the sample buffer was noted to prevent the detection of pectin esterase at certain pH values, indicating the presence of essential disulfide linkages. These pHs promote disulfide bond formation between cysteine residues in the proteins [18].

The depolymerizing activities were more pronounced particularly at pH 4.8 in the presence of β-mercaptoethanol. The extracellular pectinase from *Acrophilophora nainiana*

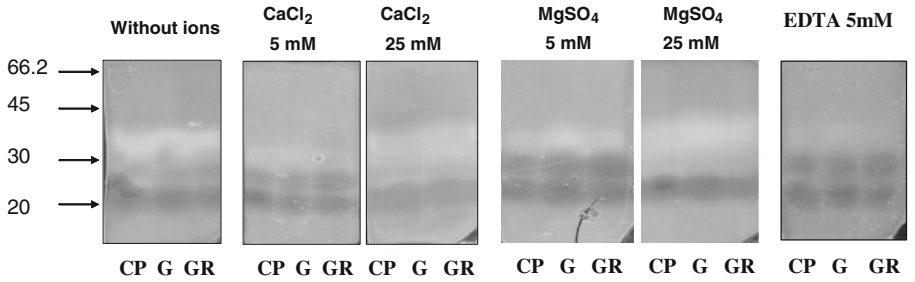

Fig. 4 Effect of the addition of $CaCl_2$, $MgSO_4$, and EDTA in the zymogram method

was activated by β-mercaptoethanol [20], while the polygalacturonase from *Sporotrichum thermophile* was inhibited by β-mercaptoethanol [21].

Several studies showed that the presence of ions in the reaction media influences pectinolytic activities. The lyases, particularly pectate lyases, have an absolute requirement for Ca^{++} ions. Inversely, pectin lyases do not require cations but are stimulated by Ca^{++} [22]. In fact, while the presence of Ca^{++} in media at pH 4.8 did not exert significant effects on the test of activity, at pH alkaline, the presence of Ca^{++} at 5 or 25 mM was necessary for the stimulation of lyase activities.

Overall, the findings of the present study clearly demonstrate that the combination of SDS-polyacrylamide gel electrophoresis with pectin–agarose overlay stained with CTAB provides a rapid, effective, and determined method for the analysis of a complex mixture of pectinases. This method can, therefore, be considered as a strong promising candidate for application in future studies aiming to detect, identify, and delineate pectin-depolymerizing activities in solid media.

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