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Full Length Research Paper

Activity staining method of chitinase on chitin agar plate through polyacrylamide gel electrophoresis

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A method for detection of chitinase activity on chitin agar plate after polyacrylamide gel electrophoresis is described. Different staining dyes such as calcofluor white M2R, fluorescein isothiocyanate, rhodamine B, ruthenium red and congo red were separately incorporated in chitin agar plates. After running polyacrylamide gel electrophoresis, the gel was transferred onto chitin agar plate containing different dyes for the activity staining. Thin layer of acetate buffer (0.2 M, pH 5) was pored on the gel, which helps faster diffusion of the enzyme from gel onto the plate. After incubation of about 7 h, bands of chitinase were visible by daylight or UV light. The method is very sensitive since it can detect even 0.5 units of chitinase. Thus, this method is sensitive, rapid, user-friendly, reliable and cost effective.

Key words: Activity staining, chitinase, dyes, sensitivity, stability.

INTRODUCTION

Chitin, a linear homopolysaccharide of *N*- acetyl glucosamine is widely distributed in nature as a principal structural component in the cell wall of fungi and, in the exoskeleton of crustacean and arthropods (Patil et al., 2000). Chitinase activity has been found with bacteria and *Streptomycetes*, plants, invertebrates, vertebrates and fungi. A great deal of interest has been generated on chitinase because of its applications in the biocontrol of plant pathogenic fungi (Ordentlich et al., 1988), molting process of insects, mosquito control (Mendonsa et al., 1996), production of chitooligosaccharide (Terayama et al., 1993), single cell protein (Vyas and Deshpande, 1991) and mycolytic enzyme preparation (Vyas and Deshpande, 1989).

Glycol chitin is a soluble modified form of chitin, which has recently become a very useful substrate for activity staining but it is costlier than acid swollen chitin. Despite the importance of chitin metabolism in nature and *in situ* gel activity staining technique (Trudel and Asselin, 1989), there is still no method available for detection of chitinase activity onto the solid plate method after polyacrylamide gel electrophoresis under native or denaturing conditions.

However, *in situ* gel activity staining method has the disadvantage that the gel cannot be used further for protein staining and also the problem of mobility of chitinase in the gel because of the presence of polysaccharide in the gel. Chitin agar plate has been used earlier for isolating chitinolytic microorganisms and observing clear zone around the colony of microorganisms (Cody, 1989; Wirth and Wolf, 1990). Attempts were therefore made to separate the crude chitinase on the polyacrylamide gel and transferred it onto the chitin agar plate. A thin layer of acetate buffer (0.2 M, pH 5) was pored on the gel, which aids in faster diffusion. The crude chitinase, which was used in this method, was produced from *Pantoea dispersa* (Gohel et al., 2004). This method is a simple, reproducible, sensitive, user-friendly, reliable and cost effective activity staining for chitinase detection in native and denatured polyacrylamide gel.

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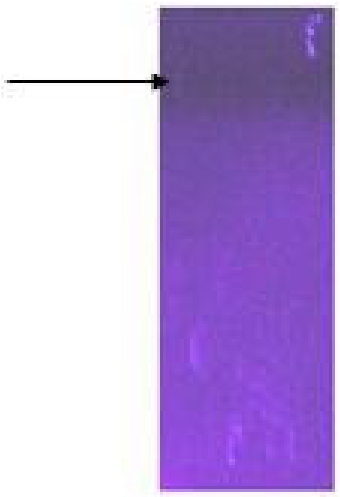

Figure 1. Chitinase activity staining of electrophoretically resolved native protein of chitin grown culture of *Pantoea dispersa* by in situ gel method with a glycol chitin used as substrate and calcofluor white M2R used for resolution of the lytic zone.

MATERIALS AND METHODS

Chemicals

Chitinase from *P. dispersa* (Gohel et al., 2004), practical grade chitin, glycol chitin, calcofluor white M2R and fluorescein isothiocyanate were obtained from Sigma Chemical Co. (St. Louis, MO). Congo red was obtained from BDH (Poole, England). Rhodamine B was obtained from RDSH (Germany). Ruthenium red was obtained from HiMedia Laboratories Ltd. (Mumbai, India).

Chitinase assay

Chitinase was assayed as described by Vyas and Deshpande (1989). The assay system consisted of 10 mg of acid swollen chitin, 50 µmoles of acetate buffer (pH 5) and suitable concentration of enzyme in a total system of 3 ml. The incubation was done at 50°C for 10 min. The product was estimated by the Nelson method (1944). One unit of chitinase activity was defined as the amount of enzyme required to liberate 1 µmole of *N*- acetyl-D glucosamine equivalent at 50°C per h.

In situ activity staining

In situ activity staining method was as described by Trudel and Asselin (1989), incorporating 0.01% (w/v) soluble glycol chitin.

Preparation of acid swollen chitin

Phosphoric acid swollen chitin (practical grade chitin) was prepared according to the method described by Hackman (1962).

Preparation of chitin agar plate with different dyes

Chitin agar plates were prepared using 0.5% acid swollen chitin or 0.5% glycol chitin (Gohel et al., 2004). Calcofluor white M2R, fluorescein isothiocyanate, rhodamine B, ruthenium red and Congo red, at 0.001% (w/v), were added separately to chitin agar plate.

PAGE (Polyacrylamide gel electrophoresis)

Native and SDS polyacrylamide gel electrophoresis were carried out at a constant current of 20 mA in 15% (w/v) gels (1.5 mm thick) by method of Sambrook et al. (1989). The gels were run at 4°C. After SDS electrophoresis, gels were incubated at 37°C for 4 h in sodium acetate buffer (0.2 M, pH 5) containing 1% (v/v) Triton X-100 to remove SDS. The gels were washed with distilled water.

Staining for chitinase activity

Gels after native and SDS PAGE were directly transferred onto each chitin agar plate containing a different dye. Thin layer of acetate buffer (0.2 M, pH 5) was put on each gel for diffusion of chitinase onto the chitin agar. The plates were incubated at 37°C. Activity band is visible as formation of dark band against a fluorescent background on chitin agar plate, with calcofluor white M2R, and fluorescein isothiocyanate after 7 h. This was observed under a hand held UV transilluminator (UVP Inc., Upland, CA, USA). Whereas chitin agar plates containing rhodamine B, ruthenium red, and Congo red showed clear distinct bands on the plate observed in daylight.

RESULTS AND DISCUSSION

We developed techniques for screening of hyperchitinase producing bacteria using acid swollen chitin with calcofluor white M2R in plate assay. Dark haloes were observed around colony against fluorescent background under UV light (Vaidya et al., 2003). The same approach was tested for the detection of chitinase activity on solid agar plate through gel electrophoresis. Trudel and Asselin (1989) have developed an activity staining method by incorporating a soluble glycol chitin in electrophoresis gel. Lytic zone was observed by UV illumination with a transilluminator after staining with calcofluor white M2R. In this method, when substrate was directly incorporated into gel the bands showed a smear instead of well-defined band. We observed retardation of mobility of enzymes (Figure 1) in the gel during the electrophoresis, which may be because of the presence of polysaccharide in the gel, which can be overcome by solid plate method.

In both SDS and native PAGE, the concentrations of chitinase (0.4 to 10 units) were loaded. After electrophoresis, native gels were transferred onto the plate containing acid swollen chitin and glycol chitin with calcofluor white. For SDS PAGE, chitinase was renatured by removing SDS using triton X 100 (0.5% v/v) treatment. The acetate buffer was layered onto the gel. The diffusion of chitinase onto plate was measured on the basis of the band observed. Without acetate buffer (0.2 M, pH 5), the time taken for diffusion was found to be 13 h whereas with acetate buffer the time taken for diffusion decreased by 6 h thus indicating an increase in diffusion rate. Both of these plates gave the same dark zone band (Figure 2).

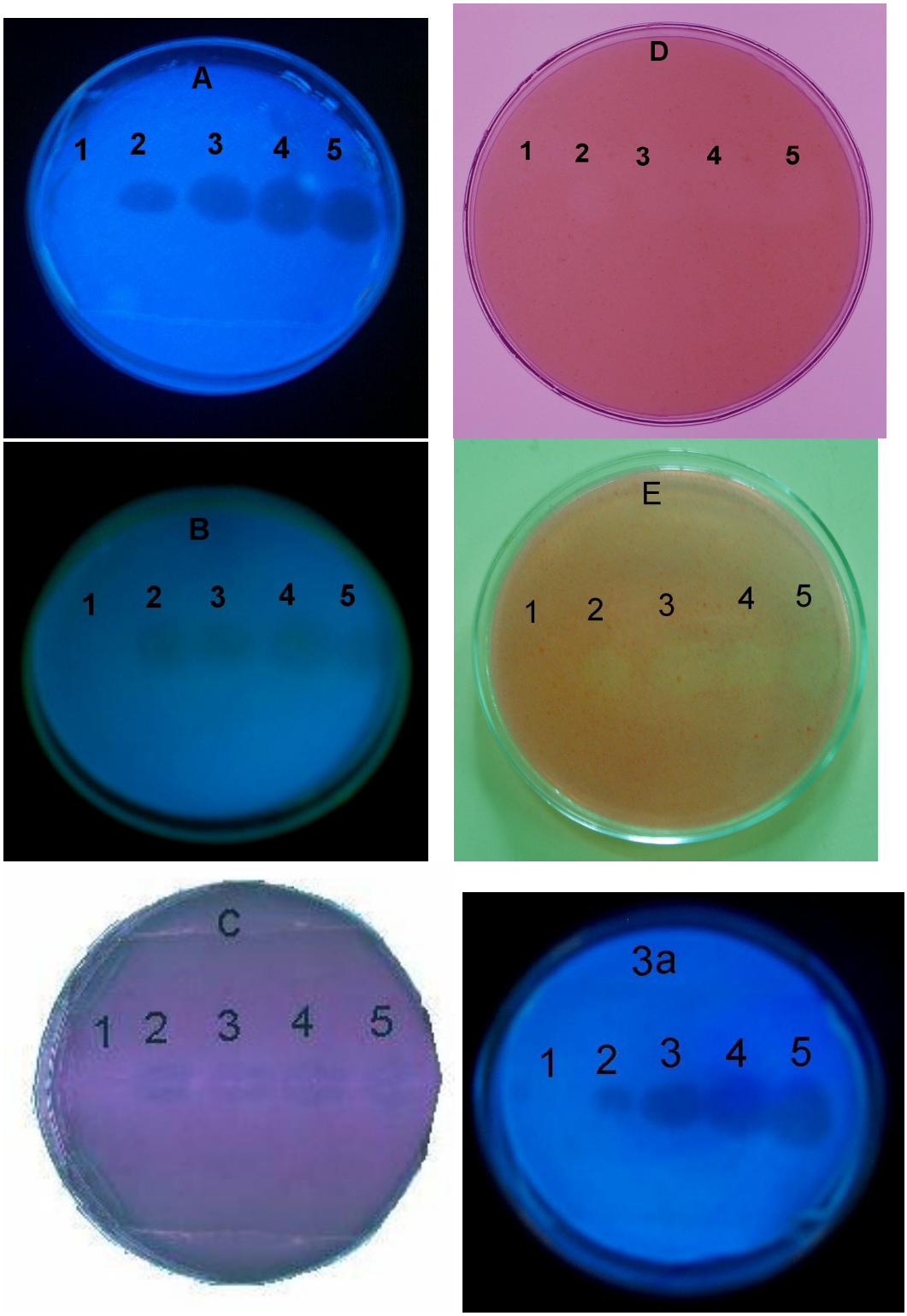

Figure 2. Chitinase activity staining of electrophoretically resolved native protein of chitin grown culture of *P. dispersa* by solid plate method, containing A) acid swollen chitin with calcofluor white; B) acid swollen chitin with fluorescein isothiocyanate; C) acid swollen chitin with rhodamine B; D) acid swollen chitin with ruthenium red; E) acid swollen chitin with congo red; and 3a) glycol chitin with calcofluor white M2R. Different units of chitinase 1) 0.4; 2) 0.5; 3) 5.0; 4) 7.5; and 5) 10 were loaded onto the gel to study the sensitivity.

As acid swollen chitin costs lesser than glycol chitin, this method is also cost effective in terms of the substrate. Different concentrations of chitinase were used in order to determine the sensitivity. The method is very sensitive since it can detect even 0.5 units of chitinase. McGrew et al. (1990) reported sensitivity of *in situ* activity staining method of 10 units when casein was used in the gel wash buffer.

In this method other than calcofluor white M2R, fluorescein isothiocyanate, rhodamine B, ruthenium red and Congo red [0.001% (w/v)] were used with acid swollen chitin as a substrate in the plates. Plates incorporated with fluorescein isothiocyanate and ruthenium red did not show significant improvement in Visualization of distinct band as compared to rhodamine B and Congo red. Whereas calcofluor white M2R shows very good distinct bands on solid plates when compared to others (Figure 2).

Calcofluor white M2R binds to the glucan chains and linear β- (1, 4)-glucosidically linked units of *N*-acetyl glucosamine. On binding to polysaccharide such as cellulose and chitin, this flourochrome highlights and emits a light blue light when exposed to UV. On degradation of this polymer to its individual subunits, this fluorescence is lost as indicated by a dark band against a fluorescent background. Calcofluor white M2R is widely used as an optical brightner in the textile industry and also as a staining dye in fluorescence microscopy (Bartnicki-Garcia et al., 1994) and for the screening of hyperchitinase producing bacteria. Calcofluor white M2R incorporated chitin plate is suitable for sensitive plate assays or semi-quantitative enzyme diffusion plate assay (Vaidya et al., 2003). The chitinase method we have developed is more sensitive, rapid, user friendly and reliable.

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