Techniques

A Technique for Detection of Chitinase, β-1,3-Glucanase, and Protein Patterns After a Single Separation Using Polyacrylamide Gel Electrophoresis or Isoelectrofocusing

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ABSTRACT

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A procedure to detect chitinase and β-1,3-glucanase isozymes and protein patterns after a single separation using native polyacrylamide gel electrophoresis (PAGE) or isoelectrofocusing (IEF) is described. After electrophoresis or isoelectrofocusing, an overlay gel containing glycol chitin as substrate for chitinase was incubated in close contact with the resolving gel. Chitinase isozymes were revealed by UV illumination after staining the overlay gel with fluorescent brightener 28. The resolving gel was then incubated with laminarin, and β-1,3-glucanase isozymes were detected by using 2,3,5-triphenyltetrazolium chloride. The resolving gel with β-1,3-glucanase bands was stained with Coomassie Brilliant Blue R 250 to reveal protein patterns. The isozymes were quantified by using native PAGE, and their pIs were estimated by IEF.

Stem injection with sporangiospores of *Peronospora tabacina* Adam and leaf inoculation with tobacco mosaic virus (TMV) both systemically induce resistance to blue mold caused by *P. tabacina* and systemically elevate chitinase (EC 3.2.1.14) and β-1,3-glucanase (EC.3.2.1.39) activities (9,13). The induction of both enzymes in tobacco appears coordinately regulated as is induction in ethylene-treated bean leaves (14). Because chitin and β-1,3-glucan are major cell wall components of many pathogenic fungi (2), it has been suggested that chitinase and β-1,3-glucanase may function in defense against fungal pathogens (1). Furthermore, these two enzymes act synergistically in the partial degradation of isolated fungal cell walls, and combinations of the two enzymes strongly inhibit growth of most fungi tested, including those that can not be inhibited by chitinase or β-1,3-glucanase alone (14). Parallel increases in the activities of β-1,3-glucanase and chitinase may be important for their optimal function in plant defense. It is also important to distinguish different isozymes to elucidate their roles in the host-pathogen interaction. Diverse roles have been suggested for different enzymes of β-1,3-glucanase (8). Techniques for detecting β-1,3-glucanase and chitinase isozymes have been developed by Pan et al (8) and Trudel and Asselin (11), respectively. This paper presents a technique that directly detects both chitinase and β-1,3-glucanase isozymes in crude extracts after a single separation on a gel by using native polyacrylamide gel electrophoresis (PAGE) or isoelectrofocusing (IEF). Chitinase and β-1,3-glucanase isozymes can be quantified by using PAGE, and protein bands can be stained with Coomassie Brilliant Blue R 250 on the same gels after staining for the isozymes. This technique facilitates studies of the coordinate induction of the two enzymes, and it reduces the time and materials required for assays. The technique also reduces the variability that results from using different separations or samples. Protein bands can be matched with enzyme activity bands on gels, and hence the technique helps the identification and purification of the enzymes.

MATERIALS AND METHODS

Materials. Turkey egg white lysozyme, fluorescent brightener 28, laminarin (β-1,3-glucan), glycol chitosan, and 2,3,5-triphenyltetrazolium chloride were from Sigma Chemical Co. (St. Louis, MO); glycol chitin was synthesized from glycol chitosan as described previously (11); acrylamide, N,N′-methylenebisacrylamide (Bis), ammonium persulfate, and N,N,N′,N′-tetramethylethylenediamine (TEMED) were from Bio-Rad (Richmond, CA); ampholines were from LKB (Bromma, Sweden).

Enzyme preparations. Burley tobacco (*Nicotiana tabacum L.* 'Samsun NN') plants were grown in a greenhouse, and the three or four lower leaves were inoculated with TMV as described (15). The TMV-infected and mock-inoculated leaves were harvested six days after inoculation. Samples were frozen in liquid nitrogen immediately after being collected and were extracted by grinding in 0.05 M sodium acetate buffer (pH 5.0) with a mortar and sea sand at 4 C (2 g of fresh leaf tissue in 2 ml of acetate buffer). The extracts were dialyzed against two changes of water, then against two changes of 0.01 M sodium acetate buffer (pH 5.0) overnight at 4 C.

Polyacrylamide IEF. An LKB 2117 Multiphor system was used to run 2.0-mm-thick 7.5% polyacrylamide gels (pH 3.5–9.5). Samples were applied to the gel surface on sample application pieces (LKB). The gels were run for 3 h at 6 C (constant power: 10 W).

Native PAGE. Polyacrylamide resolving gels (15%, 1.5 mm thick) were prepared by mixing 15.0 ml of 30% acrylamide (acrylamide/Bis; 30:0.8), 3.75 ml of 3.0 M Tris-HCl (pH 8.8) for anodic PAGE or 3.75 ml of 3.0 M acetic acid-KOH (pH 4.3) for cathodic PAGE, 1.5 ml of 1.5% ammonium persulfate, and 9.75 ml of distilled water, by degassing for 10 min, then by adding 15 μl of TEMED, and by pouring. The stacking gel included 2.5 ml of 30% acrylamide, 1.0 ml of 1.5% ammonium persulfate, 11.5 ml of distilled water, and 5.0 ml of 0.5 M Tris-HCl (pH 6.8) for anodic PAGE or 5 ml of 0.5 M acetic acid-KOH (pH 6.7) for cathodic PAGE. This was degassed for 10 min, then 15 μl of TEMED was added, and the gel was poured. The gels were run for 4 h at 8 C (constant current: 30 mA) with an LKB 2001 Vertical Electrophoresis unit.

Enzyme detection. After electrophoresis or isoelectrofocusing, PAGE or IEF gels attached to supporting glass plates were incubated in 0.1 M sodium acetate (pH 5.0) for 5 min. They were then covered with a 7.5% (0.75 mm thick) polyacrylamide overlay gel (attached to another supporting glass plate) containing 0.04% glycol chitin in 0.1 M sodium acetate (pH 5.0). The bubbles between PAGE or IEF gels and overlay gels were eliminated by gently sliding overlay gels on the top of the gels. The gels were incubated at 40 C for 1.5 h under moist conditions. Overlay gels were then incubated in freshly prepared 0.01% (w/v) fluorescent brightener 28 in 500 mM Tris-HCl (pH 8.9) at room temperature for 5 min. The brightener solution was discarded and the overlay gels were incubated in water at room temperature in the dark for 2 h, or in the refrigerator overnight. Chitinase isozymes were visualized as cleared zones by placing the overlay gels on a UV transilluminator and were photographed as described below. The overlay gels with chitinase isozymes can be stored in the refrigerator for weeks. To stain for β-1,3-glucanases, the PAGE or IEF gels were washed with water, incubated with 0.05 M sodium acetate (pH 5.0) for 5 min, and then incubated at 40 C for 30 min in a mixture containing 75 ml of 0.05 M sodium acetate (pH 5.0) and 1 g of laminarin dissolved in 75 ml of water by heating in a boiling water bath. The gels were then incubated in a mixture of methanol, water, and acetic acid (5:5:2) for 5 min, washed with water, and PAGE gels were stained with 0.3 g of 2,3,5-triphenyltetrazolium chloride in 200 ml of 1.0 M NaOH in a boiling water bath until red bands appeared (about 10 min). IEF gels were stained as described except they were heated in a microwave oven for 3 min (interrupted by shaking every 30 s). The stained gels could be dried or stained for proteins with Coomassie Brilliant Blue R 250.

To verify the chitinase and β-1,3-glucanase activities, blanks were utilized by omitting the substrates glycol chitin and laminarin from the procedure, respectively, and staining the gels as described. These blanks were run because some plant crude extracts may generate nonfluorescent bands in overlay gels without glycol chitin, which can be falsely regarded as chitinase bands, and/or contain compounds that react with the triphenyltetrazolium reagent and give false β-1,3-glucanase bands. However, no such interfering bands were found in these tobacco extracts.

To match chitinase activity bands with protein bands, the overlay gel was slowly laid on the resolving gel attached to a supporting glass plate after the resolving gel was incubated with 0.1 M sodium acetate (pH 5.0) for 5 min. The gels were incubated for 1.5 h as described. Agarose (1%) was used to seal the contact edges of the resolving and overlay gels so that the two gels could be fixed together. The gels were incubated with the brightener solution, and the chitinase activity bands were marked by piercing small holes through both gels. The resolving gel was then stained for β-1,3-glucanase and protein bands. The small holes facilitated matching chitinase activity bands with protein bands.

Enzyme quantitation. Chitinase isozyme bands on the overlay gels were photographed with an SLR camera with Kodak TMK 135 film using an orange filter. The exposure time varied from 4 to 10 s with 50 mm Macro lens at f4.5. The photographs were then scanned with an LKB Ultroscan XL Laser Densitometer. A quantitative relationship between band intensity (expressed as signal area) and amount of chitinase was established. To quantify β-1,3-glucanase isozymes on PAGE gels, the gels with β-1,3-glucanase bands were dried with a gel dryer (Model 483, Bio-Rad) and then scanned with a TLC Scanner (CS-920, Shimadzu, Kyoto, Japan) using the smaller light beam with light wavelength 555 nm (8).

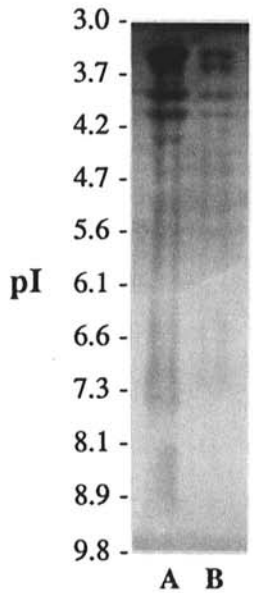

Fig. 1. Chitinase isozyme pattern on overlay gel after isoelectrofocusing (7.5%). Crude enzyme preparation (150 μg of total protein) was applied per lane. **A,** Tobacco leaves infected with tobacco mosaic virus; **B,** tobacco leaves mock-inoculated.

RESULTS

Detecting chitinase isozymes after IEF. Crude enzyme extracts were electrophoresed on 7.5% (2.0 mm thick) IEF gels with a pH range of 3.5–9.5 and then assayed for chitinase isozymes as described in Materials and Methods. The principle of the detection is as follows. Glycol chitin, which serves as substrate for chitinases (6), binds to fluorescent brightener 28 by affinity (7). After the substrate is digested by chitinases that have diffused from PAGE or IEF gels to the substrate-containing overlay gels, the resulting bands are visualized on overlay gels by UV transillumination as nonfluorescent dark bands in a fluorescent background, because

the brightener is bound only to undigested glycol chitin (11). The assay using IEF gels demonstrated that the dominant chitinase isozymes in tobacco extracts had low pI values (Fig. 1). This suggested that the dominant chitinase isozymes could be detected by the anodic PAGE system, which can be used for enzyme quantitation as shown later. The assay for chitinase via IEF gels was not quantitative (data not shown), however, the isozyme pattern revealed on IEF gels was consistent with a previous report that dominant chitinase isozymes in tobacco are acidic proteins (12).

Detecting chitinase and β-1,3-glucanase isozymes and proteins after PAGE. Because the dominant chitinase isozymes in tobacco crude extracts were acidic proteins, anodic PAGE was run to separate chitinase and β-1,3-glucanase isozymes in the crude extracts. The chitinase isozymes were visualized by using the overlay gels as described above. There were eight dominant chitinase isozymes detected in tobacco extracts, one of them appeared only in the TMV-infected leaves, and another accumulated more in the TMV-infected than mock-inoculated leaves (Fig. 2). The β-1,3-glucanase isozymes were stained with 2,3,5-triphenyltetrazolium chloride on the PAGE gels. There were two dominant β-1,3-glucanase isozymes detected in tobacco extracts, one was detected only in the TMV-infected leaves and another accumulated more in the TMV-infected than in the mock-inoculated leaves (Fig. 3). After staining for β-1,3-glucanase isozymes, the PAGE gels were then stained with Coomassie Brilliant Blue R 250. The β-1,3-glucanase bands appeared red or purple, whereas the other proteins were visualized as blue bands (Fig. 4B,C). All the protein bands on the PAGE gel stained with Coomassie Blue (Fig. 4A) could be found on the gel stained for protein bands after staining for chitinase and β-1,3-glucanase isozyme bands (Fig. 4B). This allowed matching protein bands with β-1,3-glucanase activity bands. Chitinase activity bands may also be matched with protein bands if the resolving and overlay gels are sealed together by using 1% agarose, and the activity bands are marked by piercing small holes through both gels before staining for β-1,3-glucanase and protein bands (data not shown).

While both PAGE and IEF revealed that one chitinase isozyme appeared only in the TMV-infected leaves, and another accumulated more in the TMV-infected than mock-inoculated leaves (Figs. 1 and 2), there were more chitinase isozymes detected via

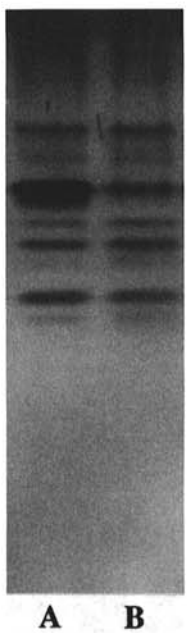

Fig. 2. Chitinase isozyme pattern on overlay gel after anodic polyacrylamide gel electrophoresis (15%). Crude enzyme preparation (150 μg of total protein) was applied per lane. **A,** Tobacco leaves infected with tobacco mosaic virus; **B,** tobacco leaves mock-inoculated.

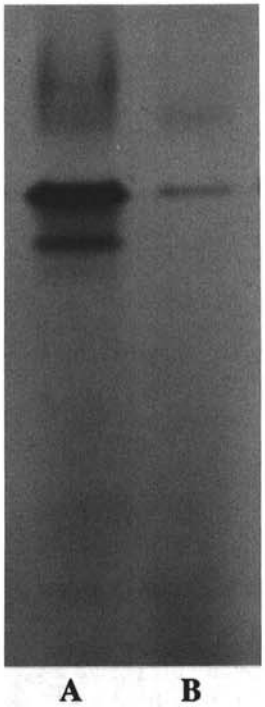

Fig. 3. β-1,3-Glucanase isozyme pattern on 15% anodic polyacrylamide gel electrophoresis (PAGE) gel after staining for chitinase isozymes. β-1,3-Glucanase isozyme pattern was revealed on the 15% anodic PAGE gel that had been used for detecting chitinase isozymes as shown in Figure 2. **A,** Tobacco leaves infected with tobacco mosaic virus; **B,** tobacco leaves mock-inoculated.

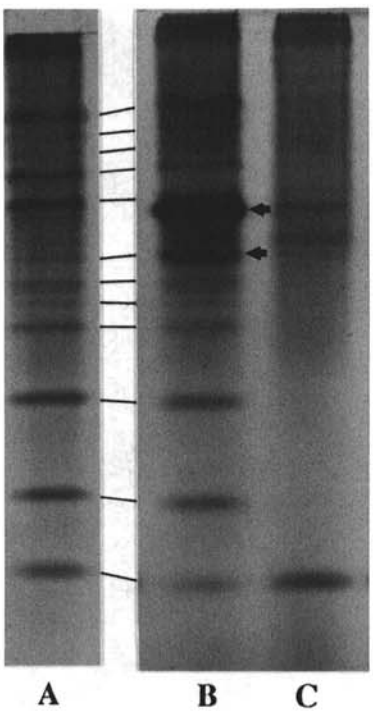

Fig. 4. Protein pattern on 15% anodic polyacrylamide gel electrophoresis (PAGE) after staining for chitinase and β-1,3-glucanase isozymes. Crude enzyme preparation (150 μg of total protein) was applied per lane. **A,** Crude enzyme preparation from tobacco leaves infected with tobacco mosaic virus was stained with Coomassie Blue for protein bands on 15% anodic PAGE gel; **B,** the same preparation was stained with Coomassie Blue for protein bands on the gel that had been used for detecting chitinase and β-1,3-glucanase isozymes as shown in Figures 2A and 3A; **C,** the preparation from mock-inoculated tobacco leaves was stained with Coomassie Blue for protein bands on the gel that had been used for detecting chitinase and β-1,3-glucanase isozymes as shown in Figures 2B and 3B. The arrows indicate β-1,3-glucanase isozyme bands, which appear purple-red after staining with Coomassie Blue.

PAGE than IEF. A possible explanation is that IEF gels have low gel concentration (7.5%), and the sieving effect has been minimized so that proteins are separated on IEF gels principally based on their pI values (3). Proteins with similar pIs cannot be separated by IEF gels utilizing a wide pH range. However, PAGE gels used in this case had a high polyacrylamide concentration (15%), and the sieving effect should contribute to protein separation on such gels, in addition to the protein charges generated based on the pIs. Therefore, PAGE may separate the proteins with similar pIs but different molecular weights, which may not be separated by IEF.

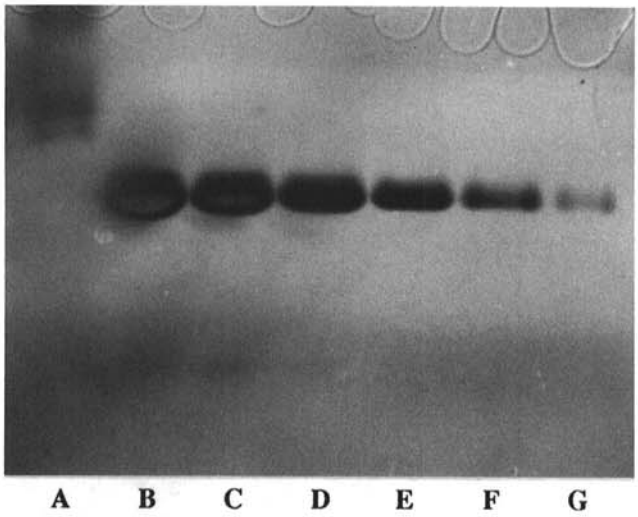

Fig. 5. Relationship between band intensity and amount of turkey egg white lysozyme, which has chitinase activity (11), loaded on 15% cathodic polyacrylamide gel electrophoresis (PAGE) gel. **A,** Extract from tobacco leaves infected with tobacco mosaic virus (150 μg of total protein); **B–G,** 80, 40, 20, 5, 1, and 0.25 μg of turkey egg white lysozyme, respectively. The chitinase activity was visualized by using 15% cathodic PAGE gel as described in Materials and Methods.

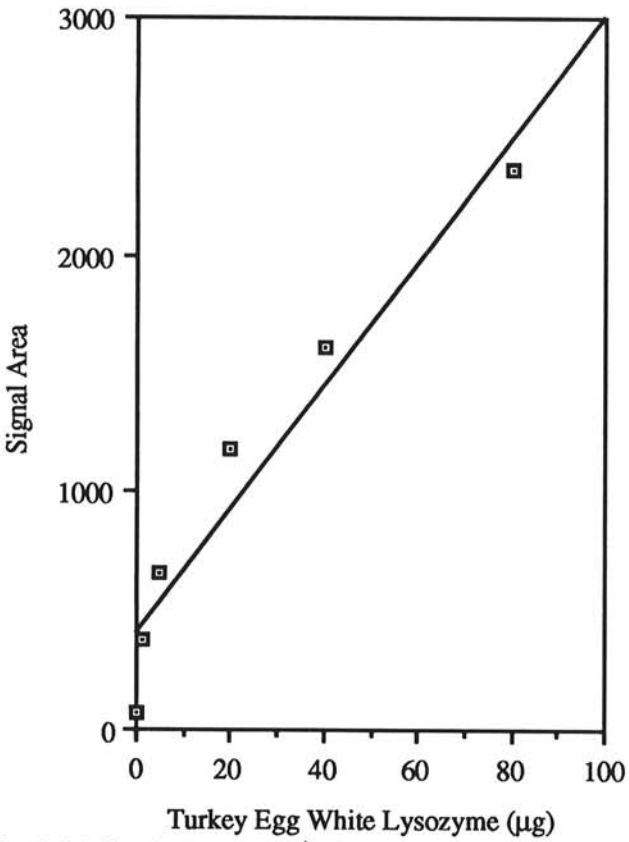

Fig. 6. Relationship between band signal area and amount of turkey egg white lysozyme loaded on 15% cathodic polyacrylamide gel electrophoresis gel. Chitinase activity of turkey egg white lysozyme was expressed as signal area after scanning the bands on Figure 5B–G with a densitometer. The relationship between enzyme activity and amount of enzyme was established by regression analysis.

Quantifying chitinase activity after PAGE. To verify that chitinase activity can be quantified after PAGE, purified turkey egg white lysozyme, which has chitinase activity (11), was electrophoresed by the cathodic PAGE system and visualized on the overlay gel. Cathodic PAGE was used because detection on IEF gel indicated that this lysozyme had a very high pI value (pH 10.5–11.0) (data not shown). A linear relationship was found between band intensity (expressed as signal area) and amount of turkey egg white lysozyme loaded (Figs. 5 and 6). Regression analysis showed that the linear relationship was highly significant within the range tested (α is <0.01, $R = 0.966$). The regression formula was $Y = 398.05 + 26.397X$ (Y: signal area; X: micrograms of turkey egg white lysozyme loaded).

DISCUSSION

The procedure described can qualitatively detect chitinase and β-1,3-glucanase isozymes and protein patterns after a single separation using native PAGE. As demonstrated, chitinase activity could be quantified by densitometry after the chitinase bands were visualized on the overlay gels and photographed. β-1,3-Glucanase can also be quantified on the PAGE gels as described previously (8). Subsequently, the protein pattern could be observed by staining with Coomassie Brilliant Blue R 250 on the same gels that had β-1,3-glucanase bands. After chitinase isozymes were detected on the overlay gels following IEF (Fig. 1), β-1,3-glucanase and protein patterns could also be detected on the same IEF gels, although the enzyme assay that used IEF is not quantitative and the isozyme bands stained with IEF are not as sharp as those stained with PAGE (data not shown). However, the pI values of chitinase or (and) β-1,3-glucanase isozymes could be estimated by using IEF gels. If both resolving and overlay gels were properly marked, chitinase and β-1,3-glucanase isozymes could be matched on PAGE or IEF gels with protein bands stained with Coomassie Brilliant Blue R 250. Because the anodic PAGE system can detect only negatively charged isozymes and the cathodic PAGE system can detect only positively charged isozymes (4,10), it was recommended that IEF be run first to obtain a full isozyme pattern and the anodic or cathodic PAGE system be used to study the isozymes based on their pIs as revealed by IEF. Chitinase and β-1,3-glucanase isozymes could be quantified by using the anodic or cathodic PAGE system, but their pI values could be estimated by IEF.

This technique detected endochitinases, because glycol chitin is a substrate for endochitinase but not for exochitinase (6). Both exo- and endo-β-1,3-glucanases could be detected by this procedure because laminarin can be used as substrate for both exo- and endo-β-1,3-glucanases, and both glucose and reducing oligomers released by the two enzymes, respectively, can be stained by the triphenyltetrazolium reagent (5).

This procedure provides a rapid, reliable, and simplified method for detecting and quantifying chitinase and β-1,3-glucanase isozymes after a single electrophoretic separation. The technique saves time and materials required for assaying the two hydrolases. Isozymes of both chitinase and β-1,3-glucanase can be identified; this can simplify enzyme purification. The technique should be especially useful in investigating the coordinated induction of the two enzymes in plant-pathogen interactions.

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