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Developing Polyclonal Antibody-based Indirect-ELISA to Detect Anthracnose Inocula Prior to Soybean Sprout Rot

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We developed a polyclonal antibody based-ELISA system to monitor inocula accurately and rapidly before onset of anthracnose on soybean sprouts. Titer of mouse antisera against conidia of *Colletotrichum gloeosporioides*, determined by indirect ELISA, was high enough to be detectable up to x25,600 dilutions. Both PAb1 and PAb2 had the highest level of reactivity to *Colletotrichum gloeosporioides*. Absorbance readings exceeded 0.15. Sensitivity of PAb to *C. gloeosporioides* was precise enough to detect spore concentration as low as 500 conidia/well by indirect ELISA. Both antibodies are very sensitive and highly specific to the target pathogen *Colletotrichum gloeosporioides*, apparently discriminating other unrelated pathogen, or epiphytes. This kit fulfills the requirements for detecting inocula before infection and onset of anthracnose. Our ELISA system should also be feasible to detect *C. acutatum* (Mungbean sprouts rot) and *G cingulata* (*C. gleosporioides*), (apple, pepper). It was remarkable that absorbance value was not reduced even after 4 consecutive washings (Fig. 4), suggesting that antigenic determinants are on the surface of conidia. Antigenic determinant was characterized by heating and enzyme treatment: Both PAb1 and PAb2 bind to protein epitope that does not contain residue of amino acid, arginine, and lysine, even though more work needs to be done.

Keywords **:** anthracnose, indirect ELISA, soybean sprout

Market value of annual consumption of bean sprout is estimated at 700 billion won in Korea. Soybean sprouts are being produced under environmental conditions favorable for pathogen proliferation. Hence, there are higher chances of epidemic.

Current practice to minimize the soybean sprouts rot is to soak the bean seed in water for 5 min, then air-dry them for 45 min, submerge the seeds again, and discard the seeds that floated (Kang et al., 2002).

Detection of specific fungus from the seed surface or of soybean sprouts in the presence of other microbes is difficult. Classical methods, such as isolation on selective media, are useful but subject to limitations, ie., many pathogens are masked by growth of faster growing fungi (Meyer et al., 2000). Therefore, rapid, reliable and accurate diagnostic tools are required to detect and identify causal organism.

Clark (1981) emphasized the potential of enzyme-linked immunosorbent assays for detecting and identifying plant pathogen in much lower concentrations than is possible by classical methods. Majority of assays and experiments have been with plant viruses, but same technique has been attempted to detect bacterial and fungal plant pathogen.

Sundaram et al. (1991) reported that polyclonal Ab based indirect ELISA, prepared against purified mycelial proteins from *Verticillium dahliae*, reacted positively with 11 of 12 isolates from potato, cotton, and soil; but negatively with one isolate from tomato and also with non-target pathogens: *Fusarium oxysporum, Colletotrichum lindemuthianum, Rhizoctonia solani, V. nigrescens* and *V. tricorpus*. Their antiserum reacted strongly with *V. dahliae* and intensely with *V. albo-atrum*.

Velicheti et al. (1993), using polyclonal Ab based DAS-ELISA, attempted to develop a method for early detection of *Phomopsis phaseoli* and *Phomopsis longicolla* of soybean. Unfortunately, their assay system yielded cross-reaction with all *Phomopsis* sp. and *Colletotrichum truncatum*, although it discriminated *Phytophthora sojae, Rhizopus* sp., *Rhizoctonia solani, Septoria glycines*, and *Cercospora kikuchii*.

As an alternative, polymerase chain reactions (PCR) have been developed by using specific primer to pathogens (Singh et al., 2000). The PCR assay is proved to be a highly sensitive method for detecting very low titers of pathogens. But enzyme-linked immunosorbent assay (ELISA) is often preferred over PCR because a specific primer should be developed for PCR test, and there are technical difficulties encountered in screening the DNA from conidia, which is target biomass in the ecological niche. Use of PCR also is

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time-consuming when testing a large number of samples (Somai et al., 2002).

ELISA method is simple to operate, specific, and highly sensitive to detection and identification of target pathogen.

In this study, we developed two polyclonal antibody-based ELISA system to detect conidia of the soybean sprout pathogen.

Materials and Methods

Antigen preparation. Anthracnose pathogen was isolated from Cheongju, Cheongyang, Daejeon, Gyeongju, Iksan, Jinju, Pohang and Sacheon. Sample slices were placed on water agar and incubated for 3 days at 27°C. The typical colonies from the samples were transferred into potato dextrose agar (24 g/L, PDA, Difco) for further studies. Other fungal and bacterial isolates were also used for screening the cross-reactivity of PAb.

Mycelial colony on the PDA plate was cultured at 27°C for 5 days under the dark condition. Large quantities of mass-produced conidia on PDA were collected by washing the colony surface on PDA plate with 2 ml of ultra pure water. Then this was filtered with cheesecloth. The spore suspension was centrifuged for 5 min at 8,000 rpm, and resuspended to about 1×10^6 conidia/ml in physiological salt solution (0.9% NaCl). The suspension, used as the immunogen, was aliquot and stored at -20°C until use.

Polyclonal antibody production. The spore suspension (300 μL) prepared following the steps above, was mixed with an equal volume of Freund's Complete Adjuvant (Sigma F-5881) and emulsified by 3-WAY STOPCOCK. The antigen-adjuvant mixture was injected into the ascites of two female (6-weeks-old) BALB/C mice (200 μL/mouse). The first injection was followed by two booster injections at a three-week interval. For the second injection, spore suspension was mixed with Incomplete Adjuvant (Gibco cat. 15720-030). The third injection was done with equal volume mixture of suspension and physiological salt solution (0.9% NaCl).

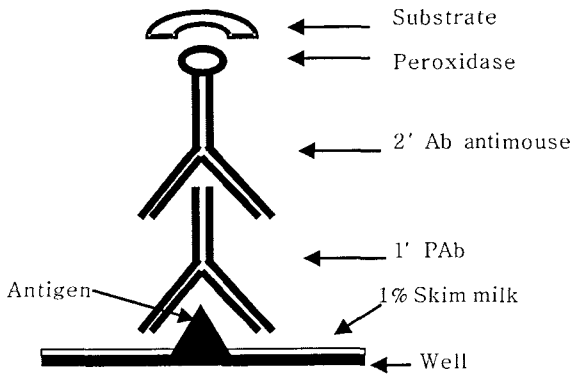

Fig. 1. Scheme of indirect ELISA.

For serum preparation. Blood sample was collected in E-tube from mouse tail at 2 days after the third injection, which was exposed to room temperature for 5 min. Anti-serum was prepared by centrifugation at 3,000 rpm for 10 min and subjected to titration by ELISA.

ELISA Method. Indirect ELISA was performed for screening of antisera. Aliquots of conidial suspension were dissolved in carbonate coating buffer (10^7 spore ml^{-1}), dispensed 50 μL per well, and kept at 4°C overnight. Thus coated wells were washed three times with PBST (0.8% NaCl, 0.02% KCl, 0.115% Na_2HPO_4, 0.02% KH_2PO_4, 0.05 % Tween 20, pH 7.4), and the uncoated well surface blocked with 1% skim milk for 30 min at room temperature. After further washing for three times with PBST, well plate was incubated at 37°C for 1 h with 50 μL antisera, appropriately diluted in 1% BSA per well. Washing three times with PBST again and then incubating at 37°C for 1 h with secondary antibody were done (Sigma A0412, goat Anti-mouse Polyvalent IgG, IgA, IgM, conjugated with peroxidase diluted to 1:10,000 in 1% BSA).

After final washing for five times, 50 μL substrate solution(o-phenylenediamine 4 mg/5 ml; PCB 0.1 M phosphate-citrate, 10

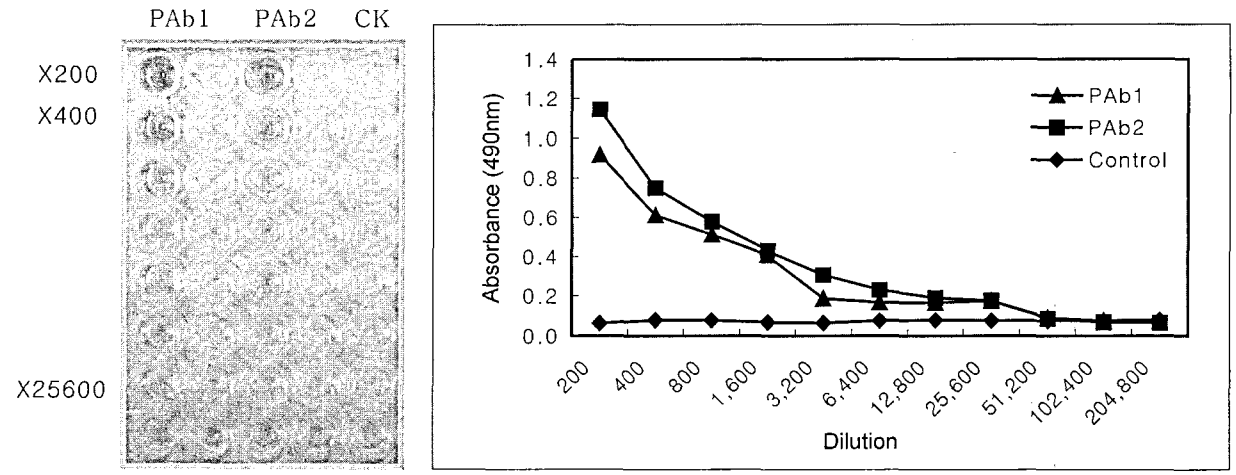

Fig. 2. Titer of polyclonal antibody against *Colletotrichum gloeosporioides*. Two-fold serial dilutions from x200 to x204, 800 are designated at left column of well plate. CK, negative control denotes the wells which were not treated with primary antibody during ELISA procedure.

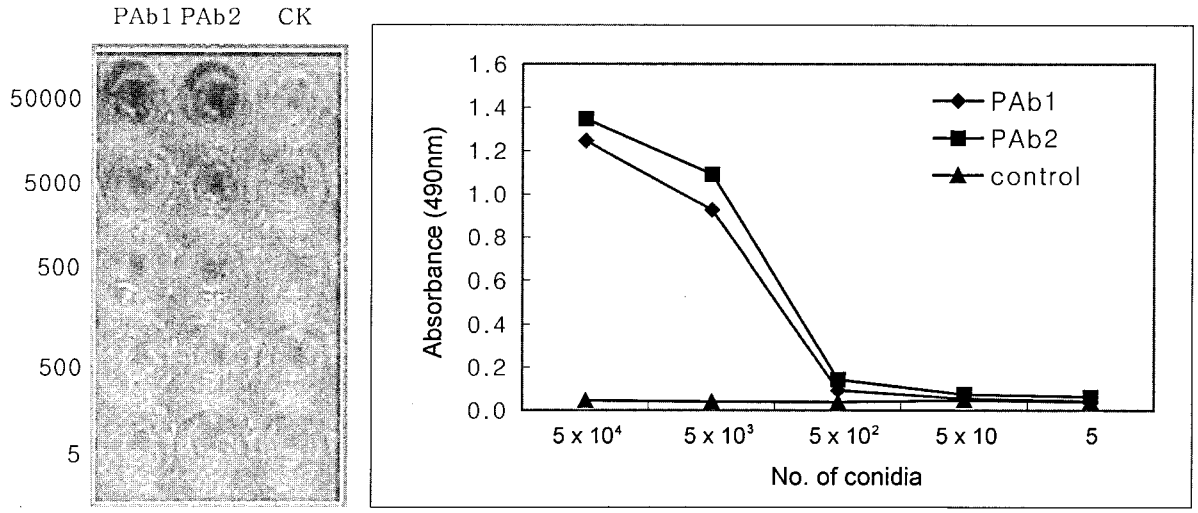

Fig. 3. Sensitivity of PAb1&2 to the threshold concentration of conidia of *Colletotrichum gloeosporioides* by indirect ELISA. Plates were coated with conidia at designated concentrations adjusted by tenfold serial dilutions and primary antiserum diluted to 1:200. Arrow indicates the absorbance value of 0.15 or higher corresponding to conidia level which is visible to naked eye. CK, refer to the footnote of Fig. 2, for negative control.

μL H_2O_2) was added per well, and incubated for 15 min. at ambient temperature. The reaction was stopped by adding 50 μL of 2 N H_2SO_4, followed by OD reading in ELISA reader (BIO-RAD, Model 550) at 490 nm (Fig. 1).

Titer of antisera was determined by adding 50 μL antisera of two-fold serial dilutions from 200 to 204, 800 in 1% BSA as the method described by Lee et al. (2004) (Fig. 2).

Sensitivity of PAb was also determined: the plates were coated with conidia, serial ten-fold dilutions from 50,000 to 5 conidia/well, and each well received 50 μL PAb (1:200 dilution) (Fig. 3).

Antigen determination. Antigen fractions were prepared as follows: An aliquot of suspension at 10^6 conidia/ml was dispensed to an E-tube, and briefly centrifuged for 30 sec. in Beckman microcentrifuge. Supernatant was dispensed to 3rd and 4th column, the pellet was resuspended in double distilled water by vortexing, and then transferred to 1st and 2nd columns for the top row of Fig. 4 (1x washing). For the second row from the top, supernatant from two washings was dispensed to 3rd and 4th columns, correspondingly, and the pellet was resuspended and transferred to 1st and 2nd columns; same set of fraction was laid out to 2 more times of washing (3x, 4x) as shown in Fig. 4. Sample fractions as mentioned above were coated to well-plate and subsequently introduced to PAb1 and PAb2, then visualized in indirect ELISA (Fig. 4).

Specificity test of PAb for *Colletotrichum gloeosporioides*. Specificity of PAb for *Colletotrichum gloeosporioides* from bean sprout against other isolates was also tested. The ELISA plates, prepared with PAb as mentioned above, were tested for reactivity to isolates of *Colletotrichum gloeosporioides*, as well as other pathogens and bacteria isolated from soybean sprouts.

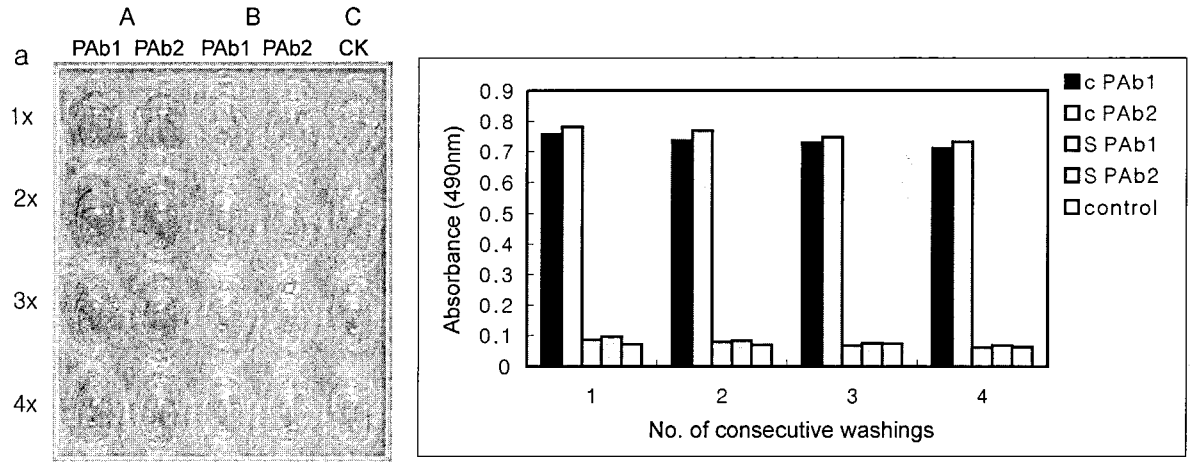

Fig. 4. Determination of antigen epitope to polyclonal antibody. A, washed conidia(c) vs. PAb1 and PAb2 (1st & 2nd column, respectively) B, surface washing(s) vs. PAb1 and PAb2 (3rd & 4th column, respectively) C, refers to the footnote of Fig. 2. for negative control. a, no. of consecutive washing.

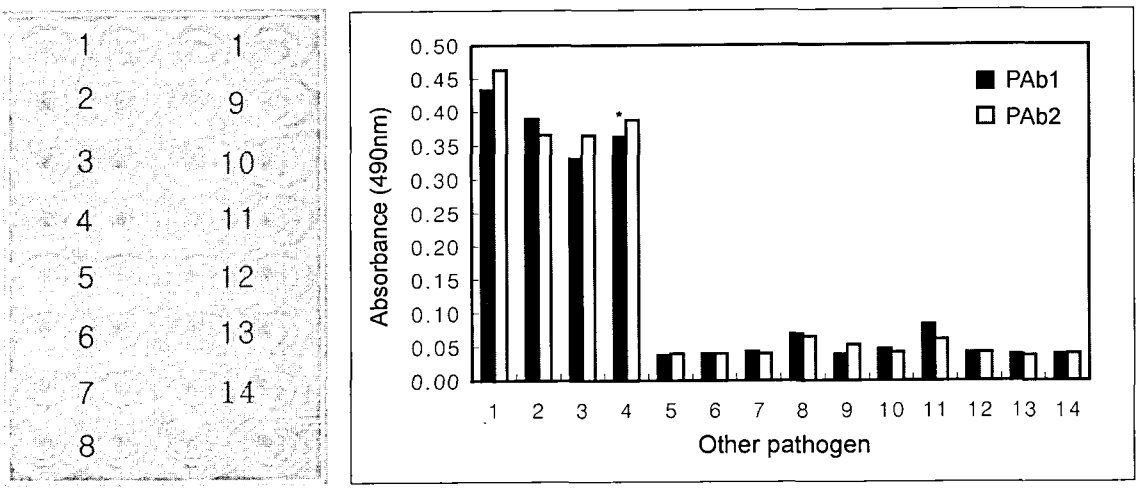

Fig. 5. Test of polyclonal antibody PAb1 and PAb2 for cross-reactivity with other pathogenic isolates. Plates were coated with fungal homogenates (50 μl per well), and antiserum was diluted 1:1,000. 1, *Colletotrichum gloeosporioides*; 2, *Colletotrichum acutatum* (mungbean sprout); 3, *Glomerella cingulata* (apple); 4, *Colletotrichum gloeosporioides*; 5, *Fusarium oxysporum*; 6, *Pythium ultimum*; 7, *Rhizoctonia solani*; 8, *Sclerotinia sclerotiorum*; 9, *Phytophthora capsici*; 10, *Embellesia allii*; 11, *Cladosporium cucumerinum*; 12, *Kluyvera ascorbata*; 13, *Sphingobacterium thalpophilum*; 14, negative control without fungal homogenates.
*; indicates the strain was reisolated from soybean sprout which was inoculated previously with *C. gloeosporioides*

Antigen characterization. To characterize the *Colletotrichum gloeosporioides* antigen, conidia immobilized in micro titer wells (50 μL) were incubated either with pronase E, protease and trypsin and cellulase at 37°C from 1 to 5 min at 1 min interval to make sure of any reaction to occur, and washed three times with PBST. Washed wells were blocked with 1% skim milk. Control wells received only PBST without enzyme but were otherwise treated similarly. The influence of those treatments was monitored by using the ELISA scheme (Fig. 5).

Heat treatments were performed to determine thermostability of the *C. gloeosporioides* antigen. Conidia were placed in microcentrifuge tubes, boiled in a water bath for 5 to 35 min, and cooled to 25°C. Both samples were coated in micro titer plate and reactivity with PAb1, PAb2 was determined by indirect ELISA (Fig. 6).

Results

Titer of Polyclonal Antibody against *Colletotrichum gloeosporioides*. For producing PAb1, PAb2 against *Colletotrichum gloeosporioides*, we used conidia as an immunogen. After immunization, the titer of mouse antisera against *Colletotrichum gloeosporioides*, determined by

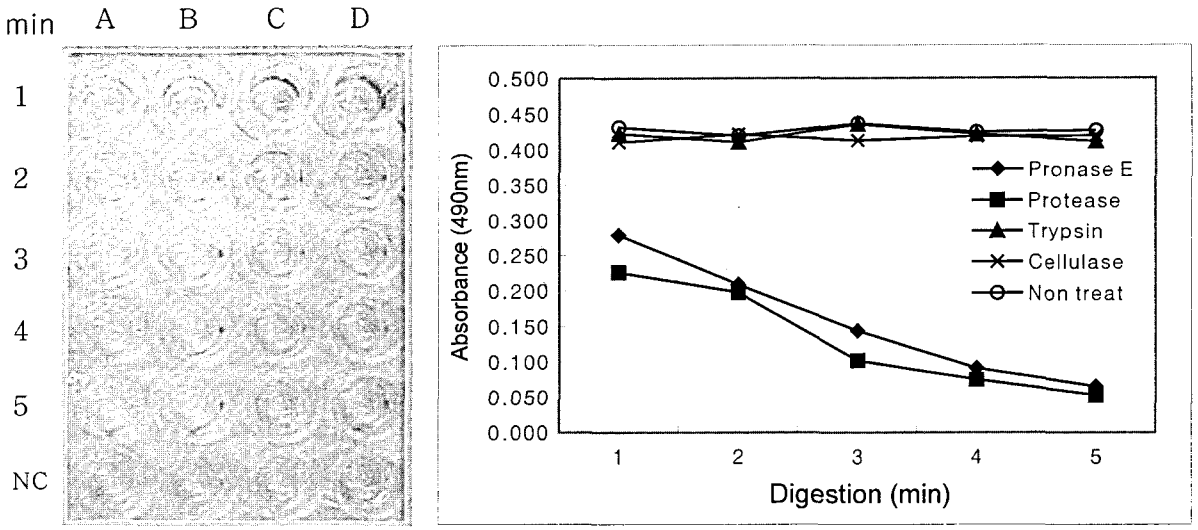

Fig. 6. Effect of enzyme treatment on binding of the PAb to immobilize conidia of *Colletotrichum gloeosporioides*. A, pronase E; B, protease; C, trypsin; D, cellulase; NC, non-treated.

indirect ELISA, was high enough to be detectable up to x25,600 dilutions (Fig. 2).

Sensitivity of PAb. Serums produced from the two mice were harvested. Both PAb1 and PAb2 had the highest level of reactivity to *Colletotrichum gloeosporioides*. Absorbance readings exceeded 0.15; sensitivity of PAb was precise enough to detect spore concentration to as low as 500 conidia/well by indirect ELISA (Fig. 3).

Determination of antigen epitope to Polyclonal Antibody. Conidia washing supernatant vs. washed conidia were coated to plate and subsequently introduced to PAb1 and PAb2, then visualized in indirect ELISA. We found interesting results that both antibody recognized only the conidial surface, not the surface washouts, i.e., supernatant. It was remarkable that absorbance value was not reduced even after 4 consecutive washings (Fig. 4), suggesting that antigenic determinants are being attached to conidia.

Specificity of PAb. The PAbs were tested against isolates of *Colletotrichum gloeosporioides*. Absorbance values exceeded 0.35 with distinct visibility, but no visible reaction was detected with other isolates, except for two other anthracnose strains: *Colletotrichum acutatum* (mungbean sprouts strain) and *Glomerella cingulata* (apple strain) and also that of reisolated strain of soybean sprout rot (Fig. 5).

Effect of enzyme treatment on binding of Polyclonal Antibody. The epitopes recognized by these PAbs were characterized by enzyme and boiling treatment. When the antigens were treated by pronase E and protease, the absorbance values for PAb1, 2 were reduced. Trypsin and cellulase treatment did not result in reduction of absorbance value (Fig. 6). Conidia antigens of *C. gloeosporioides* recognized by the PAb1, 2 were heat labile. There was a significant reduction in absorbance value, as determined by indirect ELISA, when conidia suspension was boiled for 5 to 35 min (Fig. 7).

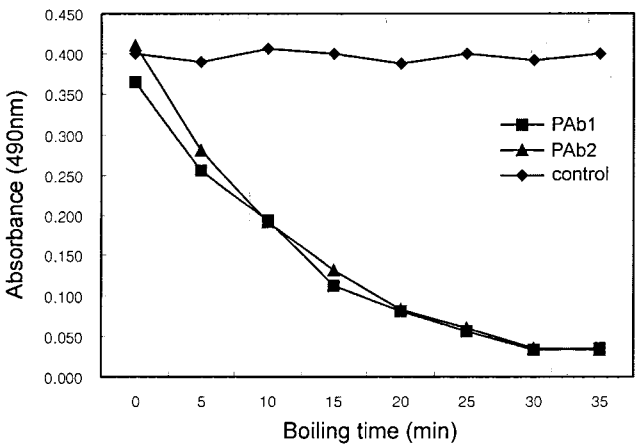

Fig. 7. Effect of boiling on binding of the PAb1 and PAb2 to immobilize conidia of *Colletotrichum gloeosporioides*. Plate were coated conidia 10^6/well and antiserum was diluted to 1:2,000.

Discussion

Inspite of huge annual market value of soybean sprouts in Korea, mass production system is not fully established. It is currently produced under conditions favorable for pathogen contamination and proliferation. Therefore, there have been public concerns about safety of soybean sprouts in the market.

It is most likely that most inoculum resources for soybean sprout rot have originated from seedlot. Therefore, it would be highly desirable to detect and disinfect the pathogen inocula at the time of seed soaking.

Unfortunately, it is very difficult to monitor inocula accurately and rapidly on soybean, before onset of anthracnose by plant clinical methods so far available. One of the approaches to achieve this goal would be to develop polyclonal antibody based ELISA kit that can specifically detect inoculum prior to infection and discriminate other epiphytic microbes on soybean sprouts. In order to determine appropriate antigens for their specific antigenicity, mice were immunized with conidia. The antiserum raised against conidia should be promising for its low cross reactivity as well as the conidia being major sources of primary inoculums of soybean sprouts anthracnose.

Both PAb1 and PAb2, developed in this study, recognized the conidia of anthracnose fungi only (Fig. 4), discriminating others associated with soybean sprouts (Fig. 5). This suggested that these PAbs could be used to develop early diagnostic kits.

It is interesting to note that mucous pinkish mass on the surface of conidia of *C. gloeosporioides* soybean sprouts strain was retained after 4 consecutive washings, compared to that of persimmon strain, which was easily washed off (Lee, J. H. personal communication).

Generally, the threshold level of conidial concentration to penetrate and infect soybean sprouts should be higher than 10^3 spores per ml (Jung and Kim, 2004). The sensitivity of PAb was determined by ELISA system by coating serial dilution of conidia concentrations. Absorbance value at 490 nm was 0.15 or higher at 500 conidia, which is distinctive and sensitive enough to observe color reaction with one's naked eye, without relying on ELISA reader, compared to 0.05 at negative control.

Specificity of our ELISA system was high enough to discriminate many other fungal pathogens. Unfortunately, our assay system yielded cross-reaction with anthracnose fungi of apple and pepper (Fig. 5) as experienced by Velicheti et al. (1993) for *Phomopsis* spp. in symptomless soybean plant.

The fact the our assay system is cross-reactive to other anthracnose fungi at practical level may not be disadvantage-

ous at all, because our ELISA system should also be feasible to detect *C. acutatum* (Mungbean sprouts rot) and *G. cingulata* (*C. gloeosporioides*), (apple, pepper).

Moreover, nomenclature of many anthracnose fungi tends to be integrated to *C. gloeosporioides*. Nonetheless, it is likely that persimmon strains, formerly *Gloeosporium kaki* and *C. gloeosporioides,* are still different from each other despite some similarity in antigenic determinant (Lee et al., 2004). There should be some characteristics involved in such diversity other than morphology and genetic similarity as mentioned earlier. Nevertheless our ELISA system might also be feasible to detect anthracnose inocula for the above host plants.

PAb1 and PAb2 antigens were characterized by heating and enzyme treatment. When conidial suspension was treated with trypsin, protease, pronase E, and cellulase, it was resistant to trypsin and cellulase; but treatment of protease, pronase E, and heat resulted in reduction of absorbance value as a function of time. This is the evidence that PAb1 and PAb2 bind to protein epitope that does not contain residue of amino acid, arginine and lysine, even though more work needs to be done.It is comparable to our previous result that antigen epitope for persimmon strain was partially resistant to trypsin (Lee et al., 2004).

In conclusion, PAb1 and PAb2 are proven to be very sensitive and highly specific to the target pathogen, *Colletotrichum gloeosporioides*, apparently discriminating other unrelated pathogen, or epiphytes. This kit fulfills the requirements for detecting inoculums before infection and onset of anthracnose, which would provide us with highly accurate information compared with PCR method or classical identification method (Singh et al., 2000).

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