MPMI Vol. 8, No. 6, 1995, pp. 899–905.

Purification and Characterization of an Acidic β-1,3-Glucanase from Cucumber and its Relationship to Systemic Disease Resistance Induced by *Colletotrichum lagenarium* and Tobacco Necrosis Virus

Cheng Ji and Joseph Kuc

Department of Plant Pathology, University of Kentucky, Lexington, KY 40546 U.S.A.
Received 26 April 1995. Accepted 14 July 1995.

An acidic β-1,3-glucanase was detected in cucumber leaves inoculated with either *Colletotrichum lagenarium* or tobacco necrosis virus (TNV) as well as in the leaves above those inoculated with the pathogens. The enzyme is extracellular and migrates in native polyacrylamide gel electrophoresis (PAGE) together with a Class III chitinase, a bifunctional chitinase/lysozyme. The β-1,3-glucanase was separated by ultra-narrow pH range IEF-PAGE or by SDS-PAGE and was purified to apparent homogeneity. Only one isoform of the enzyme was detected. Its apparent molecular mass is 38 kDa as estimated by SDS-PAGE, its isoelectric point is 3.6 and the specific activity is approximately 26 μmol glucose equivalents liberated from laminarin min^{-1} mg^{-1} protein. Partial amino acid (five peptide fragments with a total of 65 amino acids) sequencing of the β-1,3-glucanase revealed similarities of 49% to 72% to sequences of published β-1,3-glucanases from tobacco, tomato, soybean, barley, and rice plants. A time course study indicated that the increase of the β-1,3-glucanase activity was associated with induced resistance against *C. lagenarium*. The implications of these results to coordinate defense responses in plant-microbe interactions are discussed.

β-1,3-Glucanases and chitinases have been suggested to have a role in plant defense against diseases caused by fungi because their substrates, β-1,3-glucan and chitin, are major components of the cell walls of many fungi (Wessels and Sietsma 1981). The enzymes may also participate in plant defense by releasing glucan fragments (elicitors), from fungal or plant cell walls, that elicit phytoalexin accumulation in the plant (Takeuchi et al. 1990). In many higher plants, β-1,3-glucanases and chitinases accumulate coordinately following infection (Kauffmann et al. 1987; Ferraris et al. 1987; Joosten and De Wit 1989; Meins 1989), treatment with elicitors (Kombrink et al. 1988), and in response to the plant stress hormone ethylene (Mauch and Staehelin 1989). β-1,3-Glucanases and chitinases can function synergistically in degrading fungal cell walls in vitro (Mauch et al. 1988). However, the significance of this synergy in vivo with different plant-microbe systems is unclear.

Corresponding author: J. Kuc.

Cucumber provides a good model system for studying disease resistance and induced disease resistance (Kuc 1982). Local and systemic increases in chitinase and peroxidase activity induced by a necrotizing fungus, *Colletotrichum lagenarium,* tobacco necrosis virus (TNV), fungal cell wall fragments and dibasic sodium phosphate have been reported (Jenns and Kuc 1979, Boller 1988, Metraux and Boller 1986, 1988; Boller 1988; Irving and Kuc 1990). Though the relationship between chitinase activity and disease resistance in cucumber against *C. lagenarium* has been extensively investigated, there is relatively little information about the contribution of β-1,3-glucanase. Increased β-1,3-glucanase activity was reported in muskmelon foliage after infection with *C. lagenarium* (Dargent and Touze 1974); however, the increased activity was caused by an endoenzyme produced by *C. lagenarium* (Rabenantoandro et al. 1976). It is not clear from the literature whether β-1,3-glucanase is inducible and whether it functions as a defense compound against *C. lagenarium* in cucumber plants.

In this study, we present evidence for β-1,3-glucanase activity in cucumber leaves inoculated with *C. lagenarium* or TNV and in the leaves above those inoculated with the pathogens. A time course study indicated that enhanced β-1,3-glucanase activity coincided with induced systemic disease resistance. The β-1,3-glucanase has been purified and characterized in terms of isoform pattern, apparent molecular weight, pI, and amino acid sequence similarities to sequences of published β-1,3-glucanases from other plant species.

RESULTS

Induction and detection.

The β-1,3-glucanase activity in crude extracts from healthy, inoculated leaves and the leaves above the inoculated leaves was determined colorimetrically or by activity assays on native gels after electrophoreses (see Materials and Methods). β-1,3-Glucanase activity was detected in both inoculated leaves and the leaves above 7 days after inoculating foliage with *C.*

lagenarium or TNV (Fig. 1). The activity was lost after boiling the crude extracts for 10 min (Fig. 1A, B). To determine the localization of the enzyme, the intercellular wash fluid (ICF) was collected from both infected leaves and the leaves above infected leaves. β-1,3-Glucanase activity was detected in ICF and not in extracts of leaves after removal of ICF (Fig. 2), indicating that the enzyme is principally extracellular. A band with β-1,3-glucanase activity was detected on native gels (Figs. 1A, 2). After broad pH range isoelectric focusing electrophoresis (IEF), only one band with β-1,3-glucanase activity (acidic) was detected in cucumber leaf extracts (Fig. 3, lane 2), whereas several bands with β-1,3-glucanase activity (both acidic and basic) were detected in extracts (positive control, Pan et al. 1989) from tobacco leaves inoculated with *Peronospora tabacina* (Fig. 3, lane 1). To investigate whether the increase of β-1,3-glucanase activity was associated with induced resistance, a time course study was conducted. β-1,3-Glucanase activity in leaf 2 above inoculated leaf 1 was inversely related to the leaf area covered with lesions after challenge inoculation of leaf 2 (Fig. 4).

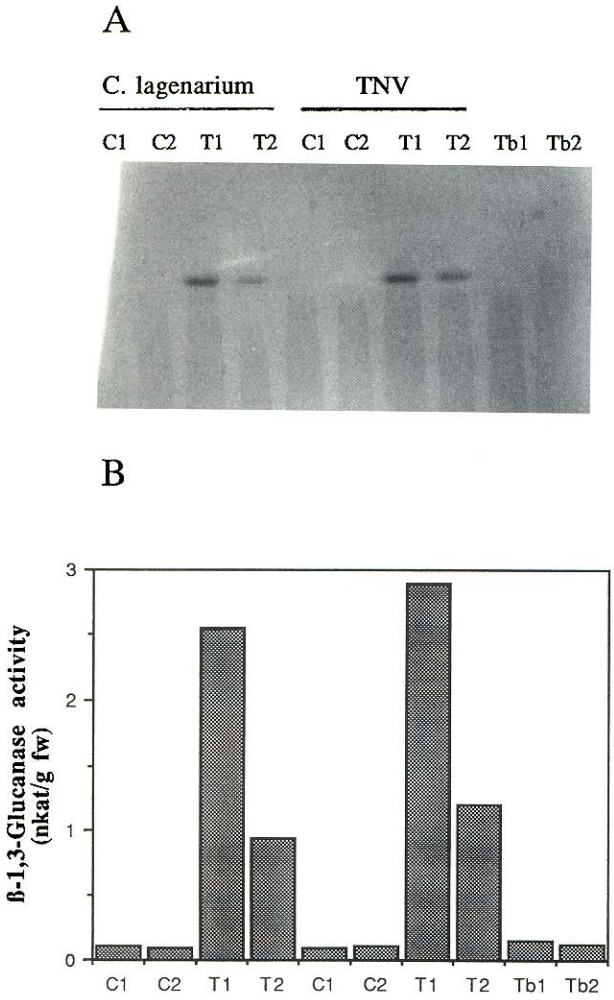

Fig. 1. Detection of β-1,3-glucanase activity in cucumber leaves 7 days after inoculation with either *Colletotrichum lagenarium* or tobacco necrosis virus (TNV). **A,** β-1,3-Glucanase activity was detected on a native polyacrylamide gel (PAGE). Crude extract (80 mg of total protein) was applied per lane; **B,** β-1,3-glucanase activity was determined colorimetrically by the standard laminarin-dinitrosalicylate method. Samples: C1 and C2, from control leaves (leaf 1 for C1 and leaf 2 for C2); T1, from leaves (leaf 1) inoculated with either *C. lagenarium* or TNV; T2, from leaves (leaf 2) above the inoculated leaves (induced leaves); Tb1 and Tb2, the same as T1 and T2 but the samples were boiled for 10 min before loading.

Purification of the β-1,3-glucanase.

Protein bands stained with Coomassie brilliant blue and a band with β-1,3-glucanase activity were compared to locate the position of β-1,3-glucanase on native gels. The band with β-1,3-glucanase activity (Fig. 5) appeared the same as the band for the bifunctional (Class III) chitinase/lysozyme reported by Metraux et al. (1989). Two possibilities were considered at this stage. One possibility was that the β-1,3-glucanase activity might be contributed by the bifunctional chitinase/lysozyme; the other was that the cucumber β-1,3-glucanase and chitinase might comigrate in native gels. Two

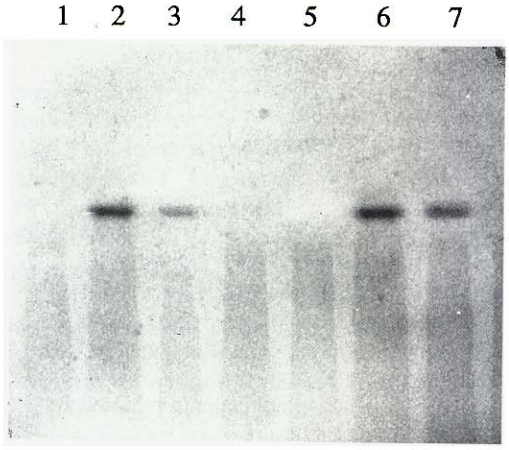

Fig. 2. Localization of β-1,3-glucanase activity in cucumber leaves. Enzyme activity was analyzed on a native polyacrylamide gel. Samples from: 1, control leaf 1; 2, leaf 1 inoculated with TNV; 3, leaf 2 above inoculated leaf 1; 4, inoculated leaf 1 after removing intercellular wash fluid (ICF); 5, leaf 2 above inoculated leaf 1 after removing ICF; 6, ICF of inoculated leaf 1; 7, ICF of leaf 2 above inoculated leaf 1.

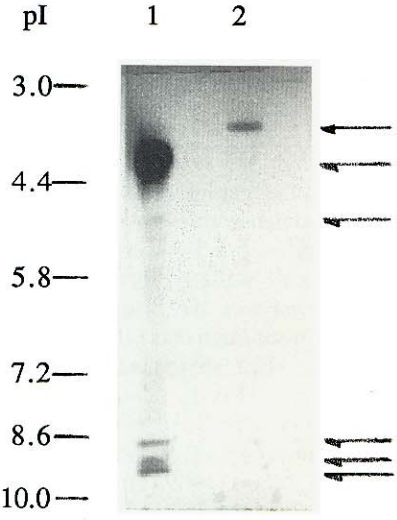

Fig. 3. Isozyme pattern of β-1,3-glucanase activity on an isoelectric focusing (IEF) polyacrylamide gel. 1, Positive control, extract of tobacco leaves that were infected with *Peronospora tabacina*; 2, extract of cucumber leaves inoculated with TNV. One hundred milligrams of total protein was applied per lane. Arrows indicate bands with activity.

experiments were conducted to determine which, if either, of the two possibilities was valid. In the first experiment, IEF-PAGE with an ultra-narrow pH range (pH 3 to 4.5) indicated that the band with β-1,3-glucanase activity could be separated from that with chitinase activity (Fig. 6). The protein band on strip A corresponding to the β-1,3-glucanase activity (strip C) did not have chitinase activity (strip B), indicating that the β-1,3-glucanase and the chitinase are different proteins. In the second experiment, the protein band detected on native gels (Fig. 5) was cut from the gel and eluted with an elution buffer (Promega). The eluted proteins were analyzed by native-PAGE as well as SDS-PAGE (Fig. 7). Confirming the result of the first experiment, there were several protein bands on the SDS gel (Fig. 7B, lane 4) while only one protein band was evident on the native gel (Fig. 7A, lane 4). The dominant protein (Fig. 7B, lane 4) was further purified (Fig. 7B, lane 5 and Fig. 7C, lane 2) and sequenced. The sequence of the N-terminal 29 amino acids was: Ala-Gly-Ile-Ala-Ile-Tyr-Trp-Gly-Gln-Asn-Gly-Asn-Glu-Gly-Ser-Leu-Ala-Ser-Thr-x-Ala-Thr-Gly-Asn-Tyr-Glu-Phe-Val-Glu. With the exception of one amino acid (no. 20, cysteine, was not detected), the sequence is identical to that of the N-terminal sequence of the bifunctional chitinase/lysozyme (Metraux et al. 1987). Therefore, the inducible β-1,3-glucanase might be one of the other protein bands. Since it was impossible to conduct enzyme activity assays to locate the β-1,3-glucanase protein on SDS gel, the β-1,3-glucanase protein was further purified (Fig. 7B, lane 6 and Fig. 7C, lane 3) by cutting the band from native IEF gel corresponding to β-1,3-glucanase activity. The molecular mass of the β-1,3-glucanase was about 38 kDa (Fig. 7B) and its pI was about 3.6 (Fig. 7C). Its specific activity was calculated as 26.4 μmol glucose equivalents released min^{-1} mg^{-1} protein with laminarin as substrate (Table 1).

Amino acid sequencing and homology analysis.

The N-terminal amino acids of the purified β-1,3-glucanase could not be sequenced, because the N-terminal was blocked. The purified protein was digested by an asparagine/aspartic acid specific endoproteinase and the resulting peptides were separated by reverse-phase HPLC. Four peptide fractions (Fractions 50, 65, 68, and 73) were selected to be sequenced. Table 2 shows the sequences. Fraction 68´ is a peptide coe-

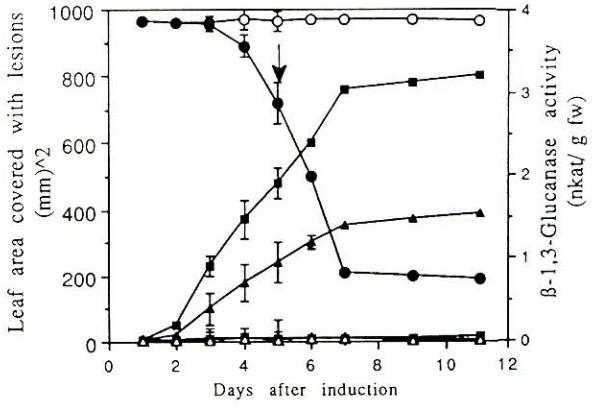

Fig. 4. Correlation of β-1,3-glucanase activity and induced resistance to *Colletotrichum lagenarium* in leaf 2 at intervals after inoculating leaf 1 with TNV. Two sets of plants were used for this experiment. For one set, leaf 2 of TNV-induced (●) or control (○) plants was challenged with *C. lagenarium* at 1-day intervals after inoculation of leaf 1 with TNV or treatment of leaf 1 with water. The leaf area covered with lesions was measured 7 days after challenge. For the other set of plants, the TNV-inoculated leaf 1 (■), leaf 2 above the TNV- inoculated leaf (▲) and leaf 1 (□) and leaf 2 (Δ) from uninfected plants were sampled at the same time intervals as above and the enzyme activities were assayed. The arrow indicates the time after induction when induced systemic resistance was consistently evident.

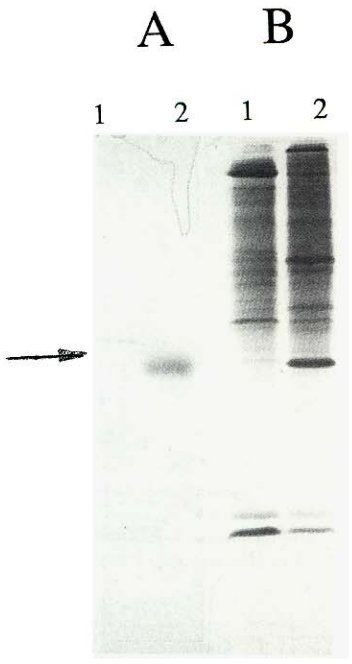

Fig. 5. Direct analysis of β-1,3-glucanase activity on a native polyacrylamide gel. Two identical gel strips were cut from the original gel after electrophoresis. On one strip (A) enzyme activity was detected. The other strip (B) was stained with Coomassie Brilliant Blue. Samples from: 1, uninoculated leaves; 2, TNV-inoculated leaves.

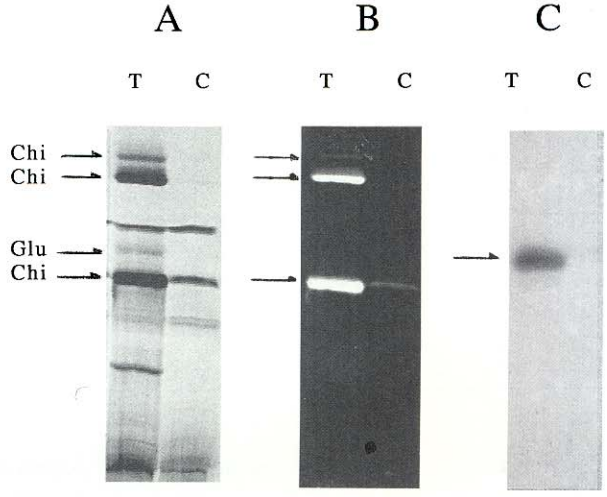

Fig. 6. Separation of β-1,3-glucanase from the bifunctional Class III chitinase/lysozyme by ultra-narrow pH (3 to 4.5) range IEF. A whole gel was cut into three identical strips after electrophoresis. One strip (A) was stained with Coomassie Brilliant Blue, a second (B) was used to determine chitinase activity (Chi) and the third (C) was used to detect β-1,3-glucanase activity (Glu). The functions for protein bands on A were identified by matching A with B and C. Samples: C, from uninoculated leaves; T, from TNV-inoculated leaves. Strip B was observed under UV radiation.

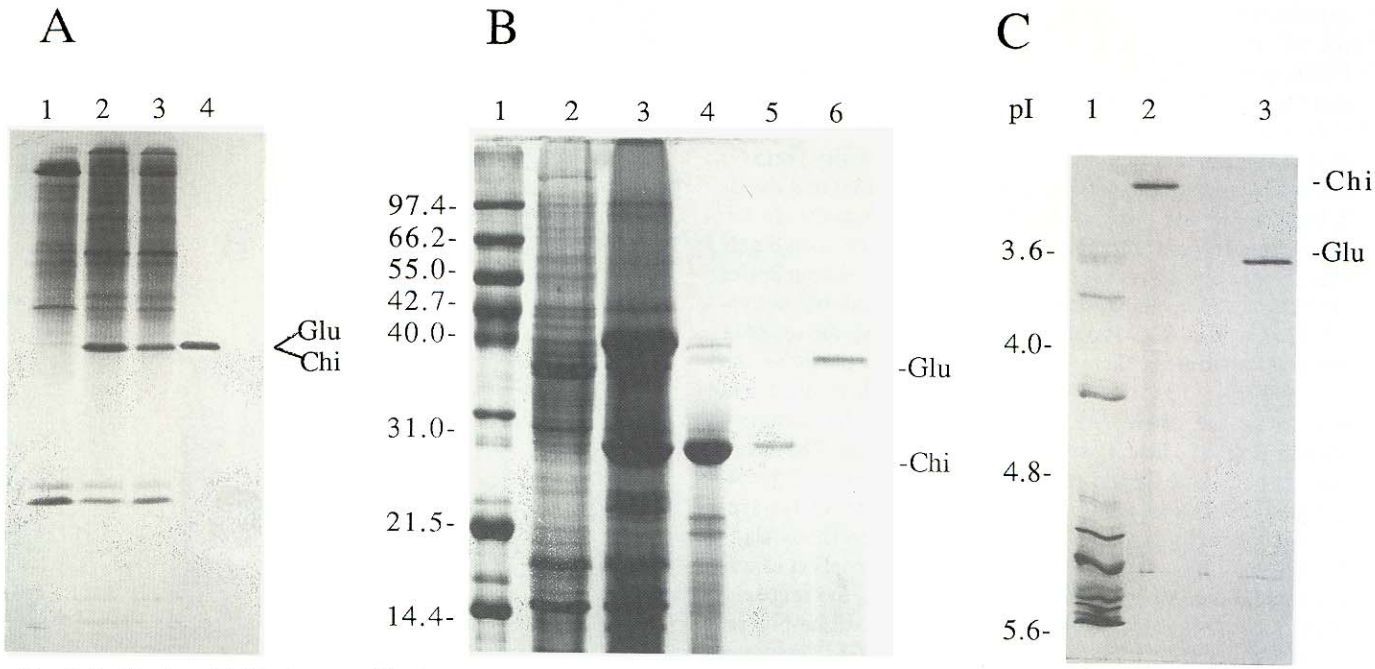

Fig. 7. Purification of β-1,3-glucanase. The band on a native gel corresponding to β-1,3-glucanase activity was cut out and protein(s) in the gel were eluted with an elution buffer (Promega). **A,** Native PAGE (15%); samples from: 1, uninoculated leaves; 2, TNV-inoculated leaves; 3, leaves above inoculated leaves; 4, the eluted proteins; **B,** SDS-PAGE (12.5%); 1, marker (Promega) for molecular weight; 2, TNV-inoculated leaves; 3, leaves above inoculated leaves; 4, the eluted proteins; 5, further purified Class III chitinase (Chi); 6, further purified β-1,3-glucanase (Glu); **C,** IEF-PAGE (7%); 1, pI marker (Sigma); 2, purified Class III chitinase; 3, purified β-1,3-glucanase. Gels were stained with Coomasie Brilliant Blue.

Table 1. Purification of β-1,3-glucanase from cucumber leaves inoculated with tobacco necrosis virus[a]

	Protein (mg)	Activity (nkat)	Specific activity (nkat/mg protein)	Recovery (%)
Crude extract	397	159	0.4	100
Electrophoreses				
Native-PAGE	6.1	57	9.4	32
IEF-PAGE	0.068	15.4	226	9.1
IEF-PAGE	0.007	3.1	440	1.8

[a] The starting material (53 g fresh weight) consisted of 26-day-old cucumber leaves 6 days after inoculation with TNV.

Table 2. Partial amino acid sequences of the cucumber β-1,3-glucanase

Fraction	50:	Asp-Asn-Ala-Asn-Ala-Trp-Ile-Gln
Fraction	65:	Asp-Glu-Asn-Leu-Lys-Phe-Ala-Pro-Pro-Glu-Val-Glu-Arg-His-Trp-Gly-Leu-Phe-Ser-Pro-Asn-Lys
Fraction	68:	Asp-Asn-Gly-Arg-Thr-Tyr-Asn-Asn-Leu-Ile-Gln-His-Val-Lys-Gln-Gly-Thr
	68′:	Asp-Tyr-Ala-Ile-Phe-Thr-Gly-The-Ser
Fraction	73:	Asp-Thr-Val-Tyr-Ala-Ala-Leu-Glu-Lys-Gly

luted with fraction 68. Figure 8 demonstrates that sequences of these five peptides (in total 65 amino acids) had 56% similarity to that of tobacco acidic β-1,3-glucanase PR-2b/a and 61% to that of tobacco vascular basic β-1,3-glucanase (Linthorst et al. 1990); 50% to that of tomato acidic β-1,3-glucanase (van Kan et al. 1992); 72% to that of soybean ethylene-inducible β-1,3-glucanase (Takeuchi et al. 1990); 51% to that of barley β-1,3-glucanase (Wang and Fincher 1992); and 49% to that of rice β-glucanase (Simmons 1991).

DISCUSSION

Several putative defense compounds have been suggested to be involved in resistance and induced resistance in cucumber plants against diseases caused by fungi, bacteria, and viruses. Associations of increased peroxidase and chitinase activities with induced resistance in cucumber are well documented (Hammerschmidt et al. 1982; Metraux et al. 1986). It is not apparent, however, that these enzymes or any other reported putative defense compound alone have a determinant role in resistance. Resistance is likely to be multicomponent (Dixon and Harrison 1990; Kuc 1990). Coordinate expressions and interactions between multiple putative defense compounds in plants during infection are likely to be critical for resistance. The characterization of the inducible cucumber β-1,3-glucanase and its association with induced disease resistance in this study provide supporting evidence for this point of view.

It is unlikely that the β-1,3-glucanase was produced by the fungus since it was increased in TNV-inoculated leaves as well as in the leaves above (Fig. 1). The bifunctional chitinase/lysozyme and β-1,3-glucanase comigrate together under nondenaturing conditions and can be separated by isoelectric points (Fig. 7C). One possibility is that the charge-to-mass ratio of the two proteins are coincidentally similar since the β-1,3-glucanase is larger and has a higher charge than the chitinase. Another possibility is that, since both enzymes are principally intercellular and tend to aggregate together under nondenaturing conditions (Fig. 7A), the two hydrolases may have a similar three-dimensional structure and might be arranged juxtapositionally in vivo after they are induced and secreted extracellularly. Such an arrangement

```
CcGn:          DNANAWIQ          DYAIFTGTS          DTVYAALEK G
NtAc:    68    SR--G-V-    192   S--L--QQE    215   -SM-F-V--A-
NtBa:    69    EH-RW-V-    193   P-SL--APN    221   -S------RS-
LeGlua   68    SKRQG-V-    191   P--L-KQQG    214   -SM-F-T--L-
GmEi:    68    ----K-V-    191   ---L-RSP-    218   -A-------A-
HvGIII:  66    SA-A--VK    183   TF-T-VPG-    210   -SI------A-
OsGns1:  67    AA-AS-VR    185   S--L--AAG    212   -AFY--MA-H-

CcGn:          DNGRTYN NLIQHVKQ  GT          DENLKFAPPEVERHWGLFSP NK
NtAc:    249   E-AQ--YE---N---SGA--    285   ---N-E GDIT-K-F----- DQ
NtBa:    255   --AA--LR-----A-E  -S    288   ---N-  N--L-K-F----- --
LeGlua   248   E-AM--YT---N---G   --    284   ---R-D GKPS-------K- DQ
GmEi:    252   --A----T--VRN---  --    286   ---Q-  Q--F-KF------IT-
HvGIII:  244   --A-A--QG--N--GN  -S    277   N---- DGDEL-KNF---K- -M
OsGns1:  246   A-A-I--Q--VN-I GR --    279   N--Q-  DAG--QN----Y- -M
```

Fig. 8. Partial amino acid sequence of cucumber β-1,3-glucanase compared to sequences of several published plant β-1,3-glucanases. Numbers indicate amino acid positions in the respective whole sequences. Amino acids identical to those of cucumber β-1,3-glucanase are designated by a dash (–): CcGn, Cucumber; NtNa, Tobacco (acidic); NtBa, Tobacco (basic); LeGlua, Tomato; GmEi, Soybean; HvGIII, Barley; OsGns1, Rice.

would support a role in plant defense for the synergism reported in vitro between the two hydrolases in fungal cell wall degradation. Although the β-1,3-glucanase activity was not enhanced as markedly as the Class III bifunctional chitinase (Fig. 7B, lane 4), the β-1,3-glucanase has a relatively high specific activity (Table 1) and the activity required for action as a defense compound per se or synergistically in vivo is not reported. It would be interesting to obtain direct evidence for the compartmental arrangement of these two hydrolases.

Homology analyses (Fig. 8) of partial amino acid sequences between cucumber β-1,3-glucanase and β-1,3-glucanases from six other plant species demonstrate the highest identity to that of soybean β-1,3-glucanase. The soybean β-1,3-glucanase has been suggested to release β-1,3-glucan from fungal cell wall and the released β-1,3-glucans may act as elicitors of phytoalexin and other stress-related compounds in soybeans (Keen and Yoshikawa 1983; Tacheuchi et al. 1990; Yoshikawa et al. 1993). The acidic cucumber β-1,3-glucanase may also release β-1,3-glucans as elicitors for other defense mechanisms.

An interesting finding reported here is that only one β-1,3-glucanase isoform (acidic) was detected in cucumber, whereas in other plant systems there are often several β-1,3-glucanase isozymes (acidic and basic). When several isozymes are detected in a plant, it is often difficult to identify the specific role of each of them in defense (Simmons 1994). We suggest that there are advantages in studying the role of β-1,3-glucanase in a plant system with a single isoform. Cucumber β-1,3-glucanase might, therefore, be a good enzyme for further study.

MATERIALS AND METHODS

Biological materials.

Cucumber plants (*Cucumis sativus* L.) cv. Wisconsin SMR-58 were grown as previously described (Dean and Kuc 1986) in a greenhouse at 23 to 33°C with a 14-h photoperiod. *Colletotrichum lagenarium* (Pass) Ell. & Halst. Race 1 was maintained on green bean juice agar at 24°C in the dark and conidial suspensions were prepared from 7-day cultures (Dean and Kuc 1986). TNV inoculum was obtained from freeze-dried cucumber leaves infected with TNV and stored at –20°C.

Plant inoculation and assessment of systemic resistance.

Plants (16 to 20 days old) with the first true leaf (leaf 1) fully expanded, and the leaf above (leaf 2) one third to one half expanded were used for the induction of systemic resistance in all experiments. β-1,3-Glucanase activity and systemic resistance were induced by infiltrating approximately 10 μl of a conidial suspension of *C. lagenarium* (5×10^5

spores ml^{-1}) into 20 sites on the lower surface of leaf 1 using an Eppendorf repeating pipette. β-1,3-Glucanase activity and systemic resistance were also induced by TNV. The TNV inoculum was prepared from 1 g of frozen infected leaf tissue and ground in 10 ml of 0.1 M cold phosphate buffer (pH 6.0) in an ice-cooled mortar. The homogenate was filtered through four layers of cheesecloth, and then diluted to 200 ml with distilled water to yield 30 to 50 lesions per inoculated leaf. The leaf to be inoculated (leaf 1) was lightly dusted with Carborundum and rubbed gently with cheesecloth previously dipped into the virus preparation (Jenns and Kuc 1979). The plants remained on greenhouse benches as it was unnecessary to place infiltrated or rubbed plants in a humidity chamber to ensure symptom development (Gottstein and Kuc 1989). Control plants were infiltrated or rubbed with water. Plants were challenged 7 days after induction for assessment of systemic resistance according to the method previously described (Dean and Kuc 1986).

Tissue collection and protein extraction.

In all experiments, tissues were collected from leaves 1 and 2 during the 11-day period following induction of leaf 1 and after challenge of leaf 2. The leaf tissues were rapidly frozen with dry ice and stored at –80°C. Frozen leaf tissues were homogenized at 0 to 4°C in 0.1 M sodium citrate buffer (pH 5.4), containing 0.1% (v/v) β-mercaptoethanol and 0.1% (w/v) L-ascorbic acid. The presence of reducing agents in the extraction buffer was essential for obtaining high activity of the enzyme. The homogenate was centrifuged at 12,000 × *g* for 30 min, and the supernatant was decanted and dialyzed against two changes of water for 24 h and then against two changes of 0.05 M sodium acetate buffer (pH 5.0) for 2 h. The dialyzate was centrifuged again at 10,000 × *g* for 10 min. The resulting supernatant was used as crude enzyme extract and for further β-1,3-glucanase purification. Intercellular wash fluid (ICF) was collected by the method of Rathmell and Sequeira (1974). The leaves were washed with distilled water, cut longitudinally into 2-cm-wide strips, and vacuum-infiltrated with water for 5 min at approximately –5 kPa. The leaf strips were blotted dry with paper towels, and centrifuged at 1,500 × *g* for 10 min in perforated polypropylene syringe tubes. The ICF accumulated in the lower chamber was collected and stored at –20°C. The tissue remaining was collected and extracted as described above. Protein concentrations were measured using the Bio-Rad protein assay kit with bovine gamma globulin as standard (Bradford 1976).

Determination of enzyme activities.

Total β-1,3-glucanase activity was colorimetrically assayed by the laminarin-dinitrosalicylate method of Abeles and Forrence (1979) with some modifications. Crude enzyme extract was precipitated with 5 volumes of cold acetone at –20°C for 30 min and centrifuged at 10,000 × *g* for 10 min. The pellet was vacuum dried and suspended in sodium acetate buffer (pH 5.0). The suspension, 62.5 µl, was added to 62.5 µl of 4% laminarin (USB) and then incubated at 40°C for 10 min. The reaction was stopped by adding 375 µl of dinitrosalicylate reagent and heating for 5 min on a boiling water bath. The resulting colored solution was diluted with 4.5 ml of water, vortexed, and its absorbance at 500 nm was determined. The blank was the suspension mixed with the dinitrosalicylate reagent to which the solution containing laminarin was added (Pan et al. 1989). One katal unit of enzyme activity was defined as the amount of enzyme that produced reducing sugar equivalent to 1 mole of glucose equivalent per second under the above conditions. The activities of β-1,3-glucanase and chitinase on gels and protein patterns were analyzed after a single separation using 15% (w/v) native-PAGE gel or 7% (w/v) IEF-PAGE gel (Pan et al. 1989) with urea (2.4 M) as an additive.

Protein purification.

The extracts used for enzyme assays described above were freeze dried, the residues were dissolved in acetate buffer, pH 5.2 (5 g fresh weight leaf tissue/ ml), and applied to gels. Proteins were first separated by 15% native-PAGE described above. A 1-cm wide gel strip was cut from the whole gel immediately after electrophoresis. The gel strip was assayed for β-1,3-glucanase activity and then matched to the remaining gel. Gel pieces were cut from the remaining gel corresponding to the position on the gel strip with demonstrated β-1,3-glucanase activity. The gel pieces were placed in distilled water for 5 min and blotted dry with paper towels. The pieces were then placed in microcentrifuge tubes with 2 volumes of protein elution buffer (Promega) and homogenized. The proteins were eluted from the homogenate for 3 h at 37°C, and the homogenate was centrifuged at 10,000 × *g* for 10 min. The supernatant was decanted, mixed with 5 volumes of cold acetone, and put at –20°C for 30 min. The mixture was centrifuged at 10,000 × *g* for 15 min. The pellet was vacuum dried and resuspended in sodium acetate buffer (pH 5.0). The purity of cucumber β-1,3-glucanase was checked by 12.5% (w/v) SDS-PAGE and 7% IEF-PAGE with a pH range of 3 to 5.5 (Pharmacia, Sweden). The above processes were repeated to further purify the β-1,3-glucanase.

Chemical amino acid sequencing.

To determine the amino acid sequence, the purified β-1,3-glucanase was subjected to IEF on 7% polyacrylamide gels. Following electrophoresis, the protein was electroblotted onto an Immobilon-P (Millipore) PVDF membrane by the method of Matsudaira (1987), but without methanol in the transfer buffer. Protein was stained with Coomassie Brilliant Blue R-250 (Bio-Rad). After the membrane was air dried, the protein band was excised. The protein was digested with an asparagine/aspartic acid-specific endoproteinase for 21.5 h at 37°C by the methods of Fernandez et al. (1994). The resulting products were separated by reverse-phase HPLC using a C18 stationary phase and a gradient of CH_3CN versus H_2O, 0.06% trifluoroacetic acid throughout. The peptides from clear and sharp fractions were sequenced using an Applied Biosystems (Foster City, CA) model 477A peptide sequencer with on-line phenylthiohydantoin-amino acid analysis.

ACKNOWLEDGMENTS

We thank Carol M. Beach, Department of Biochemistry, University of Kentucky, for amino acid sequence analyses, and Sheng Yang He and Etta Nuckles, Department of Plant Pathology, University of Kentucky, for technical assistance and useful suggestions. The research was supported in part by an assistantship to the first author from the Kentucky Agricultural Experiment Station. This is journal paper 95-12-051 of the Kentucky Agricultural Experiment Station, Lexington, Kentucky 40546.

LITERATURE CITED

Abeles, F. B., and Forrence, L. E. 1979. Temporal and hormonal control of β-1,3-glucans in *Phaseolus vulgaris* L. Plant Physiol. 45:395-400.

Boller, T. 1988. Ethylene and the regulation of antifungal hydrolases in plants. Pages 145 in: Oxford Surveys of Plant Molecular and Cell Biology, Vol. 5. B. J. Milfin ed. Oxford University Press, Oxford.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. Analyt. Biochem. 72:248-254.

Dargent, R., and Touze, A. 1974. Etude cinetigue, en microscopie electronique, des interactions entre *Colletotrichum lagenarium* et les celliles foliaires de cucumis melo. Can. J. Bot. 52:1319-1323.

Dean, R. A., and Kuc, J. 1986. Induced systemic protection in cucumber: The source of the "signal." Physiol. Mol. Plant Pathol. 28:227-233.

Dixon, R. A., and Harrison, M. J. 1990. Activation, structure, and organization of genes involved in microbial defense of plants. Adv. Genet. 28:165-234.

Fernandez, J., Andrews, L., and Mische, S. M. 1994. An improved procedure for enzymatic digestion of polyvinylidene difluoride-bound proteins for internal sequence analysis. Analyt. Biochem. 218:112-117.

Ferraris, L., Abbattista, G. I., and Matta, A. 1987. Activation of glycosidases as a consequence of infection stress in *Fusarium* wilt of tomato. J. Phytopathol. 118:317-325.

Gottstein, H. D., and Kuc, J. 1989. Induction of systemic resistance to anthracnose in cucumber by phosphates. Phytopathology 79:179-189.

Hammerschmidt, R., Nuckles, E. H., and Kuc, J. 1982. Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium*. Physiol. Plant Pathol. 20:73-82.

Irving, H. R., and Kuc, J. 1990. Local and systemic induction of peroxidase, chitinase and resistance in cucumber plants by K_2HPO_4. Physiol. Mol. Plant Pathol. 37:355-366.

Jenns, A. E., Caruso, F. L., and Kuc, J. 1979. Nonspecific resistance to pathogens induced systemically by local infection of cucumber with tobacco necrosis virus, *Colletotrichum lagenarium* or *Pseudomonas lachrymans*. Phytopathol. Mediterranea 18:129-134.

Joosten, M. H. A. J., and De Wit, P. J. G. M. 1989. Identification of several pathogenesis-related proteins in tomato leaves inoculated with *Cladosporium fulvum* (syn. *Fulvia fulva*) as β-1,3-glucanases and chitinases. Plant Physiol. 89:945-951.

Kauffmann, S., Legrand, M., Geoffroy, P., and Fritig, B. 1987. Biological function of pathogenesis-related proteins: Four proteins have β-1,3-glucanase activity. EMBO J. 6:3209-3212.

Keen, N. T., and Yoshikawa, M. 1983. β-1,3-Glucanase from soybean releases elicitor-active carbohydrates from fungus cell walls. Plant Physiol. 71:460-465.

Kombrink, E., Schroeder, M., and Hahlbrock, K. 1988. Several "Pathogenesis related" proteins in potato are β-1,3-glucanases and chitinases. Proc. Natl. Acad. Sci. USA. 85:782-786.

Kuc, J. 1982. Induced immunity to plant disease. BioScience 32:854-860.

Kuc, J. 1990. A case for self defense in plants against disease. Phytoparasitica 18:3-8.

Leah, R., Tommerup, H., Svendsen, I., and Mundy, J. 1991. Biochemical and molecular characterization of three barley seed proteins with antifungal properties. J. Biol. Chem. 266:1564-1573.

Linthorst, H. J. M., Melchers, L. S. Mayer, A., van Roekel, J. S. C., Cornelissen, B. J. C., and Bol, J. F. 1990. Analysis of gene families encoding acidic and basic β-1,3-glucanases of tobacco. Proc. Natl. Acad. Sci. USA 87:8756-8760.

Matsudaira, P. 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. 262:10035-10038.

Mauch, F., Mauch-Mani, B., and Boller, T. 1988. Antifungal hydrolases in pea tissue II Inhibition of fungal growth by combinations of chitinase and β-1,3- glucanase. Plant Physiol. 88:936-942.

Mauch, F., and Staehelin, L. A. 1989. Function implications of the subcellular localization of ethylene-induced chitinase and β-1,3-glucanase in bean leaves. Plant Cell 1:447-457.

Meins, J. F., and Ahl P. 1989. Induction of chitinase and β-1,3-glucanase in tobacco plants infected with *Pseudomonas tabaci* and *Phytophthora parasitica* var. *nicotianae*. Plant Sci. 61:151-161.

Metraux, J. P., and Boller, T. 1986. Local and systemic induction of chitinase in cucumber plants in response to viral, bacterial, and fungal infections. Physiol. Mol. Plant Pathol. 28:161-169.

Metraux, J. P., Streit, L., and Staub, T. 1988. A pathogenesis-related protein in cucumber is a chitinase. Physiol. Mol. Plant Pathol. 33:1-9.

Metraux, J. P., Burkhart, W., Moyers, M., Dincher, S., and Ryals, J. 1989. Isolation of a complementary DNA encoding a chitinase with structural homology to a bifunctional lysozyme/chitinase. Proc. Natl. Acad. Sci. USA 86:896-900.

Mucharromah, E., and Kuc, J. 1991. Oxalate and phosphates induce systemic resistance against diseases caused by fungi, bacteria and virus in cucumber. Crop Prot. Vol. 10:265-270.

Pan, S. Q., Ye, X. S., and Kuc, J. 1989. Direct detection of β-1,3-glucanase isozymes on polyacrylamide electrophoresis and isoelectrofocusing gels. Analyt. Biochem. 182:136-140.

Rathmell, W. G., and Sequeira, L. 1974. Soluble peroxidases in fluid from the intercellular spaces of tobacco leaves. Plant Physiol. 53:317-318.

Rabenantoandro, Y., Auriol, P., and Touze, A. 1976. Implication of β-1,3-glucanase in melon anthracnose. Physiol. Plant Pathol. 8:313-324.

Simmons, C. R. 1991. Structure and regulation of rice β-glucanase and α–αmylase gene families. Ph.D. thesis, University of California, Davis.

Simmons, C. R. 1994. The physiology and molecular biology of plant 1,3-β-D-glucanases. Crit. Rev. Plant Sci. 13(4):325-387.

Takeuchi, Y., Yoshikawa, M., Tanaka, K., Shibata, D., and Horino, O. 1990. Molecular cloning and ethylene induction of mRNA encoding a phytoalexin elicitor-releasing factor, β-1,3-glucanase, in soybean. Plant Physiol. 93:673-682.

van Kan, J. A. L., Joosten, M. H. A. J., and Wagenmakers, C. A. M. 1992. Differential accumulation of mRNAs encoding extracellular and intracellular PR proteins in tomato induced by virulent and avirulent races of *Cladosporium fulvum*. Plant Mol. Biol. 20 (3):513-527.

Wang, J., Xu, P., and Fincher, G. B. 1992. Purification, characterization and gene structure of β-1,3-glucanase isozyme GIII from barley (*Hordeum vulgare*), Eur. J. Biochem. 209:103-109.

Wessels, J. G. H., and Sietsma, J. H. 1981. Fungal cell wall: A survey. Pages 352-394 in: Encyclopedia of Plant physiology, New Series. Vol. 13B. W. Tanner and F. A. Loewus, eds. Springer, New York.

Yoshikawa, M., Tsuda, M., and Takeuchi, Y. 1993. Resistance to fungal disease in transgenic tobacco plants expressing the phytoalexin elicitor-releasing factor, β-1,3-glucanase, from soybean. Naturwissenschaften 80:417-420.