A Novel Fungal Protease Expressed in Endophytic Infection of Poa Species

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The fungus Acremonium typhinum produces a novel endoprotease during symbiotic endophytic infection of the grass, Poa ampla. This protease is unusual because it is highly active in the presence of sodium dodecyl sulfate. The enzyme is a thiol-containing serine protease and is localized to a crude membrane fraction. Similar protease activity has been detected in endophyte-infected Poa autumnalis and Poa sylvestris plants. Expression of this protease may be important in endophytic infection of Poa spp., because similar activity has not been detected in endophyte-infected Festuca arundinacea or Lolium perenne.

Endophytic fungal infection of grasses has been recognized for nearly 100 years and was first extensively studied by Sampson (1933, 1935, 1937, 1939). An "endophyte" is defined as "an organism that lives its life cycle within a host plant without causing disease" (Gwinn et al., 1992). The systemic endophytic infection of many forage and turfgrass species with fungi of the tribe Balansiae is widespread (for reviews, see Siegel et al., 1987; Clay, 1988, 1990). The endophytic fungi infecting commercially important forage and turfgrasses belong to the genus Epichloe and its asexual anamorphs, which have been classified as Acremonium section Albo-lanosa (Morgan-Jones and Gams, 1982). These fungi colonize the intercellular spaces of aerial plant parts and are abundant in the leaf sheaths, pith, and seeds (Hinton and Bacon, 1985). In the case of the asexual Acremonium spp., infection of the host causes no symptoms. The Acremonium spp. are transmitted exclusively through the seeds and tillers of the host plants.

Interest in the relationship between endophytic fungi and the host grasses increased with the realization that two poisoning syndromes of grazing animals, fescue toxicosis and ryegrass staggers, were associated with endophyte infection of tall fescue (Festuca arundinacea) and perennial ryegrass (Lolium perenne), respectively (Bacon et al., 1975; Fletcher and Harvey, 1981). In these forage grasses the presence of endophytic fungi often results in toxic alkaloids in the plants that can cause poisoning and even death in grazing animals. In recent years, considerable research efforts have been aimed at understanding the role of alkaloids in toxicosis. Attempts have been made to reduce the incidence of livestock toxicosis through use of endophyte-free pastures (Read and Camp, 1986).

It is interesting that endophyte-free pastures were found to have reduced stand maintenance relative to endophyte-infested pastures (Read and Camp, 1986). These results suggest that, in contrast to the detrimental effect of endophyte infection on grazing animals, there may be a beneficial effect of endophyte infection on the host plants. Some of the beneficial effects associated with endophytic fungal infection include increased insect and nematode resistance (Funk et al., 1983; Barker et al., 1984; West et al., 1988), increased drought resistance (Arachevaleta et al., 1989), and increased photosynthetic rates (Clay, 1990). Although endophytic infection with the sexual Epichloe typhina can result in a plant disease referred to as choke (Kirby, 1961), the relationship between the asexual Acremonium spp., which cause no disease symptoms, and their hosts is considered to be a mutualistic symbiosis (Clay, 1990).

We are interested in studying the physiology of the interaction of fungal endophytes with their host turfgrasses. In turfgrass and forage grasses, true inbred lines are not available. To avoid complications due to a mixture of genotypes, we have chosen to study the endophyte-grass interaction in the genus Poa. Many Poa spp. are highly apomictic, which results in a cultivar representing essentially a single genotype (Hiesey and Nobs, 1982). Apomixis is the asexual reproduction through seed, in which somatic cells give rise to unreduced eggs that undergo embryogenesis without fertilization. For seed development, however, fertilization of the endosperm is required. The highly apomictic big blue grass (Poa ampla) cv Service (PI387931), released by the Alaska Department of Natural Resources as an improved grass for roadside vegetation, has been found to be infected with the Acremonium typhinum endophyte (Sun and Breen, 1993).

We have detected a novel fungal protease that is expressed in the symbiosis of A. typhinum with the host grass P. ampla. Here we report the initial characterization of this novel protease and present evidence for similar protease activity in endophyte-infected Poa autumnalis and Poa sylvestris.

MATERIALS AND METHODS

Materials

Poa ampla cv Service plants were grown from seed. Endophyte-infected plants were obtained from the 1988 and 1990

Abbreviations: BCA, bicinchoninic acid; E-64, L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane; PDA, potato dextrose agar.
seed lots. When seed are stored long term, the viability of the endophyte is lost (Neill, 1940). Therefore, endophyte-free plants were obtained from the 1987 seed lot. As a result of apomixis, plants from the different seed lots are genetically identical. Leaf sheaths from individual seedlings were checked microscopically for the presence of the endophyte by rose Bengal staining (Saha et al., 1988). Individual seedlings were potted and maintained in the greenhouse.

Protein concentrations were determined using the BCA assay from Pierce (Rockford, IL). PMSF, E-64, BSA, and electrophoresis protein standards were from Sigma.

Culture of Acremonium typhinum

The A. typhinum endophyte can be cultured in vitro on PDA (Difco, Detroit, MI) plates (Latch et al., 1984). To isolate the endophytic fungus, small pieces of leaf sheath tissue were surface sterilized for 15 min in 1.25% sodium hypochlorite, rinsed in sterile water, and then placed on PDA plates. After 2 to 3 weeks in the dark at 24°C, fungal mycelia began to emerge from the plant tissue. The A. typhinum cultures growing on PDA plates were the source of isolated fungal tissue for protease assays.

Protein Extraction and Electrophoresis

Plant tissue or isolated fungal mycelium were homogenized with sand in 2X SDS sample buffer in a ratio of 0.1 g 400 μL⁻¹. The extracts were centrifuged to remove debris and boiled for 5 min, and aliquots of the supernatant were subjected to SDS-PAGE. SDS sample buffer (2X) is 125 mM Tris (pH 8.0), 4.6% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 20% (v/v) glycerol, and 0.002% bromophenol blue (Laemmli, 1970). SDS-PAGE was carried out using 10% polyacrylamide gels (Laemmli, 1970), which were stained with Coomassie brilliant blue.

Protease Assays

Leaf sheath extracts from endophyte-infected and endophyte-free plants or extracts of isolated fungal mycelium were obtained by homogenizing the tissue directly in 2X SDS buffer in a ratio of 0.1 g 400 μL⁻¹. The homogenate was centrifuged for 5 min, and 20-μL aliquots of the extracts were incubated with 20 μg of BSA for 20 min or 1 h at room temperature (24°C) and boiled for 5 min; aliquots were then subjected to SDS-PAGE.

For assays of protease inhibition, leaf sheaths from endophyte-infected P. ampla and mycelium from the isolated endophyte were ground in a buffer consisting of 125 mM Tris (pH 8.0) and 4.6% (w/v) SDS. Samples were centrifuged for 10 min in a microcentrifuge, and the supernatant was transferred to a new tube. Protein concentration in the supernatant was determined using the BCA assay. For each comparison, approximately 17.5 μg of protein from P. ampla and 7.5 μg of protein from the endophyte were used. More P. ampla protein was used because the majority of proteins in these samples was of plant origin.

The inhibitors PMSF and E-64 were added at 5 mM and 10 μM final concentration, respectively (Salvesen and Nagase, 1989). At these concentrations PMSF completely inhibited the Ser protease proteinase K, and E-64 completely inhibited the thiol protease papain. β-Mercaptoethanol was added at 10% (v/v) unless otherwise indicated. After the samples were incubated for 30 min at room temperature (24°C), 5 μg of BSA were added to each sample, and incubation continued for an additional 30 min. After loading dye was added to all samples, they were boiled for 5 min and subjected to SDS-PAGE.

Preparation of Fungal Membranes

To obtain crude fungal membrane preparations, mycelia were ground in 125 mM Tris-HCl (pH 8.0). The sample was filtered through Miracloth (22–25 μm) (Calbiochem, La Jolla, CA) and centrifuged at 3000g for 10 min to remove unbroken cells and cell walls. An aliquot of the supernatant was reserved, and the remainder was brought to 11.5 mL with additional buffer and centrifuged at 100,000g for 60 min to pellet membranes. The membrane pellet obtained was solubilized in 200 μL of 125 mM Tris-HCl (pH 8.0) with 4.6% SDS. The resuspended pellet was centrifuged 10 min in a microcentrifuge (13,000g), and the supernatant was saved as the crude membrane fraction. Protein concentrations in the fractions were determined using the BCA assay. Protease activity in the samples was monitored by assaying BSA degradation. Protein (5 μg) was used from each fraction. The protease was activated by adding β-mercaptoethanol to 10% and allowing the samples to incubate for 30 min at room temperature. BSA (5 μg) was added to each sample, and digestion proceeded for an additional 30 min.

Activity Gels

Protease activity was detected in SDS-gelatin gels essentially as described by Lockwood et al. (1987). The gels were 4% (w/v) polyacrylamide with 0.3% (w/v) gelatin copolymerized into the matrix. Gels were run for 16 h at 4°C with a current of 6 mA. Immediately after the run, gels were soaked in an activity buffer of 125 mM Tris (pH 8.0) and 2.5% β-mercaptoethanol for 6 h at 37°C. Because SDS does not inhibit the activity of this protease, incubation in Triton X-100 to allow renaturation of proteases was eliminated. Gels were stained with Coomassie brilliant blue and destained. Areas of protease activity appear as clear bands against the blue background.

RESULTS

Detection of a Novel Protease in Endophyte-Infected P. ampla Plants

The presence of a novel protease was originally detected by comparing SDS-PAGE profiles of extracts from endophyte-infected P. ampla with those from endophyte-free P. ampla. Figure 1 depicts the protein profiles found in leaf blades and leaf sheaths of P. ampla plants containing or lacking the fungal endophyte. The protein profiles of the endophyte-infected and endophyte-free samples were strikingly different. In the endophyte-infected leaf blade extract, there was little Rubisco large subunit (the major protein at
Figure 1. SDS-PAGE analysis of total protein extracts from endophyte-free and endophyte-infected P. ampla leaf blades and leaf sheaths. Lane 1, Endophyte-free leaf blade; lane 2, endophyte-infected leaf blade; lane 3, endophyte-free leaf sheath; lane 4, endophyte-infected leaf sheath. The protein standards and their mol wts are BSA, 66,000; egg albumin, 45,000; glyceraldehyde-3-phosphate dehydrogenase, 36,000; carbonic anhydrase, 29,000; trypsinogen, 24,000.

$M, 53,000$ as compared with the endophyte-free extract (Fig. 1, lanes 1 and 2). In the leaf blade and sheath extracts from the endophyte-infected samples, there was much less high mol wt protein than in the endophyte-free plants, and there was considerable protein staining below the dye front (Fig. 1, lanes 2 and 4). These protein profile patterns led us to suspect the presence of a protease in the endophyte-infected samples.

To test this possibility, we monitored the ability of the tissue extracts, in 2X SDS buffer, to degrade an exogenous protein, BSA. As shown in Figure 2, lane 6, incubation of BSA with the extract from the endophyte-infected tissue resulted in extensive degradation of BSA. The observed degradation of BSA was considered to be enzymic because the extract before incubation with BSA resulted in no detectable degradation (Fig. 2, lane 5). Discrete bands of BSA fragments were produced by the protease (Fig. 2, lane 6), indicating that it is an endoprotease. Although endophyte-free plants are expected to contain multiple proteases, under the conditions of our assay there was no detectable degradation of BSA (Fig. 2, lane 3). These results indicated that one or more proteases that maintain their activity in 2X SDS buffer are present only in the endophyte-infected plants.

Protease activity similar to that in infected plants was found in the fungal mycelium isolated from infected P. ampla plants. The fungal extract, in 2X SDS buffer, did degrade BSA (Fig. 3, lane 3). These results indicated that the novel protease activity observed in the grass-endophyte interaction was from the fungus.

Effect of Reductants and Inhibitors on Protease Activity

To categorize the type of protease activity produced by the fungus we determined the effects of the Ser and thiol protease inhibitor PMSF and the specific thiol protease inhibitor E-64. Proteolytic activity was present in control extracts from both P. ampla and A. typhinum (Fig. 4, lanes 1 and 2). The pattern of BSA degradation found in the two samples was identical, further confirming that the protease activity in the endophyte-infected plants was of fungal origin. In both cases, the

Figure 2. Endophyte-infected P. ampla leaf sheath extract degrades BSA. Leaf sheaths from endophyte-free and endophyte-infected plants were homogenized in 2X SDS buffer and centrifuged to remove debris, and aliquots were incubated with BSA for 20 min at room temperature. Lane 1, Endophyte-free extract; lane 2, endophyte-free extract boiled for 5 min and incubated with BSA; lane 3, endophyte-free extract incubated with BSA; lane 4, endophyte-infected extract; lane 5, endophyte-infected extract boiled for 5 min and then incubated with BSA; lane 6, endophyte-infected extract incubated with BSA; lane 7, BSA alone. The protein standards are as in Figure 1.

Figure 3. A. typhinum extract degrades BSA. A. typhinum mycelium was homogenized in 2X SDS buffer and centrifuged to remove debris, and aliquots were incubated with BSA for 20 min at room temperature. Lane 1, Fungal extract; lane 2, fungal extract boiled for 5 min and incubated with BSA; lane 3, fungal extract incubated with BSA; lane 4, BSA alone. The protein standards are as in Figure 1.

Figure 4. The fungal protease requires a reductant for activity and is inhibited by PMSF. Extracts from endophyte-infected P. ampla leaf sheaths and from A. typhinum mycelia were prepared, and aliquots were preincubated for 30 min with the inhibitors. BSA (5 $\mu$g) was added, and the incubation continued for 30 min. Lane 1, P. ampla extract plus BSA; lane 2, A. typhinum mycelial extract plus BSA; lane 3, P. ampla extract plus PMSF and BSA; lane 4, A. typhinum extract plus PMSF and BSA; lane 5, P. ampla extract minus $\beta$-mercaptoethanol plus BSA; lane 6, A. typhinum extract minus $\beta$-mercaptoethanol, plus BSA; lane 7, P. ampla extract plus E-64 and BSA; lane 8, A. typhinum extract plus E-64 and BSA; lane 9, P. ampla extract alone; lane 10, A. typhinum extract alone; lane 11, BSA alone.
addition of PMSF inhibited BSA degradation (Fig. 4, lanes 3 and 4), whereas E-64 had no detectable effect on protease activity (Fig. 4, lanes 7 and 8). Omission of the reductant β-mercaptoethanol inhibited all detectable proteolytic activity in both plant and fungal samples (Fig. 4, lanes 5 and 6). DTT could be substituted for β-mercaptoethanol (data not shown).

From these results we conclude that the fungal protease was a Ser protease, which requires a reducing agent for activity.

Fungal Protease Is a Membrane Protein

In preliminary experiments, we observed that the presence of SDS in the extraction buffer resulted in increased protease activity. This suggested that perhaps the protease was a membrane protein whose extraction was enhanced by SDS. To test this possibility we assayed the protease activity of crude membrane preparations. Soluble and membrane fractions were prepared from fungal mycelia and assayed for their ability to degrade BSA. There was no detectable activity in the 100,000g supernatant (Fig. 5, lane 2), whereas the BSA was completely degraded by the crude membrane fraction (Fig. 5, lane 3). These data indicated that the fungal protease was a membrane protein.

Visualization of the Fungal Protease Activity in an SDS-Gelatin Gel

Detection of enzyme activity following protein electrophoresis can be used to estimate the molecular mass of the active form of the enzyme. Activity gels are usually performed using native gels to avoid denaturation of the enzyme by SDS. We used SDS-PAGE gels because the fungal protease was active even in the presence of 4.6% SDS. The activity from a fungal crude membrane fraction is shown in Figure 6. Because of the high apparent molecular mass of the active protease, 4% gels were used. A single band of activity was detected that was considerably higher than the highest protein marker of 205,000 D.

Detection of Protease Activity in Other Endophyte-Infected Poa Species

To determine whether the fungal protease is unique to *A. typhinum* infecting *P. ampla*, we monitored the ability of leaf sheath extracts, in 2X SDS buffer, of other endophyte-infected grasses to degrade BSA. Extracts from endophyte-infected *P. ampla*, *P. autumnalis*, and *P. sylvestris* did degrade BSA (Fig. 7, lanes 2, 4, and 6). Protease activity during the 1-h incubation also resulted in extensive degradation of proteins in the tissue extracts (Fig. 7, lanes 1, 3, and 5). Similar protease activity was detected in extracts of the *Acremonium* endophytes isolated from these plants (data not shown). The species of *Acremonium* infecting *P. autumnalis* and *P. sylvestris* have not yet been determined.
perenne did not degrade BSA (Fig. 7, lanes 8 and 10), and the protein profile of the extracts alone did not appear degraded (Fig. 7, lanes 7 and 9). F. arundinacea is infected with Acremonium coenophialum, and L. perenne is infected with Acremonium lolii (Morgan-Jones and Gams, 1982; Latch et al., 1984).

DISCUSSION

We have detected a novel protease produced by the endophytic fungus A. typhinum. This protease is expressed during symbiosis with its host plant P. ampla. We have detected similar protease activity in extracts from two other endophyte-infected Poa spp. P. autumnalis and P. sylvestris. Activity was detected both in the plant and in the isolated fungal mycelium. We have not detected similar protease activities in extracts from endophyte-infected F. arundinacea or in endophyte-infected L. perenne. This observation suggests that expression of this protease may be important in the symbiotic relationship of Poa spp. with fungal endophytes. If the protease is important for infection of Poa spp., one implication is that Poa spp. may produce a peptide or protein that is toxic to fungal endophytes. Expression of a highly active protease may counteract such a putative toxin. Other possible functions for this protease are in reduction of host structural barriers to infection or in nutrition of the fungus, which must obtain all its nutrients from the apoplast of the plant. Further characterization and localization of the protease will be necessary to understand its role in endophytic infection of Poa.

Protease activity was localized to a crude membrane fraction. Other membrane-bound proteases have been reported to be important in two host-parasite interactions and in the invasiveness of tumor cells. The major surface protein of the promastigote stage of Leishmania spp., mammalian protozoan parasites, is a membrane-anchored acid proteinase (Etges et al., 1986; Chaudhuri et al., 1989). Surface expression of this protease is believed to protect the parasite from degradation by macrophages (Chaudhuri et al., 1989). Expression of a surface protease of the plague bacterium Yersinia pestis has been linked to virulence of the bacterium and presumably acts by interfering with the host defenses to the spread of the bacterium (Sodeinde et al., 1992). A sulfhydryl-sensitive, 170-kD membrane-bound protease is expressed in an invasive human tumor cell line (Aoyama and Chen, 1990). The invasiveness of these tumor cells is believed to be due to their surface proteolytic activity, which degrades surrounding connective tissue (Aoyama and Chen, 1990). Localization of the A. typhinum protease to a specific membrane system will aid in understanding its importance in the plant-fungus interaction.

In addition to its possible importance in the symbiosis of Acremonium endophytes with Poa species, the protease is interesting because of its unusual characteristics. The protease is highly active in conditions considered to be denaturing, i.e. 2X SDS buffer (4.6% SDS = 160 mM). In this regard, the A. typhinum protease is similar to proteinase K from the fungus Trichirachium album (Ebeling et al., 1974), which is also highly active in the presence of SDS. In size and membrane localization, however, the A. typhinum protease is distinct from proteinase K, which is a secreted M, 18,500 protein (Ebeling et al., 1974).

Based on its inhibition with PMSF but not E-64 and its requirement for a reductant, the A. typhinum protease appears to be a Ser protease with an essential thiol group. Other Ser proteases with essential thiol groups have been described from Streptomyces rectus (Mizusawa and Yoshida, 1973), Bacillus thuringiensis (Stepanov et al., 1981), Thermoactinomyces vulgaris (Stepanov et al., 1981), and Thermomyces lanuginosus (Hasnain et al., 1992). These proteases are all secreted proteins.

The A. typhinum protease migrated in an SDS-gelatin gel with an apparent molecular mass considerably greater than 205,000 D. Although both the sample and the gel contained SDS, we cannot eliminate the possibility that the high apparent molecular mass is due to aggregation or to the presence of a multimeric enzyme. In general, SDS is considered to disrupt protein-protein interactions such as occur in multimeric enzymes. There are, however, exceptions for which proteins are known to aggregate in the presence of SDS. Germin, a protein found in germinating wheat (Triticum aestivum) seedlings, was found to migrate as a homopentamer in SDS gels. The polymeric form could be converted to a monomer of 26,000 D by heating before electrophoresis (Grzelczak and Lane, 1984). Alternatively, the protease may be an extremely large monomeric protein or may be modified in a manner that retards its migration in SDS gels. Glycosylation or lipid additions could be expected to cause anomalous electrophoretic migration. Indeed, membrane proteins are often glycosylated (Hubbard and Ivatt, 1981). Further characterization of the protein will be required to determine whether the electrophoretic migration is an anomaly of protein modification or whether it truly reflects the size of the protease.

For turfgrass species, symbiosis with fungal endophytes is highly beneficial. Many commercial turfgrass varieties now contain endophytes, and turfgrass breeders are attempting to obtain new grass-endophyte combinations and to broaden the host range of desirable endophytes. Kentucky bluegrass (Poa pratensis) is a commercially important turfgrass species for which endophyte-infected varieties have not yet been found. Efforts to incorporate endophytes into P. pratensis through breeding or direct inoculation have not been successful. In this report we have described a novel fungal protease that is expressed in endophytic infection of three Poa spp. Because we have not detected similar protease activities in endophyte-infected F. arundinacea or L. perenne, expression of the novel fungal protease may therefore be an important factor in developing effective endophytic infection of Poa spp.

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