A Protease Activity Displaying Some Thrombin-like Characteristics in Conditioned Medium of Zinnia Mesophyll Cells Undergoing Tracheary Element Differentiation

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ABSTRACT

The normal development of tracheary elements (TE) requires a selective degradation of the cytoplasm without loss of the extracellular wall that remains behind as the water-conducting units of xylem. Using zinnia-(*Zinnia elegans L.* cv. Green Envy) cultured mesophyll cells that synchronously transdifferentiate into TEs, extracellular and intracellular proteases, respectively, have been shown to both trigger death and to execute autolysis as the final component of a programmed cell death (PCD). We report here the appearance in the medium of an unusual proteolytic activity correlated with the PCD process just prior to the autolysis. The activity has a pH optimum of 5.5–6.0 and displays some thrombin characteristics. This protease activity has 1) a 10-fold higher affinity towards a thrombin-specific chromogenic substrate than toward a trypsin-specific chromogenic substrate; 2) a 1000-fold lower sensitivity to soybean trypsin inhibitor (STI) compared to trypsin; and 3) limited ability to cleave the protease-activated receptor-1, the native thrombin substrate. However, the addition of partially purified fraction containing the thrombin-like protease activity to the medium of PCD-competent cells does not prematurely trigger PCD, and the thrombin-specific peptide inhibitor phenylalanine-proline-aspartic acid-chloromethylketone fails to inhibit PCD or tracheary element (TE) formation. This suggests that this protease activity may play a role within the cells in execution of the autolysis or in the collapse of the tonoplast rather than as an extracellular proteolytic activity participating in the chain of events leading to cell death.

Key words: Programmed cell death; Thrombin; Tracheary elements; Zinnia.

Terminal differentiation of tracheary elements (TE) culminates in cytoplasmic autolysis to leave behind

a functional cellular corpse and is the final manifestation of a programmed cell death (PCD). These hollow TEs form end-on-end to produce the fluid-conducting vessels of xylem and their formation requires strict orchestration of the formation of rigid secondary walls with the cytoplasmic autolysis. The profile of hydrolases that are involved in TE differentiation is critical to the initiation and further execution of the process and probably involves novel activities given the specialized tasks they perform. In TE PCD, the hydrolase profile must serve the task of removing the cytoplasm completely while leaving the final vascular walls intact.

Much of what we know and understand about the molecular biology of tracheary element formation has been derived from observations and manipulations of synchronously differentiating zinnia cells in culture (Fukuda and Komamine 1980; Burgess and Linstead, 1984; Fukuda, 1992; Groover and others 1997; 1999). Primary cultures of mesophyll cells from zinnia leaves are established and initiated to transdifferentiate into TEs over a 96-hour period. These cells begin preparation for the final step of autolysis early by synthesizing a number of hydrolases and sequestering them into the large central vacuole (Ye and Varner 1996). It is hypothesized that when synthesis of the secondary cell wall is visibly completed, a serine protease (designated ''trigger'' protease) is secreted into the medium to a threshold level at which it triggers an abrupt collapse of the central vacuole and consequent release of sequestered hydrolases (Groover and others 1999; Kuriyama and Fukuda 1999; Obara and others 2001). Autolysis is completed within 2 h and represents the final manifestation of this PCD. Thus, it appears that TE differentiation involves both proteases that trigger death as well as proteases that execute the removal of the cytoplasm.

We detected the appearance of an unusual proteolytic activity in the medium just prior to the onset of PCD displaying some characteristics of the mammalian thrombin. Based on its characteristics presented in this report, it is suggested that this proteolytic activity may have an intracellular site of action and may serve as a downstream effector protease. However, because of its low abundance and unique substrate specificity, we do not rule out the possibility that it plays a regulatory role. We describe here the partial purification and characterization of this protease activity as the preliminary step towards its cloning and test of functionality in TE formation.

MATERIALS AND METHODS

Plant Growth and Establishment of Mesophyll Cell Culture

Conditions for growing zinnia (*Zinnia elegans L.* cv. Green Envy; Strokes seeds, Buffalo, NY, USA) plants and establishing mesophyll cell cultures were as previously described (Yu and others 2002; Groover and others 1997, 1999). Cultures displaying 4–10% TE at the beginning of the third day were used because they showed at least a 25% TE count on the fourth day and thus were considered suitable for further experimentation.

Human Fibroblast Cell Culture

Flag-tagged-protease-activated receptor-1 (PAR1) transfected human fibroblast cells were cultured in DMEM (Dubecco modified Eagle's medium), high glucose (GIBCO BRL) with penicillin, G418, fetal bovine serum, hygromycin and HEPES at 37°C (Trejo and others 2000).

Chemicals and Antibodies

Anti-thrombin, hirudin, aprotinin, soybean trypsin inhibitor (STI), trypsin, thrombin, and anti-FLAG M1 monoclonal antibody were purchased from Sigma (St. Louis, MO, USA), STI-Sepharose and avidin-Sepharose from Pierce Chemicals (Rockford, IL, USA), FPR-cmk (phenylalanyl-Prolyl-Arginine-chloromethylketone) and HDFPR-cmk from Bachem (Torrance, CA, USA), Chromozym TH (Tosyl-glycyl-prolyl-arginine-4-nitranilide acetate), chromozym TRY (Carbobenzoxy-valyl-glycyl-arginine-4-nitrilanilide acetate) and α-benzoyl-R-4-nitranilide from Roche (Indianapolis, IN, USA). Q-Sepharose was purchased from Pharmacia. HRP-conjugated, anti-mouse goat polyclonal antibody was purchased from BIO-RAD (Hercules, CA, USA), α-thrombin was provided by Dr. JoAnn Trejo (University of North Carolina at Chapel Hill).

Protease Activity Assay

The presence of hormones in the zinnia medium had no effect on the proteolytic activity. Thus, all assays were carried out in hormone-free zinnia medium, pH-5.5 at 25°C using a Shimadzu UV 3000 spectrophotometer. The release of the chromophore, 4-nitranilide, from Chromozym TRY, Chromozym TH or α-BAN (α-Benzoyl-arginine-4-nitranilide), trypsin, thrombin and general proteolytic substrates, respectively, by the proteases was

monitored at 405 nm. The absorption coefficient for 4-nitraniline is 10.4 mmol^{-1} cm^{-1} as provided by the manufacturer (Roche, Indianapolis, IN, USA). The release of the chromophore was used for the calculation of activity, which is expressed as arbitrary units. For determination of pH optimum, either citrate or MES (methyl-ethyl-sulfonate) buffers were added to hormone-minus zinnia medium.

Isolation and Partial Purification of Protease from Conditioned Medium

Three- and four-day-old cultures were removed from the conditioned medium by filtration through glass-fiber filters followed by centrifugation at 47,000 × g for 20 min. The medium was further filtered through a 0.22 nm filter to remove small particles and was kept frozen at −20°C until use. The proteolytic activity in the conditioned medium was not affected by freezing (data not shown). Frozen conditioned medium (1000-2000 ml) was thawed and loaded onto a 1 × 10 cm Q-Sepharose column equilibrated with hormone-minus zinnia medium containing 1 mM DTT (1,4-dithiothreitol), pH-5.5, at a flow rate of 10 ml/min. Protease activity was not detected in the column flow through. The column was washed at a flow rate of 1.2 ml/min with 50 ml of equilibrating buffer. The Q-Sepharose column was then eluted with a linear gradient of 0–0.7 M NaCl in the equilibrating buffer in a volume of 85 ml. The column was then eluted with a gradient of 0.7–1.0 M NaCl in a volume of 18 ml, in the equilibrating buffer, at a flow rate of 1.2 ml/min. Under these conditions the protease activity was eluted from the Q-Sepharose at 0.3 M NaCl, in a sharp peak (12 mL).

Cell Surface ELISA

The genetic modification of the fibroblast cell line, the methodological rationale and experimental protocol were already published (Prickett and others 1989; Hung and others 1992; Trejo and others 2000). After establishing the cell layers in 24-well culture dishes containing confluent fibroblast layers the cell layer growth was checked on the second day. Each well was washed with 350 μl PBS (10 mM sodium phosphate pH 7.0, 150 mM sodium chloride, 1 mM calcium chloride). Fresh DMEM medium adjusted to pH 6.0 or pH 7.0 was added to the well according to the experiment setup. Fresh medium, α-thrombin (10 nM) or partially purified zinnia protease (relative conditioned medium units) was added to zinnia cell cultures adjusted to pH 6 or 7. Cells were than incubated at room temperature

for 30 minutes. The ELISA procedure was carried out according to Trejo and Coughlin (1999).

## TUNEL Assay for Detection of DNA Fragmentation, a Hallmark of PCD

Fifty-h PCD-competent zinnia cells were assayed for DNA fragmentation analysis as previously described (Groover and others 1999; Yu and others 2002). Cells incubated with 1% trypsin or with fresh zinnia medium, respectively, served as positive and negative controls. After 5 h of treatment, cells were assayed for terminal deoxynucleotidytransferase-mediated dUTP nicked end labeling (TUNEL). Death rate was also determined by staining with 0.33% (w/v) Evan's Blue. Death rate was calculated as the sum of dead cells and TEs versus total number of cells. Cells with fragmented nDNA, as indicated by fluorescent green nuclei, were expressed as the percentage of total cells, including necrotic cells. Fixed, permeabilized cells were incubated with DNase I to induce DNA strand breaks to serve as a positive control in each experiment. The negative control was cells treated only with terminal transferase.

RESULTS

Appearance of Proteolytic Activity in the Culture Medium

The time course of the cellular events leading to the formation of TEs was previously described (Groover and others 1997, 1999). Cells expand between 24 and 48 h after induction. TEs first appear around 67 h and by 96 h approximately 35% of the cells have completed PCD. The population of dead cells (which include TEs) at 96 h is 40–60% (data not shown).

We set forth to purify a secreted protease involved in triggering death in TE-competent cells, a protease shown to be inhibited by soybean trypsin inhibitor (Groover and others 1999). In the process, we compared the activity of different substrates for extracellular protease activity. Because our initial assessment was that the trigger protease was trypsin-like due to its inhibition by STI, we focused on chromogenic substrates for serine proteases such as Chromozym-TRY and α-BAN. Furthermore, because the trigger protease shares functional properties with thrombin, an extracellular serine protease that triggers a programmed cell death (Smirnova and others 1998), we included in our survey Chromozyme-TH, a thrombin-specific substrate.
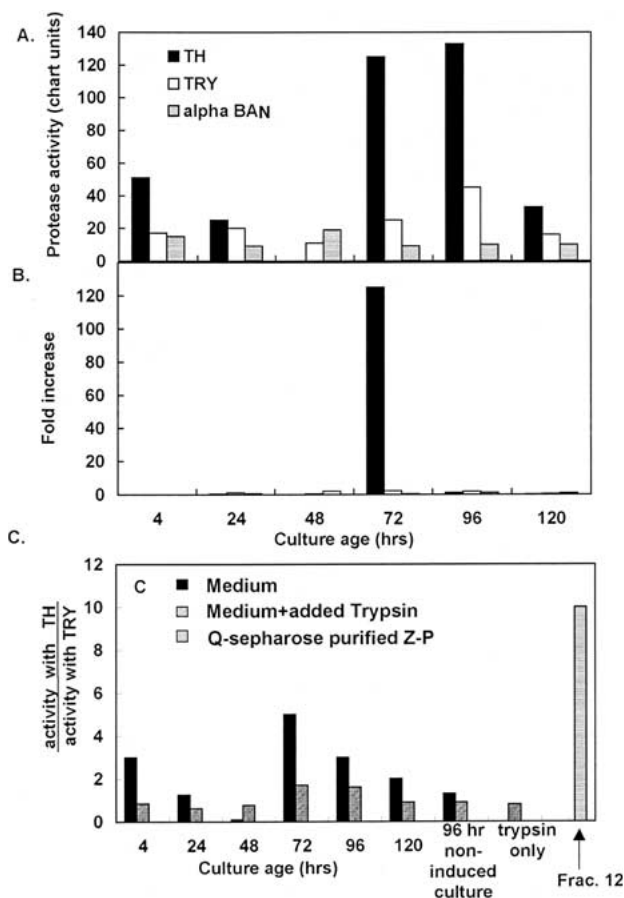
Figure 1. Proteolytic activity in conditioned medium of zinnia cell culture during terminal differentiation. (**A**) Dependence of protease activity on culture age. At 4 h after culturing mesophyll cells (zero time) and at one day intervals thereafter, the protease activity in the culture medium was determined using the substrates Chromozym TH, Chromozym TRY, and α-BAN as described in Materials and Methods. (**B**) The fold change in activity over a 24-hr window was plotted to illustrate the dramatic increase in Chromozym TH activity at the time when most cells begin autolysis. (**C**) The ratio between the protease activity using Chromozym TH and that using Chromozym TRY as substrates in: 1) medium of 96 h non-induced cell culture, 2) fresh zinnia medium supplemented with trypsin, and 3) the zTPA eluted from Q-Sepharose column (Fraction 12, see Figure 3). The experiment was repeated three times and the results shown are from a representative one. It should be noted here that cells proliferate in non-induced cell culture medium but do not differentiate or undergo PCD.

The proteolytic activity in the conditioned medium (zTPA from now on), displayed a higher activity toward the Chromozym-TH than toward the Chromozym-TRY or α-BAN substrates. Figure 1A shows the dependence on the age of the cell culture of the proteolytic activity in the conditioned medium as assayed with Chromozym TH, or Chromozym TRY

(Cappiello and others 1996; Matsuura and others 1998). Usually, there was little detectable activity with either of the substrates in media of freshly isolated cells. The activity with Chromozym TH as substrate steadily declined and was undetectable by 48 h. By 72 h there was a sharp increase in protease activity, particularly that which preferred the thrombin-specific substrate, Chromozym TH. It is clearly seen from Figure 1A that the sharp increase of the protease activity which prefers Chromozym TH as substrate proceeded the increase of that which prefers Chromozym TRY by about 20 hrs. The extent of increase of the protease activity which prefers Chromozym TH as substrate is best illustrated by plotting the fold change in activity over time (Figure 1B). This activity remained high at 96 h then returned to baseline levels, whereas the trypsin-like activity was much lower and did not show a dramatic fold-change over time. It should be noted here that the former activity was rather low in noninducing medium. Noninductive culture did not show thrombin-like protease activity (data not shown).

The reciprocal experiment of following zTPA activity intracellularly over time was uninformative due to a high basal protease activity (Ye and Varner 1996) and chromogenic interference by cellular pigments.

pH Optimum of the Thrombin-like Protease

The pH of the zinnia culture medium is 5.5–5.6 throughout the 120-h culture period. Thus, it was important to assess whether the protease that displayed high affinity toward Chromozym TH detected in the conditioned medium was an acidic one operating at its optimal pH or whether it was in effect a neutral protease leaking from the cells displaying aberrant activity with respect to substrate specificity at acidic pH. The pH optimum of the proteolytic activity was found to be 5.5–6.0 (Figure 2) with two buffer systems, suggesting that it was indeed an acidic protease. A similar pH-dependent activity profile was obtained with Q-Sepharose purified enzyme from the 72 or 96 h conditioned medium (data not shown). Thus, it is most likely an acidic protease active either in the extracellular milieu or in an acidic compartment such as the vacuole. In the latter case, its presence in the medium would be due to cell autolysis.

Partial Purification of the Zinnia Protease by Ion-exchange Chromatography

The proteolytic enzyme was concentrated and partially purified using ion exchange chromatography
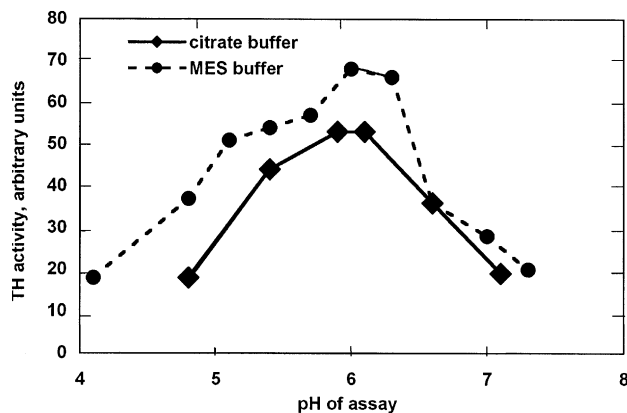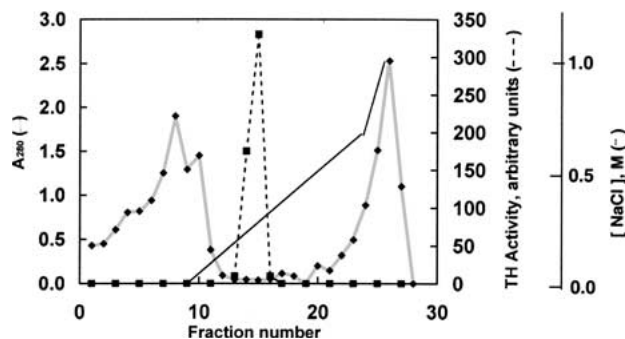
Figure 2. The dependence of the thrombin-like protease activity present in 72 h conditioned medium on pH of the assay mixture. Hormone-free zinnia medium was buffered with citrate acid or MES buffer. Chromozym TH was used as the substrate. The protease activity in 72-h conditioned medium is shown in relative units. The experiment was repeated three times and the results shown are from a representative one.

Figure 3. Elution profile of the zTPA protease activity from Q-Sepharose. The proteolytic enzyme in the conditioned medium was concentrated and purified using ion exchange chromatography on Q-Sepharose as described in Materials and Methods. The experiment was repeated three times and the results shown are from a representative one.

(Q-Sepharose). The elution profile is shown in Figure 3. All of the enzyme activity found in the conditioned medium was eluted at around 0.3 mM NaCl and recovered in two fractions. Often, the eluted activity was 150% of that in the conditioned medium, indicating the presence of a possible inhibitor in the conditioned medium which could be removed through ion exchange fractionation. The protein content in these fractions was too low to be determined by any existing procedures without sacrificing the entire pool of the purified enzyme. However, we estimated that the degree of purification exceeded 100-fold. The eluted enzyme was stable at 4°C and survived several cycles of freezing and thawing. This enzyme preparation was used to determine the interaction with inhibitors and its ability to enhance TE formation.

Effects of Conditioned Media and pH on zTPA Protease

A recent inventory of *Arabidopsis* serine proteases does not include an obvious thrombin homolog (Beers and others 2004). It was therefore essential to determine whether the preference of zTPA towards Chromozym TH as a substrate over that of Chromozym TRY was an intrinsic characteristics of the enzyme or an effect of the medium on the enzyme by either its acidic nature or by a factor present in the conditioned medium. This was tested by comparing the activity of pure trypsin added to conditioned or fresh medium or a buffer system at pH 7.5 and assayed with the two substrates. The rationale was that if a factor present in the conditioned medium was responsible for the increased affinity of the trypsin-like protease towards the thrombin-specific substrate, then trypsin would be affected similarly in displaying an increased affinity towards this substrate when assayed in conditioned medium. The activity of purified trypsin at pH 5.5 was 1/10 of that at pH 7.5, its optimal pH value. If the pH of the medium affected the activity, one would expect trypsin activity to be higher in conditioned or fresh medium (both pH 5.5) as compared to just the buffers at pH 7.5. The ratio of the activities with either of the two substrates for the added trypsin was about 1 when assayed in conditioned or fresh medium (Figure 1C), indicating that the increased activity was not due to a secreted factor. The partially purified protease (described below) when assayed at pH 5.5 or pure thrombin assayed at pH 7.5 displayed a 10:1 and 9:1 ratio of Chromozym TH: Chromozym TRY activities, respectively (Table 1).

The average ratio of the activities with the two substrates Chromozym TH: Chromozym TRY in the conditioned medium was 6:1 and that of the enzyme which eluted from Q-Sepharose chromatography (fraction #12 as described further below), was 10:1. The lower ratio obtained with unfractionated conditioned medium (6:1) as compared with that displayed by fraction #12 eluted from the Q-Sepharose column (10:1) was most likely due to the presence of other proteases which prefer the trypsin-specific substrate, thereby lowering the average ratio of activities with the two substrates.

Table 1. Substrate and Inhibitor Specificity of Zinnia Protease (zP), Trypsin, and Thrombin

| Protease tested	pH of assay	Substrate Specificity TH/TRY	Substrate	Trypsin inhibitor	Anti thrombin	Hirudin	Aprotinin	FPRcmk
zP	5.5	10:1[a]	TH	50% at 2 mg/ml assay[a]	Not effective[d]	33% at 3 units/ml assay[a]	100% at 7.5 μM[a]	90% at 1.0 nM[c]
Trypsin	7.5	1:10[b]	TH	100% at 0.875 μg/ml assay[b]	80% at 4 μg/ml assay[e]	100% at 2.5 units/ml assay[c]	99% at 7.5 nM[b]	100% at 1.0 nM[c]
			TRY	100% at 0.5 μg/ml assay[b]		95% at 2.5 units/ml assay[c]	99% at 38 nM[b]	100% at 1.0 nM[c]
α-thrombin	7.5	8:1[a]	TH	30% at 0.5 mg/ml assay[a]	80% at 0.4 μg/ml assay[e]	80% at 0.5 units/ml assay[c]	Not effective[a]	
Thrombin	7.5	9:1[a]	TH		90% at 10 μg/ml assay[e]			100% at 0.10 nM[c]

Partially-purified zP and commercially-available trypsin and thrombin, were assayed individually with or without the indicated inhibitors at the indicated pH's. ChromzymTH, TH; chromozymTRY, TRY. [a]Characteristics common to all proteases; [b]Characteristics unique to trypsin; [c]Characteristics common to thrombin and zP; [d]Characteristics unique to zP; [e]Characteristics common to trypsin and thrombin.

To characterize zTPA activity sufficiently so that it can be identified in subsequent efforts to clone the corresponding gene encoding it, it was important to establish a fixed reference point for comparison. To do that, we compared zTPA activity to two well-characterized serine proteases that share characteristics with zTPA activity and use the ratio of activity on two substrates as the zTPA signature. The results in Table 1 summarize the differences among zTPA, trypsin, and thrombin with respect to the ratio of activities with Chromozym TH and Chromozym TRY. The average ratio of activities obtained with pure trypsin or pure thrombin assayed at pH-7.5 was 1:10 and 9:1, respectively (Table 1). Thus, the protease present in conditioned medium and thrombin have, respectively, 100 and 90-fold higher affinity to Chromozym TH compared to trypsin.

Effect of Trypsin- and Thrombin-specific Inhibitors on the Activity of the Zinnia Protease

Groover and coworkers (1999) previously showed that STI inhibited TE formation and added trypsin can trigger PCD. Based on these results without further characterization of the enzyme, the authors concluded that a serine, trypsin-like protease controlled the initiation of autolysis. As described above, the protease activity present in conditioned zinnia cell culture medium displayed thrombin-like characteristics at least with respect to substrate specificity (Table 1). We further characterized the zinnia protease using trypsin and thrombin inhibitors to interpret the results of these inhibitors on PCD during TE formation (described below). The zinnia protease (zTPA) was partially purified by ion exchange chromatography (Q-Sepharose) and further concentrated 1000-fold as described in Materials and Methods. The effect of trypsin- and thrombin-specific inhibitors was tested against zTPA and compared to trypsin and thrombin (Table 1). Because these inhibitors act noncompetitively, the results are presented as the concentration of the inhibitor that caused the highest inhibition of activity.

At maximum inhibition, STI was 1000 times more effective in inhibiting trypsin than zTPA or thrombin. However, the thrombin-specific inhibitor FPR (1-letter amino-acid code)-cmk, an active site serine residue-specific inhibitor, was only 10 times more effective against thrombin than either zTPA or trypsin. Anti-thrombin and hirudin, two specific inhibitors of thrombin (Wunderwald and others 1982; Markward and Walsmann 1967) were only
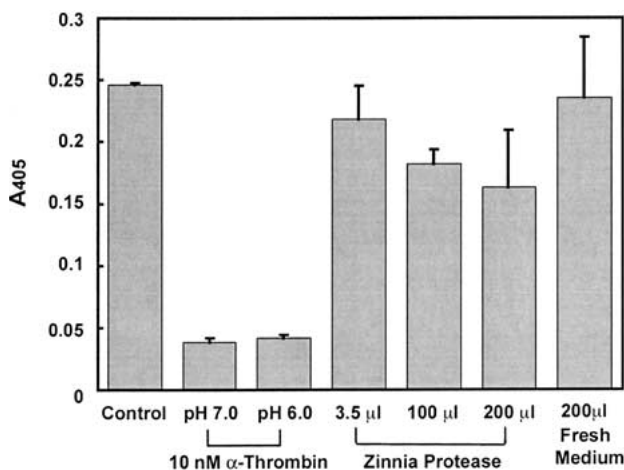
Figure 4. Cleavage of PAR1 by zTPA. The ability of zTPA and α-thrombin to cleave the thrombin substrate, PAR1, was determined as described in Materials and Methods. The cleavage and activation of the PAR1 were marked by loss of surface binding of anti-FLAG antibodies recognizing the activation peptide epitope as detected by ELISA. Error bars represent the SD of triplicate samples. The experiment was repeated two times and the results shown are from a representative one.

10-fold more effective with thrombin than with trypsin and zTPA. Aprotinin, a known inhibitor of trypsin (Fioretti and others 1988), was 1000-fold more effective with trypsin than with the zTPA and was ineffective against thrombin.

zTPA is similar to thrombin based on aprotinin sensitivity, a known thrombin inhibitor, however, it is more like trypsin based on anti-thrombin or FPRcmk sensitivity. Thus, it is concluded that zTPA is an uncharacterized serine protease having partial thrombin properties.

Cleavage of PAR1 by zTPA

For the reasons described above, we extended the characterization of zTPA activity using the natural substrate of the thrombin, protease-activated receptor-1 (PAR1) located on the plasma membrane of blood platelets (Vu and others 1991). PAR1 is cleaved by thrombin between an arginine and serine on the N-terminal exoplasmic domain, thereby activating platelet morphogenesis and in other cell types, triggering PCD. Cleavage induces a G-protein response which involves PAR1 endocytosis. Trypsin does not cleave PAR-1 and therefore is unable to induce PAR1 internalization. We hypothesized that zTPA might display low proteolytic activity on PAR1 based on its shared characteristics with thrombin. We used an established proteolytic assay to test

zTPA for thrombin-like activities *in vivo* (Ishii and others 1993; Trejo and others 1999). The assay employed human fibroblast cells transformed to express a membrane PAR1 tagged with a FLAG epitope allowing indirect quantitative estimation of thrombin activity. The amount of surface receptor was determined by ELISA. Cleavage of the tagged PAR1 by thrombin causes a loss of the immunological flag (Ishii and others 1993).

We modified the protocol by conducting the assay at pH-6.0 which is closer to the pH optimum of the zTPA. We also assayed at pH 7.0 to estimate the content of PAR1 expression and the effect that the lower pH might have on the cells. As shown in Figure 4, zTPA cleaved PAR1 in a dose-dependent manner, albeit nonlinearly. The cleavage was not as efficient as that by thrombin. The control, on the other hand, completely lacked PAR1 cleavage activity.

Effect of zTPA on Premature Programmed Cell Death

The coincident appearance of the proteolytic activity with the onset of autolysis at 72-h cells raised the possibility that zTPA triggers death. We have found that 50-h-old cells were as good as 67-h-old cells for demonstrating the effect of trypsin or STI on cell wall thickening (3rd day and 4th day) and TUNELing. Thus, the effect of zTPA on TE formation and PCD was tested on 50-h, PCD-competent cells. Enhanced appearance of TE in those cells as compared to untreated 50-h PCD-competent cells would suggest that zTPA was involved with the PCD process leading to TE formation. zTPA was added to 50-h PCD-competent cells at an activity level that was 20-200-fold over that present in the 72-h, conditioned medium. The percentage of TUNELing (a hallmark of PCD), and of dead cells were determined (Figure 5). The partially purified protease neither enhanced DNA fragmentation as determined by the TUNEL assay, nor increased cell death of PCD-competent cells as compared with the untreated PCD-competent cells. The partially purifed zTPA had no effect on the percentage of the TE formation in 72-h and 96-h cells (respectively, 22 and 46 h after treatment, data not shown).

Effect of the Specific Thrombin Inhibitor FPRcmk on TE Formation

Groover and coworkers (1999) previously showed that STI inhibited TE formation leading these authors to conclude that a trypsin-like protease con-
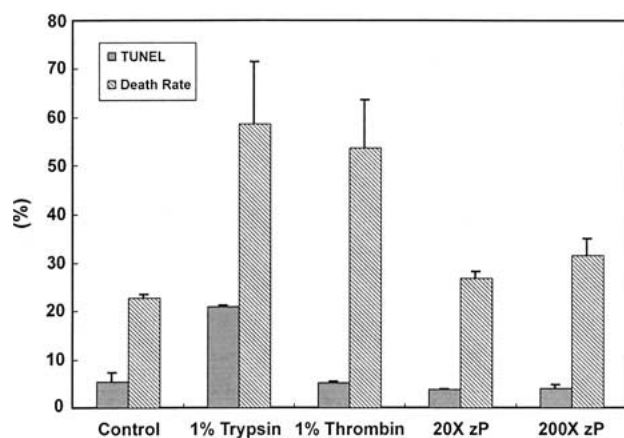
Figure 5. The effect of zTPA on TE formation and TUNEL of PCD-competent cells. Fifty-h, PCD-competent cells were used. zTPA was added to give a final activity corresponding to 20 or 200-fold (indicated 20X zTPA or 200X zTPA, respectively) over that found in the conditioned medium. Trypsin and thrombin were added at the indicated concentrations. Cells were collected after 5 h to assay the death rate and TUNEL. Error bars represent the SD from replicate samples in one experiment. The experiment was repeated three times and the results shown are from a representative one.

trolled the initiation of autolysis. FPR-cmk is a potent *in vitro* inhibitor of thrombin known to specifically react with the active site serine residue (Rahr and others 1994). FPR-cmk also inhibited trypsin and zTPA albeit at a concentration 10-fold higher than for thrombin (1.0 nM, see Table 1). We tested whether exogenous application of this inhibitor would inhibit TE formation. FPR-cmk did not inhibit TE formation when applied at 100 nM, a concentration 100-fold higher than that needed to inhibit zTPA (data not shown).

DISCUSSION

A preponderance of evidence suggests that proteases play both regulatory and effector roles in the programmed cell death of terminally differentiated TEs (for example, Ye and Varner 1996; Beers and Freeman 1997). The involvement of trypsin-like protease in TE formation in zinnia mesophyll cell culture was shown by Groover and Jones (1999) and designated "trigger protease" because exogenous trypsin triggers premature death, and STI added to the culture medium blocks death.

We fortuitously revealed the presence of a protease in cultures of zinnia mesophyll cells that displayed several characteristic features of the animal protease, thrombin: 1) selectivity towards the thrombin-specific substrate, Chromozyme TH; 2) an inhibitor profile similar, but not exactly that of thrombin; and 3) the ability to cleave the thrombin-specific substrate PAR1, although not as efficiently as thrombin. Thus, we suggest that the zTPA is not identical to mammalian thrombin but rather shares some similar characteristics with it and we have designated it thrombin-like.

The sharp increase in zTPA activity in the culture medium in parallel with TE differentiation, the extremely low abundance of zTPA and the apparent narrow substrate specificity suggest that zTPA plays a regulatory role. It should be added that cyclosporin A, which inhibited TE formation and also blocked PCD triggered by betulinic acid (Yu and others 2002), also affected the thrombin-like protease activity (data not shown).

The pH optimum of zTPA (5.5–6.0) suggests that it operates in an acidic environment such as the extracellular milieu, either as the initiator of the cascade of events leading to the TE's PCD or somewhere downstream. Accordingly, it was expected that concentrated zTPA added to competent cells would result in enhancement of PCD and that exogenous FPR-cmk would arrest PCD. The results shown in Figure 5 do not meet these expectations, thus we suggest that zTPA may have a role in the intracellular rather than extracellular chain of events leading to TE PCD. The discovery of a thrombin-like proteolytic activity that does not seem to execute cell autolysis does not contradict the concept of a trigger protease (Groover and others 1999).

The majority of protease activities in plant cells may be localized to the vacuole which is acidic in nature (reviewed by Callis 1995). Various hydrolases including nucleases (Ito and Fukuda, 2002) and proteases (Ye and Varner 1996; Beers and Freeman 1997) are synthesized before the active degeneration of cellular contents and are thought to reside in the vacuole of TEs or activated after the vacuole collapses. Sharing similar characteristics with thrombin, that is, the ability to exert some hydrolytic activity toward PAR1, thrombin's natural substrate, and the high affinity toward Chromozym TH, suggests that zTPA may function similarly to thrombin by activation of gates leading to the collapse of the tonoplast (Kuriyama 1999). On the other hand, its participation in house cleaning at the final stage following the loss of selective permeability of the tonoplast that occurred after the cells commenced secondary wall thickening should not be ruled out. Its presence and function in the vacuole would explain the lack of effect on TE formation when provided in the medium or when

attempting to inhibit its activity by adding the inhibitor FPR-cmk to the medium.

REFERENCES

Beers EP, Freeman TB. 1997. Proteinase activity during tracheary element differentiation in zinnia mesophyll cultures Plant Physiol 113:873–880.

Beers EP, Jones AM, Dickerman A. 2004. The S8 serine, C1A cysteine, and A1 aspartic protease families in Arabidopsis Phytochemistry 651:43–58.

Burgess J, Linstead P. 1984. *In-vitro* tracheary element formation: structural studies and the effect of tri-iodobenzonic acid Planta 160:481–489.

Callis J. 1995. Regulation of protein degradation Plant Cell 7:845–857.

Cappiello M, Vilardo PG, Lippi A, Criscuoli M, Corso A Del , Mura U. 1996. Kinetics of human thrombin inhibition by two novel peptide inhibitors (Hirunorm IV and Hirunorm V) Biochem Pharmacol 52:1141–1146.

Fioretti E, Angeletti M, Fiorucci L, Barra D, Bossa F, Ascoli F. 1988. Aprotinin-like isoinhibitors in bovine organs Biol Chem Hoppe Seyler 369 .Suppl:37–42.

Fukuda H. 1992. Tracheary element formation as a model system of cell differentiation Int Rev Cytol 136:289–332.

Fukuda H, Komamine A. 1980. Establishment of an experimental system for the study of tracheary element differentiation from single cells isolated from the mesophyll of *Zinnia elegans* Plant Physiol 65:57–60.

Groover A, Dewitt N, Heidel A, Jones AM. 1997. Programmed cell death of plant tracheary elements differentiating *in vitro* Protoplasma 196:197–211.

Groover A, Jones AM. 1999. Tracheary element differentiation uses a novel mechanism coordinating programmed cell death and secondary cell wall synthesis Plant Physiol 119:375–384.

Hung DT, Vu TH, Nelken NA, Coughlin SR. 1992. Thrombin-induced events in non-platelet cells are mediated by the unique proteolytic mechanism established for the cloned platelet thrombin receptor J Cell Biol 116:827–832.

Ishii K, Hein L, Kobilka B, Coughlin SR. 1993. Kinetics of thrombin receptor cleavage on intact cell J Biol Chem 268:9780–9786.

Ito J, Fukuda H. 2002. ZEN1 is a key enzyme in the degradation of nuclear DNA during programmed cell death of Tracheary Elements Plant cell 14:3201–3211.

Kuriyama H. 1999. Loss of tonoplast integrity programmed in tracheary element differentiation Plant Physiol 121:763–774.

Markwardt F, Walsmann P. 1967. Purification and analysis of the thrombin inhibitor hirudin Hope Seylers Z Physiol Chem 348:1381–1386.

Matsuura K, Yamamoto K, Sinohara H. 1994. Amidase activity of human Bence Jones proteins Biochem Biophys Res Commun 204:57–62.

Obara K, Kuriyama H, Fukuda H. 2001. Direct evidence of active and rapid nuclear degradation triggered by vacuole rupture during programmed cell death in zinnia Plant Physiol 125:615–626.

Rahr HB, Sorensen JV, Danielsen D. 1994. Markers of coagulation and fibrinolysis in blood drawn into citrate with and without D-Phe-Pro-Arg-chloromethylketone (PPACK) Thromb Res 73:279–284.

Smirnova IV, Zhang SX, Citron BA, Arnold PM, Festoff BW. 1998. Thrombin is an extracellular signal that activates intracellular death protease pathways inducing apoptosis in model motor neurons J Neurobiol 36:64–80.

Trejo A, Coughlin SR. 1999. The cytoplasmic tails of protease-activated receptor-1 and substance P receptor specify sorting to lysosome *versus* recycling J Biol Chem 274:2216–2224.

Trejo J, Altschuler Y, Fu HW, Mostov KE, Coughlin SR. 2000. Protease-activated receptor-1 down-regulation: a mutant HeLa cell line suggests novel requirements for PAR1 phosphorylation and recruitment to clathrin-coated pits J Biol Chem 275:31255–31265.

Uren AG, O'Rourke K, Aravind L, et al. 2000. Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT Lymphona Molecular Cell 6:961–967.

Vu TK, Hung DT, Wheaton VI, Coughlin SR. 1991. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation Cell 64:1057–1068.

Williams EB, Krishnaswamy S, Mann KG. 1989. Zymogen/enzyme discrimination using peptide chloromethyl ketones J Biol Chem 264:7536–7545.

Wunderwald P, Schrenk WJ, Port H. 1982. Antithrombin BM from human plasma: an antithrombin binding moderately to heparin Thromb Res 25:177–191.

Ye Z-H, Varner JE. 1996. Induction of cysteine and serine proteinases during xylogenesis in *Zinnia elegans* Plant Mol Biol 30:1233–1246.

Yu X-H, Perdue TD, Heimer YM, Jones AM. 2002. Mitochondrial involvement in tracheary element programmed cell death Cell Death Differ 9:189–198.