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Transient and Specific Expression of a Cysteine Endopeptidase Associated with Autolysis during Differentiation of *Zinnia* Mesophyll Cells into Tracheary Elements

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Endopeptidase activities during the differentiation of *Zinnia* cells into tracheary elements (TEs) were examined with several peptidyl 4-methyl-7-coumarylamido (MCA) as substrates. The activity that hydrolysed carbobenzoxy-Phe-Arg-MCA (Z-Phe-Arg-MCA) at pH 5 increased in a differentiation-related manner: this activity, which was not observed in freshly isolated mesophyll cells was induced by the combination of α-naphthaleneacetic acid (NAA) and 6-benzyladenine (BA) that is necessary for differentiation of TEs, but not by NAA or BA alone. The activity in cells cultured in TE-inductive medium that contained both NAA and BA increased very rapidly between the 48th and 60th hours of culture, when the number of TE increased rapidly. A protease responsible for this activity with a molecular mass of 30 kDa was partially purified from cells which had been cultured in the TE-inductive medium and included many immature TEs. Strong inhibition by [L-3-*trans*-carboxyoxiran-2-carbonyl]-L-Leu-agmatin (E-64), and activation by dithiothreitol (DTT) indicated that this protease belongs to a family of cysteine endopeptidases (EC 3.4.22).

Key words: Autolysis — Cell death — Cysteine endopeptidase (EC 3.4.22) — Tracheary element differentiation — *Zinnia elegans*.

The genetically programmed death of cells and tissues plays an essential role in the development in multicellular organisms. In higher plants, differentiation of parenchyma cells into tracheary elements (TEs) is one type of final differentiation that is accompanied by cell death (Fukuda 1992). During differentiation to TEs, cells first actively synthesize secondary walls. Then autolysis occurs and the vacuole and other cytoplasmic organelles, including the nucleus, plastids, mitochondria, Golgi apparatus and the endoplasmic reticulum, are disrupted to yield a hollow tube (O'Brien 1981). Although this autolytic process has been studied morphologically and histochemically by light and electron microscopy, the molecular basis of the autolytic process is still unknown. This process should involve increases in the activities of hydrolytic enzymes that degrade large molecules in cells. Elevated activities of hydrolytic enzymes, such as acid phosphatase and glycosidases, in the xylem as compared to other tissues have been demonstrated in histochemical studies (Gahan 1981). However, the true relationship of hydrolytic enzyme activities and TE differentiation has not been clarified because of the limitations of the plant system, in which only a small part of the cell population differentiates and biochemical analysis is, therefore, difficult.

Fukuda and Komamine (1980) established a system of TE differentiation in vitro in which single mesophyll cells isolated from *Zinnia* leaves redifferentiate into TEs semisynchronously and at high frequency. This system has contributed to recent progress in the study of TE differentiation (Fukuda 1992, 1994). The *Zinnia* system has allowed detailed characterization of the enzymes involved in secondary wall synthesis, such as xylan synthase (Suzuki et al. 1992), wall-bound peroxidase (Sato et al. 1993, 1995) and

Abbreviations: BA, 6-benzyladenine; Boc-, *t*-butyloxycarbonyl; Bz-, benzoyl; DTT, dithiothreitol; E-64, [L-3-*trans*-carboxyoxiran-2-carbonyl]-L-Leu-agmatin; MCA, 4-methyl-coumaryl-7-amido; NAA, α-naphthaleneacetic acid; PMSF, phenylmethylsulfonyl fluoride; Suc, succinyl; TE, tracheary element; TLCK, *N*-α-*p*-tosyl-L-lysine-chloromethyl ketone; Z-, carbobenzoxy.

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coenzyme A methyltransferase (Ye et al. 1994). A few novel genes that are expressed prior to morphological changes have also been isolated from *Zinnia* cells that are differentiating to TEs (Demura and Fukuda 1993, 1994, Ye and Varner 1993, 1994).

In early studies of the hydrolytic enzymes, Thelen and Northcote (1989) found that activities of DNase and RNase were closely associated with TE differentiation in *Zinnia* cells. One of the nucleases has been partially purified and characterized. In addition to nucleases, proteases can also be anticipated to play essential roles in the rapid degradation of cell contents during the autolysis that is associated with TE differentiation. Thomas et al. (1977) showed that the activity of leucine aminopeptidase was higher in immature TEs than in mature TEs or parenchyma cells in root tips of lima bean, although higher activity of this enzyme was not observed in induced wound vessel members. However, no other proteases have been analyzed in attempts to characterize the autolysis in TE differentiation.

In this paper, we demonstrate for the first time the involvement of a cysteine endopeptidase in the autolysis that accompanies differentiation of *Zinnia* cells to TEs.

Materials and Methods

Cell culture—Mesophyll cells were isolated from 14-day-old seedlings of *Zinnia elegans* cv. Canary bird and cultured in liquid medium as described by Fukuda and Komamine (1980, 1982). The differentiation-inductive medium (D medium) contained 0.1 mg liter^{-1} NAA and 1 mg liter^{-1} BA. Three media (CN, CB, CO) were used as control media in which TEs were not formed. CN medium contained 0.1 mg liter^{-1} NAA; CB medium contained 1 mg liter^{-1} BA; CO medium was hormone-free medium. The differentiation rate was defined as the number of tracheary elements divided by the number of non-differentiating living cells plus the number of tracheary elements.

Preparation of solution of a crude enzyme—Cells cultured for various periods of time were collected and stored at −80°C. The stored cells (1.0×10^6 cells) were ultrasonically homogenized in 1 ml of homogenization buffer [0.1 mM sodium phosphate (pH 7), 1 mM DTT, 1 mM EDTA]. The homogenate was centrifugated at $10,000 \times g$ for 20 min, and the resultant supernant was used for assays of endopeptidase activity as the solution of crude enzyme.

Assays of endopeptidase activity—Endopeptidase activities were assayed with various peptidyl MCA componds as substrates. The reaction mixture consisted of 50 μl of the solution of crude enzyme, 500 μl of 0.2 M buffer solution (sodium acetate at pH 5, sodium phosphate for pH 7, or a mixture of phosphoric, acetic and boric acids and NaOH at pH 10), and 447 μl of H_2O. Each of substrates was dissolved in dimethylsulfoxide to give concentration of 10 mM, 3 μl of which was added to 997 μl of the reaction mixture (final conc., 30 μM). The increase in emission at 460 nm with excitation at 380 nm was immediately monitored for 1 min at 25°C. The number of moles of 7-amino-4-methylcoumarin (AMC) formed was determined from a standard curve obtained with known ammounts of AMC. One unit of activity was defined as the amounts of enzyme that liberated 1 μmole of AMC per minute. Protein was quantitated by the method of Bradford (1976) with γ-globlin as the standard.

Purification of a protease—A solution of crude enzyme was prepared from 1.3×10^8 cells that had been cultured for 72 h in D medium. Solid ammonium sulfate was added to the solution to give 90% saturation. After centrifugation, the precipitated protein was dissolved in 3.5 ml of 20 mM sodium phosphate buffer (pH 7.0) and the solution was desalted by gel filtration on a PD-10 column (Pharmacia). The desalted solution of enzyme was then applied to a column ($\phi 30 \times 30$ mm) CM-Sepharose CL-4B that had been equilibrated with 20 mM sodium phosphate buffer (pH 7.0). The column was washed with 20 mM sodium phosphate buffer (pH 7.0), and eluted with 0.5 M NaCl in 10 mM sodium phosphate buffer (pH 7.0). The buffer in the fractions eluted with 0.5 M NaCl was replaced by 50 mM sodium phosphate buffer (pH 7.0) that contained 1.7 M ammonium sulfate on a filter (Molcut, Millipore). The sample was applied to a column of Phenyl Superose attached to an FPLC system (Pharmacia). The column was eluted first with 50 mM sodium phosphate buffer (pH 7.0) that containd 1.7 M ammonium sulfate, then with a gradient of 1.7 to 0 M ammonium sulfate in 50 mM sodium phosphate buffer (pH 7.0). Fractions corresponding to the first peak of activity were collected and concentrated with a Molcut filter. A Superose 12 column attached to an FPLC system was equilibrated with 50 mM sodium phosphate buffer (pH 7.0) that contained 0.15 M NaCl. The peak from column chromatography on Phenyl Superose was loaded and eluted with the same buffer as used for equilibration at a flow rate of 0.3 ml min^{-1}. A standard curve for the estimation of molecular mass was obtained with an LMW kit (Pharmacia)

Chemicals—All MCA substrates, leupeptin and pepstatin A were purchased from Peptide Institute Inc (Osaka, Japan). E-64 was purchased from Sigma, and PMSF was from Merck.

Results

Characterization of proteolytic activities in a crude extract of cultured Zinnia cells—Preliminary results obtained with hemoglobin and gelatin as substrates indicated that a rapid increase occured between 48 and 68 h in *Zinnia* cells cultured in the TE-inductive medium, and suggested the involvement of proteases in TE differentiation. In order to characterize the proteolytic activity that was expressed in

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association with TE differentiation, we examined the endopeptidase activity in crude extracts of cultured *Zinnia* cells using various MCA compounds as substrates. Z-Phe-Arg-MCA, Boc-Val-Leu-Lys-MCA and Bz-Arg-MCA were found to be efficient substrates for proteases in crude extracts of cultured *Zinnia* cell. Hydrolytic activities in cells cultured in D medium were much higher than those in cells cultured in the control medium (CN medium) under all conditions except when activity was measured with Bz-Arg-MCA as the substrate at pH 10 (Fig. 1). The dependence on pH of the hydrolytic activity against Boc-Val-Leu-Lys-MCA was similar to that against Z-Phe-Arg-MCA for cells cultured both in D and in CN medium. PMSF strongly inhibited the hydrolytic activity against Z-Phe-Arg-MCA at both pH 5 and pH 7, in particular in the case of cells cultured in D medium, but it hardly inhibited hydrolysis of Bz-Arg-MCA at pH 7 and pH 10 (Fig. 2). DTT strongly promoted the hydrolytic activity against Z-Phe-Arg-MCA, in particular at pH 5, while it did not have any promotive effect on the hydrolysis of Bz-Arg-MCA (Fig. 2). These results indicated that the main proteolytic activity in cells cultured in D medium was effective against Z-Phe-Arg-MCA. It had a pH optimum at pH 5, was activated by DTT, and was suppressed by PMSF. This activity was also inhibited completely by leupeptin (data not shown). The hydrolytic activity against Bz-Arg-MCA might have been due to a protease(s) different from the protease that hydrolyzed Z-Phe-Arg-MCA, because the former was high under neutral and basic conditions. Moreover, it was not suppressed by PMSF and it was found in cells cultured both in D and in CN medium.

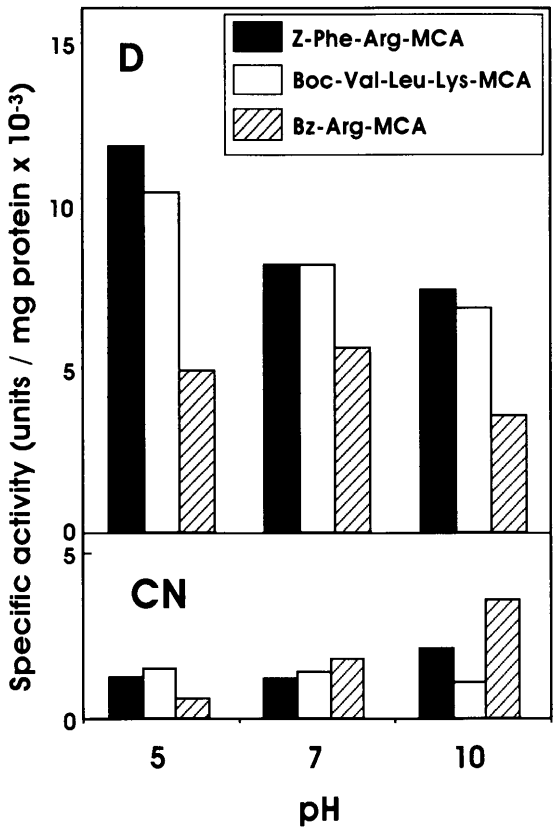

Fig. 1. Hydrolytic activities against Z-Phe-Arg-MCA, Boc-Val-Leu-Lys-MCA and Bz-Arg-MCA in extracts of *Zinnia* cells cultured in D and CN medium for 72 h. The solutions of crude enzyme from cultured cells were diluted with 0.1 M sodium acetate at pH 5 or with 0.1 M sodium phosphate at pH 7 or with a mixture of phosphoric, acetic and boric acids with NaOH at pH 10, and assayed with each MCA substrate. The increase in emission at 460 nm with excitation at 380 nm was immediately monitored for 1 min. In experiment shown in Figure 1 to Figure 3, the formation of TEs in D medium started about 54 h after the start of cultures, and the differentiation rate reached maximum about 80 h.

Expression of proteolytic activities during cell culture —Effects of plant hormones on the induction of proteolytic activities were examined with cells that had been cultured for 72 h in D medium (Fig. 3). Hydrolytic activity against Z-Phe-Arg-MCA at pH 5 was very high in cells that had been cultured in D medium. By contrast, there was no significant difference in hydrolytic activity against Bz-Arg-MCA at pH 10 between cells cultured in D and CN media or at pH 7 among cells cultured in any tested medium.

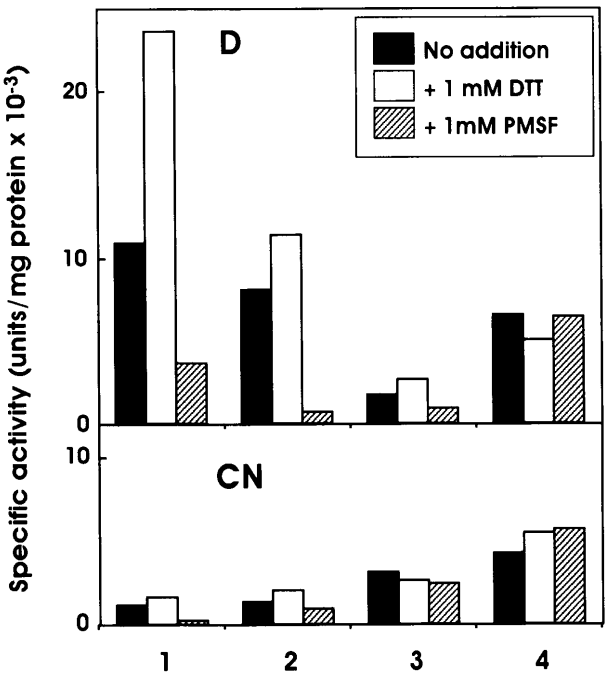

Fig. 2 Effects of DTT and PMSF on hydrolytic activities against Z-Phe-Arg-MCA and Bz-Arg-MCA in Zinnia cells cultured for 72 h in D and CN medium. The hydrolytic activities were measured with Z-Phe-Arg-MCA at pH 5 (1) and pH 7 (2) and with Bz-Arg-MCA at pH 5 (3) and pH 7 (4) in the presence of 1 mM DTT or 1 mM PMSF.

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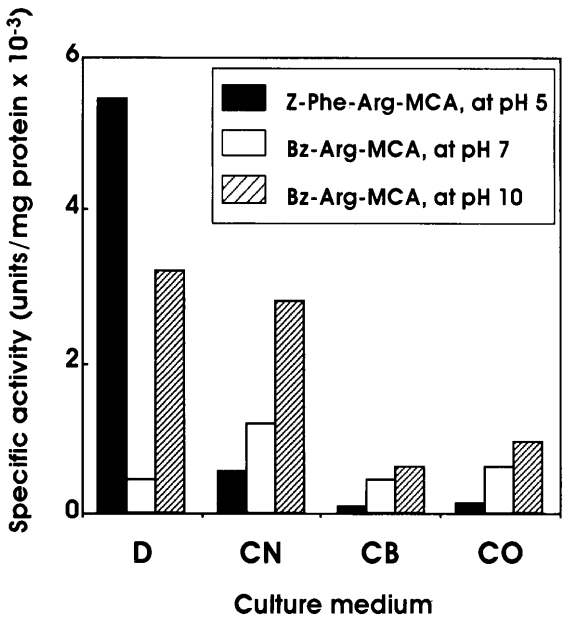

Fig. 3 Effects of hormones on hydrolytic activities against Z-Phe-Arg-MCA and Bz-Arg-MCA in *Zinnia* cells cultured for 72 h. *Zinnia* mesophyll cells were cultured in CO, CN, CB and D media. No TEs were observed in CN, CB and CO medium.

These results suggest that the elevated hydrolytic activity against Z-Phe-Arg-MCA at pH 5 is related to TE differentiation. Next, the time courses of changes in the three different activities were examined in cells cultured in D and CN medium (Fig. 4). In this experiment, the formation of TEs in D medium started about 48 h after the start of cultures, 46% of cells had differentiated at 60 h and the differentiation rate reached maximum (55%) at 72 h (Fig. 4-A). The hydrolytic activity against Bz-Arg-MCA at pH 7 was low and constant during culture both in D and in CN medium (Fig. 4-B). The hydrolytic activity against Bz-Arg-MCA at pH 10 showed a transient increase at 72 h of culture in cells cultured in D medium and those cultured in CN medium (Fig. 4-C). By contrast, the time course of changes in the hydrolytic activity against Z-Phe-Arg-MCA at pH 5 was quite different in cells cultured in D and CN media (Fig. 4-A). Freshly isolated mesophyll cells had no hydrolytic activity against Z-Phe-Arg-MCA. The activity in cells cultured in D medium increased very rapidly between 48 and 60 h of culture, with a subsequent rapid decrease, while the activity in cells cultured in CN medium did not show a marked increase. This dramatic and transient increase in the activity coincided with the timing of the rapid increase in the number of TEs. This pattern of expression of hydrolytic activity against Z-Phe-Arg-MCA strongly suggested that the protease(s) responsible for this activity is specific to TE differentiation.

Partial purification of a differentiation-specific protease—To purify the protease(s) with hydrolytic activity against Z-Phe-Arg-MCA at pH 5, we used a crude extract of cells that had been cultured for 72 h in D medium as starting material (Fig. 5 and Table 1). The protein in the crude extract, after precipitation by ammonium sulfate (90% saturation), was applied to a column of CM-Sepharose and hydrolytic activity against Z-Phe-Arg-MCA was eluted with 10 mM sodium phosphate buffer (pH 7) that contained 0.5 M NaCl (Fig. 5A). This step was so efficient that the specific activity of the protease increased from 22 mU mg^{-1} to 742 mU mg^{-1} (Table 1). The eluate from the CM-Sepharose column was applied to a Phenyl Superose column, which yielded a major peak of activity (Fig. 5B). The peak fraction was subjected to column chromatography on Superose 12 and a single peak of the activity was obtained (Fig. 5C). The molecular mass of the protein that corresponded to this peak was estimated to be approximately 30 kDa. SDS-PAGE indicated that several polypeptides were still present in this peak fraction (data not shown). The series of column-chromatographic steps increased the specific activity against Z-Phe-Arg-MCA 421-fold (Table 1).

Characterization of the partially purified protease—The purified fraction was used for characterization of the protease. The effects of various inhibitors and DTT on the activity of the purified protease are summarized in Table 2.

Table 1 Purification of a cysteine protease from cells cultured for 72 h in D medium

Step	Protein (mg)	Total activity (units × 10^{-3})	Yield (%)	Specific activity (units/mg × 10^{-3})	Purification (-fold)
Crude extract	51.4	421	100	8.19	1
$(NH_4)_2SO_4$ precipitation	7.48	166	39.4	22.2	2.71
CM-Sepharose	0.446	331	78.6	742	90.6
Phenyl Superose	0.0574	55.6	13.2	969	118
Superose 12	0.0015	5.17	1.22	3,450	421

In this experiment, the formation of TEs in D medium started about 60 h after the start of cultures, 26% of cells had differentiated at 72 h and the differentiation rate reached maximum several hours later.

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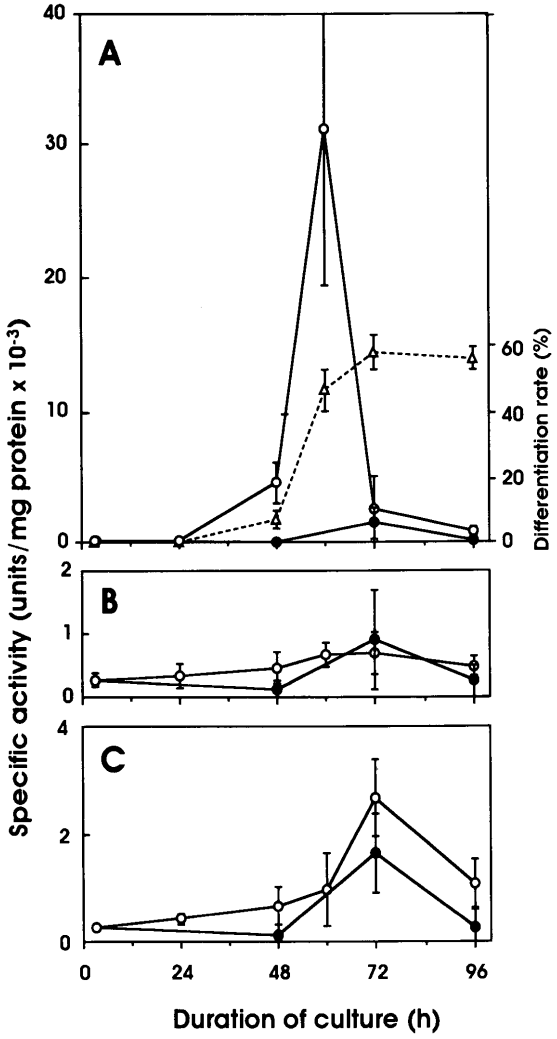

Fig. 4

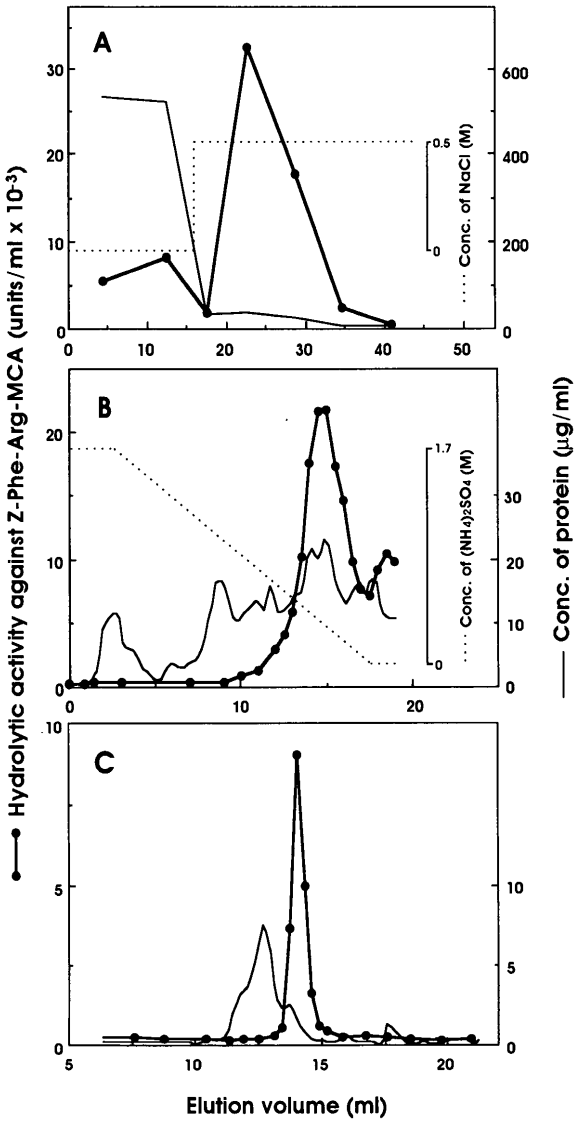

Fig. 5

Fig. 4 Time course of changes in hydrolytic activities against Z-Phe-Arg-MCA and Bz-Arg-MCA during culture of *Zinnia* cells. Hydrolytic activities in cells cultured in D medium (open circles) and CN (filled circles) medium were measured with Z-Phe-Arg-MCA at pH 5 (A) and with Bz-Arg-MCA at pH 7 (B) and pH 10 (C). Changes in differentiation rate during the culture are shown by triangles in A. Each point represents the mean value from three experiments with cells derived from different culture tubes.

Fig. 5 Purification of a protease that hydrolyzed Z-Phe-Arg-MCA from cells that had been cultured for 72 h in D medium. In this experiment, the formation of TEs in D medium started about 60 h after the start of cultures, 26% of cells had differentiated at 72 h and the differentiation rate reached maximum several hours later. A. Ion-exchange column chromatography on CM-Sepharose. A solution of crude enzyme was prepared from cells that had been cultured for 72 h in D medium. Protein was concentrated by precipitation with ammmonium sulfate, and desalted by gel filtration. The desalted enzyme solution was then applied to column of CM-Sepharose (ϕ30 × 30 mm) that had been equilibrated with 20 mM sodium phosphate buffer (pH 7.0). The column was washed with 20 mM sodium phosphate buffer (pH 7.0) and eluted with 0.5 M NaCl in 10 mM sodium phosphate buffer (pH 7.0). B. Hydrophobic-interaction chromatography on Phenyl Superose. After chromatography on a CM-Sepharose column, the eluate in 0.5 M NaCl was applied to a Phenyl Superose column. The protein was eluted first with 50 mM sodium phosphate buffer at pH 7 that contained 1.7 M ammonium sulfate, and then with a gradient of 1.7–0 M ammonium sulfate in 50 mM sodium phosphate buffer at pH 7. C. Gel filtration on Superose 12. The peak fraction after column chromatography on a Phenyl Superose was loaded onto a column of Superose 12 that had been equilibrated with 50 mM sodium phosphate buffer at pH 7 plus 0.15 M NaCl. The column was eluted isocratically at a flow rate of 0.3 ml min^{-1} with the same buffer as had been used for equilibration. Hydrolytic activity against Z-Phe-Arg-MCA at pH 5 in each fraction was measured.

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Table 2 Effect of protease inhibitors and DTT on the activity of the partially purified protease

Addition	Conc. (mM)	Relative activity
Pepstatin A	0.01	81
1,10-phenanthroline	1	247
PMSF	1	35
TLCK	0.1	0
Leupeptin	0.1	0
E-64	0.02	2
	0.1	0
DTT	0.1	240
	0.3	312
	1	336
	3	387
	10	346

Proteolytic activity was measured with Z-Phe-Arg-MCA as the substrate at pH 5, in the presence of the indicated concentrations of agents and was nomalized as the percentage of the activity of non-treated samples. Each value is the average of values obtained from two samples.

The activity was hardly inhibited by 0.01 mM pepstatin, an inhibitor of aspartic protease, or by 1 mM 1,10-phenanthroline, an inhibitor of metallo-protease. PMSF, an inhibitor of serine and cysteine proteases, at 1 mM diminished the activity by 65%. By contrast, the proteolytic activity was completely inhibited by 0.02 mM E-64, a specific inhibitor of cysteine proteases. The proteolytic activity was also completely inhibited by 0.1 mM TLCK and 0.1 mM leupeptin, which had been found to inhibit some cysteine proteases from plants (Ryan and Walker-Simons 1981). In addition, DTT of 0.3 to 10 mM increased the activity more than 3-fold. These results indicate that the activity in the purified fraction was a cysteine-type protease activity. The purified fraction was also tested for its activity to hydrolyze various MCA substrates (Table 3). This protease hydrolyzed all substrates tested but was most effective against Boc-Val-Leu-Lys-MCA and Z-Phe-Arg-MCA. These characteristics reflect those of the main activity that was specific to differentiation in crude extracts.

Table 3 Substrate specificity of the purified protease

Substrate	Relative activity
Z-Phe-Arg-MCA	100
Boc-Val-Leu-Lys-MCA	106
Bz-Arg-MCA	32
Z-Arg-Arg-MCA	19
Suc-Gly-Pro-MCA	18
Suc-Ala-Ala-MCA	14
Suc-Ala-Pro-Ala-MCA	14
Boc-Phe-Ser-Arg-MCA	8

Proteolytic activity was measured with one of the indicated MCA substrates at pH 5 and was nomalized as the percentage of the activity obtained with Z-Phe-Arg-MCA. Each value is the average of values obtained from two samples.

Discussion

In the present report, we show for the first time that a particular endopeptidase is expressed specifically during TE differentiation. We examined endopeptidase activities during TE differentiation of *Zinnia* cells, using several MCA compounds as substrates. These compounds have been used as a specific substrate for various proteases from animals, with Z-Phe-Arg-MCA, Boc-Val-Leu-Lys-MCA and Bz-Arg-MCA being specific substrates for cathepsin L, plasmin and trypsin, respectively. Cultured *Zinnia* cells had hydrolytic activities against all these MCA substrates. The hydrolytic activity against Bz-Arg-MCA in crude extracts of *Zinnia* cells was elevated under neutral and basic conditions and was not suppressed by PMSF. The hydrolytic activity against Z-Phe-Arg-MCA had a pH optimum of pH 5, and it was enhanced by DTT and suppressed by PMSF (Fig. 1, 2). These results indicate that different kinds of protease are present in crude extracts of cultured *Zinnia* cells. Among the activities, the hydrolytic activity against Z-Phe-Arg-MCA was expressed specifically in cells cultured in the presence of both NAA and BA, which induce TE differentiation, and not in cells cultured in the presence of NAA or BA alone (Fig. 3). Although freshly isolated mesophyll cells did not have this activity, the activity in cells cultured in TE-inductive medium increased very rapidly between 48 and 60 h of culture, when the number of TEs also increased rapidly, with a sudden subsequent decrease (Fig. 4). These results strongly suggest that a protease(s) that hydrolyzes Z-Phe-Arg-MCA is expressed in a close association with TE differentiation.

The hydrolytic activity against Z-Phe-Arg-MCA had a pH optimum around pH 5 both in crude extracts and in preparations of the partially purified protease (Fig. 1; data not shown). Proteases with low pH optima are localized in the central vacuoles of plant cells (Boller and Kende 1979). Burgess and Linsted (1984) observed the disruption of vacuoles at the late stage of differentiation of *Zinnia* cells into TEs by electron microscopy. This disruption of vacuoles occurred several hours after secondary wall thickening became visible and was followed by the loss of cytoplasm (A. Minami unpublished observation). After disruption of vacuoles, dramatic changes in ultrastructure of the nucleus, chloroplasts, mitochondria, and endoplasmic reticulum take place (Y. Watanabe, personal communication). These observations suggest that disruption of vacuoles which

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results in the release of hydrolytic enzymes with low pH optima into the cytoplasm, is a critical event in autolysis. The hydrolytic activity against Z-Phe-Arg-MCA in cells cultured in D medium increased very rapidly between 48 and 60 h of culture, at a time that corresponded to the time just before the disruption of vacuoles in differentiating TEs. The large amount of this hydrolytic activity and its broad substrate-specificity suggest that the role of the activity in autolysis is the rapid hydrolysis of many kinds of protein. We assume that this protease is newly synthesized and accumulated in the vacuole and, after disruption of vacuole, it is distributed throughout the entire protoplast so that the synchronous and rapid degradation of cell contents can be achieved.

The hydrolytic activity against Z-Phe-Arg-MCA was purified 421-fold from cells that had been cultured for 72 h in D medium. Strong inhibition of the activity by E-64 and activation by DTT indicated that this protease was a cysteine protease. In higher plants, cysteine proteases are the most abundant proteases among four classes of protease (cysteine, serine, metallo-, and aspartic proteases; Ryan and Walker-Simons 1981), and they have been studied extensively in relation to plant development such as germination (Hammerton and Ho 1986, Mitsuhashi et al. 1986) and senescence (Carroso and Carbonell 1990, Pladys and Vance 1993). Recently, genes encoding cysteine proteases were isolated and their expression of various developmental stages in plants were analyzed (Dietrich et al. 1989, Yamauchi et al. 1992, Watanabe et al. 1991). Granell et al. (1992), who studied GA-induced development of fruit and senescence of pea ovaries, found by in situ hybridization, that mRNA for *tpp*, which encodes a cysteine protease, was localized not only in senescing tissue but also in differentiating sclerenchymal cells in which autolysis was taking place. This localization coincides with that of neutral gelatin-degrading activity (Vercher et al. 1989). At later stages of anther development in tobacco flower, connective and stomium tissues are degraded and lost. A hybridization assay in situ by Koltunow et al. (1990) showed that TA56, which was isolated as an anther-specific gene, and which encodes a cysteine protease, was expressed in these tissues just before their degradation.

Genes specific to TE differentiation have been isolated by differential screening (Demura and Fukuda 1993, 1994) and subtraction techniques (Ye and Varner 1993, 1994) from cultured *Zinnia* cells. One of cDNA clones, p48h-17, has been suggested to have a similarity to a cysteine protease, although the nucleotide sequence remains to be determined (Ye and Varner 1993). The gene corresponding to p48h-17 might encode the protease shown that was partially purified in the present study.

In conclusion, our findings clearly indicate that a cysteine protease is involved in autolysis during TE differentiation. This protease may be a useful marker for the studies of the autolytic process during TE differentiation.

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