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A New Endopeptidase Activity Induced in the Last Stage of Tracheary Element Differentiation of *Zinnia* Cells

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Abstract

Endopeptidase activities during *in vitro* tracheary element (TE) differentiation of *Zinnia* cells were investigated using peptidyl 4-methylcoumaryl-7-amide (MCA) compounds as substrate. Endopeptidase activities against Boc-Val-Leu-Lys-MCA, with an acidic pH optimum and against Boc-Phe-Ser-Arg-MCA, with an alkaline pH optimum were found to be induced preferentially in cells cultured in TE differentiation-inductive medium, although the former activity appeared earlier than the latter one during the culture. Each activity was eluted as a single peak in DEAE-Sepharose column chromatography. The nature of Boc-Val-Leu-Lys-MCA hydrolyzing activity was similar to that of a papain-like cysteine protease as reported previously by our group. On the other hand, the partially purified Boc-Phe-Ser-Arg-MCA hydrolyzing activity was completely inhibited by leupeptin and weakly by E-64, but not by pepstatin A, 1,10-phenanthroline, or PMSF, suggesting that this activity results from a cysteine protease(s). These results reveal the Boc-Phe-Ser-Arg-MCA hydrolyzing activity is due to a novel cysteine protease with an alkaline pH optimum which may function during TE differentiation.

Key words: Boc-Phe-Ser-Arg-MCA, cysteine endopeptidase, tracheary element differentiation, *Zinnia*.

Abbreviations

Boc-, t-butyloxycarbonyl; Boc-FSR-MCA, t-butyloxycarbonyl-L-phenylalanyl-L-seryl-L-arginine 4-methyl-coumaryl-7-amide; Boc-VLK-MCA, t-butyloxycarbonyl-L-valyl-L-leucyl-L-lysine 4-methyl-coumaryl-7-amide; DTT, dithiothreitol; E-64, [L-3-*trans*-carboxyoxiran-2-carbonyl]-L-leucyl-agmatin; MCA, 4-methyl-coumaryl-7-amide; PMSF, phenylmethylsulfonyl fluoride; TE, tracheary element; Z-, benzyloxycarbonyl; Z-FR-MCA, benzyloxycarbonyl-L-phenylalanyl-L-arginine 4-methyl-coumaryl-7-amido.

Introduction

Tracheary element differentiation involves the formation of secondary walls and autolysis. In the last stage of TE maturation, cells degrade their protoplast without any help from other cells. This developmentally programmed cell death is a typical example of plant programmed cell death (Kuriyama and Fukuda, 2002). *In vitro* TE differentiation of isolated zinnia (*Zinnia elegans*) mesophyll cells (Fukuda and Komamine, 1980) has greatly contributed to study the molecular mechanisms in programmed cell death during TE differentiation (Reviewed by Fukuda, 2000).

We previously reported that an endopeptidase activity hydrolyzing Z-Phe-Arg-MCA at pH 5 was induced transiently and specifically in TE differentiation of *Zinnia* cells (Minami and Fukuda, 1995). The activity was partially purified by a series of column chromatography, and found to be a cysteine endopeptidase with apparent molecular mass of 30 kDa, which had an acidic pH optima. These characters were shared in papain-like cysteine proteases. Similar cysteine proteases were also found in differentiating TEs in *Zinnia* culture by Ye and Varner (1996). These papain-like proteases were encoded by two similar genes, p48h-17 (Ye and Varner, 1993, 1996) and ZCP4 (Demura *et al.*,

2002), which were expressed specifically in developing TEs and transiently at the time of autolysis. In addition to these cysteine proteases, serine proteases with molecular masses of 40, 59-60 and 144 kDa were identified by the activity-gel assay of crude extracts of cultured cells including differentiating TEs (Ye and Varner, 1996; Beers and Freeman, 1997; Groover and Jones, 1999). The papain-like proteases are accumulated in the vacuole of developing TEs and the rupture of the vacuole allows the proteases to release into the cytoplasm to attack the cell organelles (Kuriyama and Fukuda, 2002). Thus, although the study on the papain proteases progresses, other proteases remain to be solved.

In this study, therefore, we explored endopeptidase activity using various peptidyl-MCA compounds as substrates, and found a novel cysteine endopeptidase activity hydrolyzing Boc-Phe-Ser-Arg-MCA at alkaline pH, which was induced in the last stage of TE differentiation.

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 l^{-1} NAA and 1 mg l^{-1} BA. The non-inductive medium (CN medium) contained 0.1 mg l^{-1} NAA solely. 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 crude enzyme

Cells cultured for various periods of time were collected and stored at -80℃. The stored cells ($1.0x10^6$ 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 centrifuged at 10,000*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 compounds 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 for pH 4-6, sodium phosphate for pH 6.5-7.5, or Tris-HCl pH 8-9), 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 concentration, 30 μM). The increase in emission at 460 nm with excitation at 380 nm was immediately monitored for 1 min at 25℃. The number of moles of 7-amino-4-methylcoumarin (AMC) formed was determined from a standard curve obtained with known amounts of AMC. One unit of activity was defined as the amount of enzyme that liberated 1 μmole of AMC per minute. Protein concentration was quantitated by the method of Bradford (1976) with γ-globlin as a standard.

Separation of endopeptidase activities by column chromatography

A solution of crude enzyme which was prepared from $1.0x10^6$ cells that had been cultured for 72 h in D medium was desalted by gel filtration on a PD-10 column (Pharmacia). The desalted solution of enzyme was then applied to a DEAE-Sepharose CL-4B (Pharmacia) column (ϕ 15x35 mm). The column was washed first with 20 mM Tris-HCl (pH 7.5), then with a linear gradient of 0 to 0.5 M KCl in 20 mM Tris-HCl (pH 7.5).

Chemicals

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

Results

Changes in endopeptidase activities during TE differentiation

As the first step to explore the endopeptidase activity that was involved in TE differentiation, we examined the changes in endopeptidase activities against various MCA substrates with crude extracts from both cells cultured in D medium (D-cells) and cells cultured in CN medium (C-cells). A crude enzyme solution of *Zinnia* cells preferred MCA compounds with a basic amino acid residue at P1 site as a substrate (data not shown). When three tripeptidyl MCA substrates with a basic amino acid residue at P1 site, Boc-Val-Leu-Lys-MCA (Boc-VLK-MCA), Boc-Gln-Arg-Arg-MCA and Boc-Phe-Ser-Arg-MCA (Boc-FSR-MCR) were used, hydrolyzing activities against Boc-VLK-MCA and Boc-FSR-MCR were induced in TE-differentiation-specific manner (**Fig. 1**). A hydrolytic activity against Boc-VLK-MCA in the crude extract from D-cells was higher than that from C-cells, in all the pH region, especially in pH 5 to pH 7.5 (**Fig. 2A**). On the contrary, the Boc-FSR-MCA hydrolyzing activity in D-cells was higher in alkaline

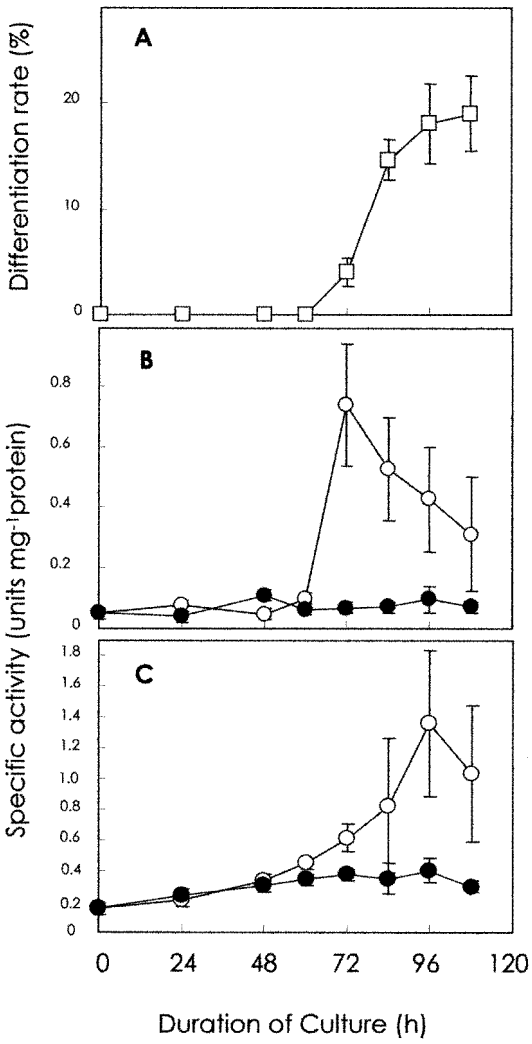

Fig. 1 Changes in endopeptidase activities during culture of *Zinnia* cells. Hydrolytic activities in cells cultured in D medium (open circles) and CN medium (filled circles) were measured with Boc-Val-Leu-Lys-MCA at pH 5 (B) and with Boc-Phe-Ser-Arg-MCA at pH 8 (C). Changes in differentiation rate during the culture are shown by squares in A. Each point represents the mean value from three experiments with cells derived from different culture tubes.

region over pH 7 than that in C-cells (**Fig. 2B**). We examined the time course of hydrolyzing activities against Boc-VLK-MCA and Boc-FSR-MCA (**Fig. 1**). In this culture, TEs appeared at 72 h and approximately 20% of cells differentiated at 96 h of culture (**Fig. 1A**). While no Boc-VLK-MCA hydrolyzing activity was induced in C-cells, it transiently increased at 72 h in D-cells (**Fig. 1B**). Similarly, Boc-FSR-MCR hydrolyzing activity was much higher in D-cells than in C-cells after 60 h of culture (**Fig. 1C**). Interestingly, the peak of Boc-VLK-MCA hydrolyzing activity preceded that of Boc-FSR-MCR hydrolyzing activity.

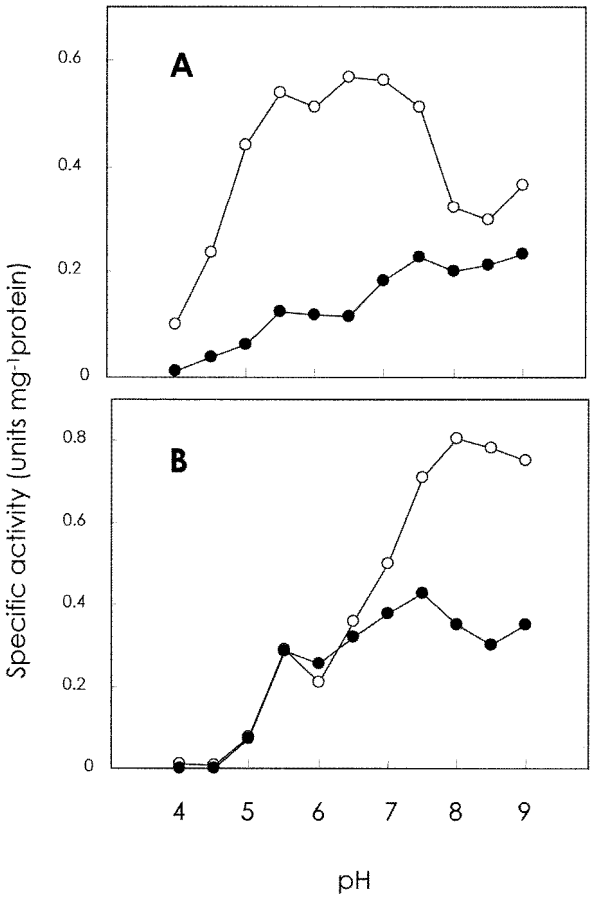

Fig. 2 pH profiles of hydrolytic activities against Boc-Val-Leu-Lys-MCA (A) and Boc-Phe-Ser-Arg-MCA (B) in cells cultured for 72 h in D medium (open circles) and CN (filled circles) medium.

Characterization of hydrolyzing activities against Boc-VLK-MCA and Boc-FSR-MCR

To confirm that hydrolyzing activities against Boc-VLK-MCA and Boc-FSR-MCR were derived from different enzymes, the crude enzyme from D-cells was subjected to DEAE-Sepharose CL-4B column chromatography and elution profiles of both activities were examined (**Fig. 3**). The Boc-VLK-MCA-hydrolyzing activity was eluted in a wide range of KCl concentration. A large peak at 320 mM KCl with a shoulder at 400 mM KCl, and a small peak at 140 mM KCl were observed. This elution profile may suggest the existence of many endopeptidases participating in hydrolysis of Boc-VLK-MCA at pH 5. In contrast, the Boc-FSR-MCR- hydrolyzing activity was eluted as a single peak at 280 mM KCl. This result clearly indicates that hydrolyzing activities against Boc-VLK-MCA and Boc-FSR-MCR results from different endopeptidases. Using a partially purified endopeptidase (280 mM KCl fraction in DEAE-Sepharose CL-4B column chromatography), the Boc-FSR-MCA-hydrolyzing endopeptidase was further characterized.

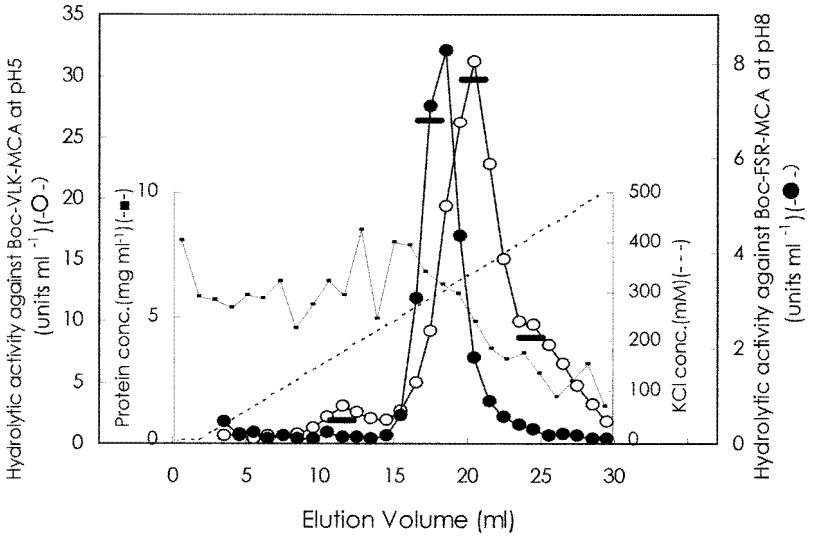

Fig. 3 Elution profile of hydrolytic activities against Boc-Val-Leu-Lys-MCA and Boc-Phe-Ser-Arg-MCA in DEAE-Sepharose CL-4B column chromatography. A crude enzyme solution of cells that had been cultured for 72 h in D medium was desalted and applied to DEAE-Sepharose CL-4B column. The proteins were eluted by a 0-500 mM KCl linear gradient. Protein concentration in each fraction (square) was measured by Bradford's method (Bradford, 1976). Endopeptidase activities were measured with Boc-Val-Leu-Lys-MCA at pH 5 (open circles) and with Boc-Phe-Ser-Arg-MCA at pH 8 (closed circles).

Table 1 Effects of protease inhibitors and DTT on endopeptidase activities separated by a DEAE-Sepharose CL-4B column.

Addition	mM	Relative activity			
		260 mM KCl	320 mM KCl	400 mM KCl	140 mM KCl
		Boc-FSR-MCA pH8	Boc-VLK-MCA pH5	Boc-VLK-MCA pH5	Boc-VLK-MCA pH5
pepstatin A	0.01	100	26	3	100
1,10-phenanthroline	2	88	53	33	129
PMSF	2	93	44	32	118
leupeptin	0.1	0	0	0	0
E-64	0.02	90	3	4	0
	0.1	73	3	4	N. D.
	0.2	53	N. D.	N. D.	N. D.
DTT	1	96	123	214	53

The crude enzyme from D-cells was subjected to DEAE-Sepharose CL-4B column chromatography and elution profiles of both activities were shown in **Fig. 3**. Activity of the partially purified endopeptidase (280 mM KCl fraction) and the Boc-FSR-MCA-hydrolyzing endopeptidase (320, 400, 140 mM KCl fractions) was measured in the presence of the indicated concentrations of agents, and was normalized at the non-treated samples. N. D., not determined.

Effects of protease inhibitors such as E-64, leupeptin, PMSF, pepstatin A, and 1,10-phenanthroline on the endopeptidase were examined (**Table 1**). PMSF (a serine protease inhibitor), pepstatin A (an aspartic protease inhibitor), and 1, 10-phenanthroline (a threonine protease inhibitor) did not inhibit substantially the Boc-FSR-MCR-hydrolyzing activity. In contrast, a cysteine protein inhibitor, leupeptin at 0.1 mM completely inhibited the activity. However, the inhibition of the activity by another cysteine protease inhibitor, E-64 was partial, and only 10% and 47% of the activity was inhibited by E64 at 0.02 mM and 0.2 mM, respectively. Dithiothreitol (DTT) does not affect the activity. These results suggest

that the Boc-FSR-MCR-hydrolyzing endopeptidase is a cysteine protease that is inhibited strongly by leupeptin and weakly by E-64. On the other hand, although the Boc-VLK-MCA-hydrolyzing activity in the three fractions (140 mM, 320 mM, and 400 mM KCl fractions) were completely suppressed by 0.1 mM leupeptin and 0.02 mM E-64, sensitivities to the other inhibitors and DTT varied among fractions. These results suggested that each fraction contains different cysteine endopeptidases hydrolyzing Boc-VLK-MCA or both the same cysteine endopeptidase and different other types of Boc-VLK-MCA-hydrolyzing endopeptidases (**Table 1**).

Discussion

In this study, we found endopeptidases hydrolyzing Boc-VLK-MCA at an acidic pH and Boc-FSR-MCA at an alkaline pH that were expressed in TE differentiation-specific manner. Although we could not purify the Boc-VLK-MCA-hydrolyzing enzyme, the following series of experiments strongly suggested that a major part of the activity resulted from a cysteine endopeptidase.

The characteristics of the cysteine endopeptidase, the acidic pH optimum (**Fig. 2**), the TE-specific expression pattern of the activity (**Fig. 1**), and the strong inhibition by leupeptin and E-64 (**Table 1**) were quite similar to the papain-type endopeptidase hydrolyzing Z-Phe-Arg-MCA (Z-FR-MCA), which we previously reported (Minami and Fukuda, 1995). Indeed, the purified papain-type enzyme could hydrolyze Boc-VLK-MCA (Minami and Fukuda, 1995). Molecular analysis by our and Ye's groups has indicated that the papain-type enzyme is encoded by *p48h-17* and *ZCP4* genes (Ye and Varner, 1993, 1996; Demura *et al.*, 2002). Therefore, a major part of the Boc-VLK-MCA-hydrolyzing activity is thought to be due to p48h-17 and ZCP4 enzymes.

On the other hand, the enzyme hydrolyzing Boc-FSR-MCA at an alkaline pH was novel. Boc-FSR-MCA is broadly used as a substrate of trypsin-type enzymes with an alkaline pH optimum (Kawabata *et al.*, 1988). However, inhibitor experiments suggested that Boc-FSR-MCA-hydrolyzing activity is rather due to a cysteine protease. This enzyme is also unique in that it exhibits different sensitivity to two cysteine protease inhibitors, leupeptin (severe) and E-64 (weak) (**Table 1**). These characteristics strongly suggest that the enzyme hydrolyzing Boc-FSR-MCA in TE-cell culture is a new enzyme that differs from papain-type cysteine proteases.

We now know that the vacuole rupture is a critical event of programmed cell death during TE differentiation, and soon after the vacuole collapses simultaneous degradation of cellular contents starts (Kuriyama and Fukuda, 2002). Many hydrolytic enzymes that have an acidic pH optimum, including the papain-like cysteine proteases are induced prior to TE morphogenesis and accumulate in the vacuole (Kuriyama and Fukuda, 2002). In the late stage of TE differentiation, the vacuole collapses rapidly and the enzymes that have been sequestered in the vacuole are released into the cytoplasm with the large amount of acidic vacuolar solution, allowing the acid enzymes to attack various organelles efficiently (Obara *et al.*, 2001). The Boc-FSR-MCA-hydrolyzing activity appeared specifically in culture containing developing TEs and peaked in the late stage of TE differentiation. Because this enzyme functions under an alkaline or neutral condition, it may not play a role in degradation of proteins after the vacuole ruptures in the late stage of TE differentiation. Therefore, this enzyme may act at a cellular event promoting autolysis in the cytoplasm prior to vacuolar rupture, for example, at initiating the vacuole rupture. In TE differentiation, vacuolar collapse was controlled and critical event for autolysis. Acid hydrolases were accumulated into vacuole, after the vacuole collapse, they were distributed into whole protoplast and rapidly degraded the cell contents. It is not plausible that the Boc-FSR-MCA-hydrolyzing enzyme of which pH optimum is alkaline is a member of vacuolar hydrolases. In maturation process of TE differentiation, hydrolases including papain-like proteases are released from maturing TEs into extracellular space, which should be harmful to surrounding cells. The Boc-FSR-MCA-hydrolyzing protease may be localized in apoplast of maturing TEs or nondifferentiating cells and degrade released hydorolytic enzymes. Recently, Endo *et al.* (2001) found that TED4 protein is secreted into the apoplastic space from developing TEs and xylem parenchyma cells and inhibits apoplastic proteolytic activity at maturation stage. Therefore the Boc-FSR-MCA-hydrolyzing enzyme may be secreted out of the cells to function in the apoplast. However, because the pH of the apoplast is normally acidic, it is unlikely that the acting site of the enzyme is the apoplast. Another possibility is that this enzyme may function in turnover of proteins in xylem parenchyma cells. In *Zinnia* culture, not only TEs but also xylem parenchyma cells are differentiated. Xylem parenchyma cells produce phenylalanine actively as the precursor of lignin monomers, which are supplied to TEs (Hosokawa *et al.*, 2001). The production of phenylalanine might be supported partly by rapid degra-

dation of proteins by the enzyme hydrolyzing Boc-FSR-MCA.

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