Enzymatic characterization of germination-specific cysteine protease-1 expressed transiently in cotyledons during the early phase of germination*

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*The nucleotide and amino acid sequences reported in this article for daikon DRCP26 have been submitted to DDBJ/EMBL/GenBank with accession number AB721307.

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Papain-like cysteine protease activity that shows a unique transient expression profile in cotyledons of daikon radish during germination was detected. The enzyme showed a distinct elution pattern on DEAE-cellulose compared with cathepsin B-like and Responsive to dessication-21 cysteine protease. Although this activity was not detected in seed prior to imbibition, the activity increased markedly and reached a maximum at 2 days after imbibition and then decreased rapidly and completely disappeared after 5 days. Using cystatin-Sepharose, the 26 kDa cysteine protease (DRCP26) was isolated from cotyledons at 2 days after imbibition. The deduced amino acid sequence from the cDNA nucleotide sequence indicated that DRCP26 is an orthologue of *Arabidopsis* unidentified protein, germination-specific cysteine protease-1, belonging to the C1 family of cysteine protease predicted from genetic information. In an effort to characterize the enzymatic properties of DRCP26, the enzyme was purified to homogeneity from cotyledons at 48 h after imbibition. The best synthetic substrate for the enzyme was carbobenzoxy-Phe-Arg-4-methylcoumaryl-7-amide. All model peptides were digested to small peptides by the enzyme, suggesting that DRCP26 possesses broad cleavage specificity. These results indicated that DRCP26 plays a role in the mobilization of storage proteins in the early phase of seed germination.

Keywords: cotyledon/cysteine protease/daikon radish/GCP-1/germination.

Abbreviations: ACTH, adrenocorticotrophic hormone; Boc, *t*-butyloxycarbonyl; CBCP, cathepsin B-like cysteine protease; DRCP26, 26 kDa daikon radish cysteine protease; E-64, *trans*-epoxysuccinyl-L-leucylamide-(4-guanidino)butane; GCP-1, germination-specific cysteine protease-1; MCA, 4-methylcoumaryl 7-amide; *pyr*, L-pyroglutamyl;

RD21, Responsive to dessication-21; *Suc*, succinyl; *Z*, carbobenzoxy.

Proteases play a key role in many aspects of plant physiology and development (1). Cysteine proteases in particular are involved in the mobilization of storage proteins during germination, and their expression can be regulated by various types of environmental stresses (2, 3). Recent genetic studies have indicated the involvement of cysteine proteases in programmed cell death and disease resistance, in addition to germination (4–8). The most investigated cysteine proteases are members of the papain-like cysteine protease family (MEROPS classification: clan CA, family C1). A number of genes encoding papain-like cysteine proteases have been identified in diverse plant species. In *Arabidopsis thaliana*, the papain-like cysteine protease family comprises ~30 genes, and their expression is regulated particularly during germination and senescence. Cathepsin B-like cysteine protease (CBCP) was first identified as a gibberellin-regulated gene during wheat germination (9). After imbibition, CBCP begins to be expressed in aleurone cells surrounding the endosperm, and plays a role in the mobilization of seed storage proteins and the support of seedling growth. In rice, oryzain α and γ are expressed only in seeds, but not in shoots or roots, and expression continues during germination with maximum expression being reached at 5 days after imbibition (10). TPE4A is also induced 3 days after imbibition during the germination of pea seed (11). In most cases, the expression of papain-like cysteine protease was investigated at the mRNA level. Cysteine proteases are synthesized as inactive precursors containing a propeptide at the N-terminus, and subsequently activated by the removal of the propeptide. Therefore, expression of enzyme activity is finally determined by activation of the proenzyme. Furthermore, cystatin, an endogenous cysteine protease-specific inhibitor, also plays a role in the regulation of cysteine protease activity (5, 12).

In an effort to delineate the activity profiles of papain-like cysteine protease in cotyledons of daikon radish during germination, E-64-sensitive protease activity was isolated from Responsive to dessication-21 (RD21) and CBCP by various chromatographic procedures and changes in enzyme activity of each fraction were analysed (13, 14). Cysteine protease activity that increased during germination could be isolated by

cystatin-Sepharose and the enzyme was identified by amino acid sequencing. Using this approach, the expression profiles of active CBCP and RD21 were investigated in cotyledons during germination at the protein level (13, 14). The level of CBCP transcript in cotyledons was highest at 2 days after imbibition and decreased gradually during days 3–8, whereas CBCP enzyme activity reached a maximum at 5 days after imbibition and subsequently decreased. CBCP and RD21 activity in daikon cotyledons is mainly regulated by an autocatalytic activation process of the pro-enzyme, although the respective mRNA expression is initiated by imbibition. Thus, the expression level of papain-like cysteine protease mRNA and enzyme activity is not always correlated. These results indicate that an understanding of both the transcriptional and post-transcriptional regulatory systems for cysteine proteases is necessary to delineate their roles in germination. Furthermore, characterization of enzyme properties, and in particular cleavage specificity, is important in identifying the physiological substrates of the enzymes. van der Hoorn *et al.* developed a method for protease activity profiling of plant papain-like cysteine proteases using a combination of biotinylated irreversible protease inhibitors and mass spectrometry (15). Using this method, active forms of XCP2, RD21, CBCP, AALP, TPE4A and aleurain-like proteases were identified in *A. thaliana*. Comprehensive analyses of transcriptome, proteome and activity profiling can reveal the presence of mRNA, protein and enzyme activity with high sensitivity. However, information concerning protease enzyme cleavage specificity is not obtained. Furthermore, there remain many cysteine protease-like proteins identified where active protease activity has yet to be demonstrated.

Recently, we identified E-64-sensitive carbobenzoxy-Phe-Arg-4-methylcoumryl-7-amide (Z-Phe-Arg-MCA) cleaving activity that is transiently expressed in the early phase of germination of daikon radish, with maximum expression being reached at 1–2 days after imbibition, followed by complete disappearance of activity at 5 days after imbibition. In this article, we purified the protein associated with this activity and its cDNA was cloned. Our results clearly indicated that this protease [26 kDa daikon radish cysteine protease (DRCP26)] is a daikon orthologue of the uncharacterized gene product germination-specific cysteine protease-1 (GCP1) (At4g36880, GCP1, UniProt ID: Q94B08) of *A. thaliana*. This article represents the first report detailing the enzymatic characterization of GCP1.

Experimental Procedures

Materials

Peptide 4-methylcoumryl 7-amide (MCA) substrates, glucagon, adrenocorticotrophic hormone (ACTH 1–24), leupeptin and *trans*-epoxysuccinyl-L-leucylamide-(4-guanidino) butane (E-64) were purchased from the Peptide Institute (Osaka). Sephacryl S-100 was purchased from GE Healthcare (Uppsala, Sweden). Egg cystatin was purified by the method of Anastasi *et al.* (16) and immobilized onto Sepharose 4B (17). All other chemicals used were of analytical grade.

Plant

Daikon radish (*Raphanus sativus*) seeds were allowed to imbibe water on filter paper at 20°C in the dark. After 2 days, embryos were grown at 20°C under long-day conditions (16 h light, 8 h dark) until collected. For enzyme purification, cotyledons at 2 days after imbibition were collected and stored at –30°C until use.

Enzyme assay

Endopeptidase activity was assayed using carbobenzoxy-Phe-Arg-4-methylcoumryl 7-amide (Z-Phe-Arg-MCA), unless otherwise indicated. The reaction mixture contained 0.1 M acetate buffer (pH 5.5) and 50 µM Z-Phe-Arg-MCA. The reaction was initiated by the addition of enzyme solution. Following incubation at 37°C for 10–20 min, the reaction was terminated and the MCA liberated was determined fluorometrically, as earlier described (18). One unit (U) was defined as the activity that produced 1 µmol of MCA per min at 37°C. Protein concentration was determined by the method of Bradford using BSA as a standard (19).

Expression analysis of Z-Phe-Arg-MCA cleaving activity following germination

Cotyledons (20 g) from germinating embryo (8, 24, 36, 48, 72, 144 h after imbibition) were homogenized with 100 ml of 20 mM acetate buffer (pH 6.5) containing 1 mM β-mercaptoethanol and 0.5 mM EDTA (buffer A). Following centrifugation at 20,000 × *g* for 10 min, the supernatant was fractionated by ammonium sulfate precipitation (60% saturation) and then dialysed against buffer A. The dialysate was applied to a DEAE-cellulose column (1.5 × 3 cm) and eluted with 20 ml of buffer A (unbound fraction) followed by 20 ml of buffer A containing 0.5 M NaCl (bound fraction). Protease activity using Z-Phe-Arg-MCA as substrate was assayed at pH 5.5 in the absence or presence of E-64 (50 µg/ml). The DEAE-cellulose unbound fraction was concentrated by ultrafiltration and then subjected to Sephacryl S-100 gel filtration.

Sequence analysis of Z-Phe-Arg-MCA cleaving enzyme

The fraction possessing Z-Phe-Arg-MCA cleaving activity (0.5–2.0 µmol/min) was incubated with cystatin-Sepharose (50 µl) at 4°C with continuous mixing. Following incubation for 16 h, cystatin-Sepharose was collected by centrifugation at 3,000 × *g* for 1 min, washed by centrifugation with 1 ml of buffer A containing 0.5 M NaCl and 0.5% Triton X-100 five times, and finally washed twice with 1 ml of buffer A. The cystatin-Sepharose was then suspended in 50 µl of SDS-PAGE loading buffer and treated at 95°C for 5 min. Following centrifugation, the supernatant was subjected to SDS-PAGE (12% gel) (20), and then electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Immobilon™, 0.45 µm Millipore, Bedford, MA, USA) according to the manufacturer's instructions. The protein band was detected by staining with Ponceau 3R, cut into small pieces and then applied to an automated protein sequencer (Shimadzu PPSQ-10, Kyoto, Japan).

Purification of Z-Phe-Arg-MCA cleaving activity from daikon radish

All purification procedures were performed at 4°C, unless otherwise indicated. Cotyledons of daikon radish (300 g, 48 h after imbibition) were homogenized in 1 l of buffer A using a Waring blender. The homogenate was centrifuged at 12,000 × *g* for 10 min at 4°C. The supernatant was fractionated using ammonium sulfate (60% saturation). The precipitate was dissolved in buffer A and dialysed against the same buffer. Following centrifugation at 12,000 × *g* for 10 min at 4°C, the resultant supernatant was concentrated by ultrafiltration (Amicon YM-10 filter). The concentrate was then applied to a DEAE-cellulose column (2.5 × 20 cm) equilibrated with buffer A and eluted with the same buffer. Z-Phe-Arg-MCA cleaving activity was eluted as two peaks with buffer A. The activity in the second peak was concentrated and ammonium sulfate was added to a final concentration of 1 M. Following centrifugation, the concentrate was applied to a HiLoad 16/10 phenyl-Sepharose column equilibrated with buffer A containing 1 M ammonium sulfate. Bound proteins were eluted using a linear gradient of ammonium sulfate (1–0 M) in the same buffer. The fraction possessing activity was concentrated, applied to a Sephacryl S-100 gel filtration column (2.0 × 100 cm) and then eluted with buffer A containing 0.1 M NaCl. The fraction possessing activity was eluted as a single peak, concentrated by ultrafiltration, dialysed against buffer A and then applied to a Mono-Q

column (0.8 × 1.5 cm) equilibrated with buffer A. The activity was eluted using a linear gradient of NaCl (0–0.2 M) in the same buffer. The purity of the enzyme in the fraction possessing activity was examined by SDS–PAGE. The fraction showing a single protein band on SDS–PAGE was used as a final preparation.

Cleavage specificity towards glucagon, ACTH (1–24) and somatostatin-28

For the cleavage specificity analysis, 5 nmol of peptide substrates, glucagon, ACTH (1–24) and somatostatin-28 were digested with purified enzyme (40 pmol) at 37°C for 20 h. The mixture was then acidified using trifluoroacetic acid and separated by RP–HPLC on a Gemini-NX5u C18 (150 × 4.6 mm) column (Phenomenex, Torrance, CA, USA), as earlier described (21). Purified peptide fragments were applied to glass fibre discs that had been coated with polybrene and then analysed using an automated protein sequencer.

Isolation of cDNA encoding catalytic domain of DRCP26

To isolate cDNA encoding the catalytic domain of daikon radish DRCP26, we designed PCR primers corresponding to the N-terminal sequence (KEALPETVDWR) of the purified enzyme and a sequence (VRGNTISSV) in the C-terminal region of *Arabidopsis* GCP-1. The sequences of the sense and antisense primers were 5'-AAAGAGGCGCTTCCAGAGACGGTTGAT TGG-3' and 5'-TCAAACACTGCTGATAGTATTTCCACGAA N-3' (terminal codon underlined), respectively. Total RNA was isolated from cotyledons of daikon radish at 2 days after imbibition using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. One microgram of total RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Wako Chemicals, Osaka, Japan) and a random hexamer. PCR was performed using 30 cycles of denaturation at 95°C for 1 min, annealing at 53°C for 30 s, followed by extension at 72°C for 1 min. The DNA sequence of the PCR product of expected size (720 bp) was determined.

Expression analysis of DRCP26 transcript during germination

The expression of DRCP26 transcripts in cotyledons (1, 2, 3, 4, 5, 6 and 8 days after imbibition) was analysed by RT–PCR as mentioned earlier. Actin 2 transcript was amplified by PCR using 5'-GCTGTT CTCTCCCTGTACGCCAGTG-3' as the sense primer and 5'-CCA GCAGCTTCCATTCCACGAACG-3' as the antisense primer. PCR was performed using 28 cycles (95°C, 30 s; 53°C, 30 s; 72°C, 30 s), and identification of the PCR products was confirmed by sequencing.

Degradation of storage proteins by DRCP26

Cotyledons (0.6 g) from germinating embryo (14, 40, 52 and 72 h after imbibition) were homogenized with 5.0 ml of buffer A. The homogenate (240 µg protein) was incubated with DRCP26 (1.5 mU) at 37°C in the absence and presence of E-64. After 24 h, the reaction mixture was mixed with SDS–PAGE loading buffer, treated at 95°C for 5 min and subjected to 12% SDS–PAGE.

Results

Z-Phe-Arg-MCA cleaving activity expressed transiently in the early phase of germination

Previously we showed that three enzymes, oligopeptidase B (OPB, serine protease), CBCP and RD-21, which can hydrolyse Z-Phe-Arg-MCA, are present in daikon radish cotyledons at 3, 5 and 8 days after imbibition (14). E-64 inhibits both CBCP and RD21 activity, but not OPB activity. These activities were separated by stepwise elution using NaCl with DEAE-cellulose chromatography (14). OPB, CBCP and RD21 were eluted by 50, 200 and 400 mM NaCl, respectively. Figure 1A shows a comparison of DEAE-cellulose elution profiles of Z-Phe-Arg-MCA cleaving activity in daikon radish cotyledons at 1 and 5 days after imbibition. After elution with buffer A containing 50 mM NaCl, CBCP and RD21 were eluted using a linear gradient of NaCl

(0.05–0.5 M) in buffer A. Although all Z-Phe-Arg-MCA cleaving activity from cotyledons at 5 days after imbibition bound to DEAE-cellulose, two activity peaks (I and II) were found in the unbound fraction in cotyledons at 1 day after imbibition. In contrast, RD21 and CBCP activities of cotyledons at 1 day after imbibition were lower than those in cotyledons at 5 days after imbibition. The Z-Phe-Arg-MCA cleaving activity of fractions I and II was completely inhibited by E-64 and cystatin, indicating that these enzymes are papain-like cysteine proteases. Figure 1B shows the elution profiles of fractions I and II on Sephacryl S-100 gel filtration. The Z-Phe-Arg-MCA cleaving activity in fraction I was eluted slightly faster than that of fraction II. The molecular mass of the protein associated with the Z-Phe-Arg-MCA cleaving activity of fractions I and II was estimated to be 25 and 20 kDa, respectively.

In an effort to identify the enzyme associated with the Z-Phe-Arg-MCA cleaving activity at the amino acid sequence level, the enzyme was further purified by phenyl-Sepharose chromatography (Fig. 2A and B), and the papain-like cysteine protease was then isolated using cystatin-Sepharose. Egg white cystatin is a tight-binding inhibitor of papain-like cysteine proteases (16). The Z-Phe-Arg-MCA cleaving activity of fractions I and II was completely absorbed by cystatin-Sepharose (data not shown), indicating that the Z-Phe-Arg-MCA cleaving activity is derived from papain-like cysteine proteases. Proteins bound to cysteine-Sepharose were eluted by heat treatment in SDS solution. As shown in Fig. 2C, a 26-kDa protein was detected in both fractions as a major cystatin-binding protein. The amino-terminal sequence of the 26 kDa protein in fractions I and II was determined (KEALPETVDWRQKGAVNAIKNQGT) and found to be identical in both fractions. These results strongly suggested that the Z-Phe-Arg-MCA cleaving activity in fractions I and II was derived from the same 26 kDa cysteine protease. The sequence shows a high degree of identity (83%) with the amino-terminal sequence (GKEVPETVDWRQKGAVNPIKDQGT) of *Arabidopsis* GCP1 (UniProt ID: Q94B08), corresponding to amino acid residues 142–165 as predicted from the cDNA (DDBJ accession number: AY043294). The amino-terminal sequence of the 26 kDa protein from cotyledons of daikon radish also shows high identity with the sequence of pseudotzain (amino acid residues: 129–152; DDBJ accession number: U41902) from *Pseudotsuga menziesii* (22), cysteine protease COT44 (97–120; UniProt ID: P25251) from *Brassica napus* (23), triticain-alpha (127–150; DDBJ no: AB267407) from *Triticum aestivum* (24) and oryzain-beta (137–160; DDBJ no: HQ827837) from *Oryza officinalis* (10). The amino acid identity of the N-terminal sequence of the 26 kDa protein with the corresponding region of pseudotzain, COT44, triticain-alpha and oryzain-beta is 67, 79, 71 and 67%, respectively.

Change in Z-Phe-Arg-MCA cleaving activity in cotyledons during germination

As mentioned earlier, the Z-Phe-Arg-MCA cleaving activity of the 26 kDa cysteine protease was separated

Fig. 1 Identification of E-64-sensitive Z-Phe-Arg-MCA activity transiently expressed in the early phase of germinating embryo.

(A) DEAE-cellulose column chromatography of daikon radish cotyledon extract at 1 and 5 days after imbibition. Extract of cotyledons (100 g) was applied to a DEAE-cellulose column (0.9 × 16 cm) equilibrated with buffer A [20 mM acetate buffer (pH 6.5) containing 1 mM β-mercaptoethanol and 0.5 mM EDTA] and washed with the same buffer. Oligopeptidase B was eluted with buffer A containing 50 mM NaCl (indicated by arrow), and RD21 and CBCP were eluted using a linear gradient of NaCl (0.05–0.5 M) in the same buffer. The elution starting points with buffer A containing 50 mM NaCl and the linear gradient of NaCl are indicated by arrows. (B) Sephacryl S-100 gel filtration of fractions I and II. Fractions I and II separated by DEAE-cellulose chromatography were applied to a Sephacryl S-100 column (2.0 × 100 cm) and eluted with buffer A containing 0.1 M NaCl. The column was calibrated using BSA (68 kDa), ovalbumin (45 kDa), soybean trypsin inhibitor (25 kDa) and cytochrome c (12 kDa).

Fig. 2 Identification of Z-Phe-Arg-MCA cleaving activity in fractions I and II. Fractions I (A) and II (B) obtained from DEAE-cellulose column chromatography were applied to a phenyl-Sepharose column and eluted using a linear gradient of ammonium sulfate (1.0 M) in buffer A as described in 'Experimental procedures' section. (C) The fraction with activity (indicated by a horizontal bar) was concentrated and incubated with cystatin-Sepharose. After washing with buffer A containing 0.5 M NaCl and 0.1% Triton X-100, the gel was rinsed with buffer A. Cystatin-Sepharose-bound proteins were eluted by SDS-heat treatment and then analysed by SDS-PAGE as described in 'Experimental procedures' section. The SDS-polyacrylamide gel was stained with Coomassie Brilliant Blue (CBB) R-250. For sequence analysis, cystatin-Sepharose-bound proteins resolved by SDS-PAGE were electroblotted onto a PVDF membrane and stained with Ponceau 3R. The 26 kDa band of fractions I and II was subjected to peptide sequence analysis.

from OPB, CBCP and RD-21 by DEAE-cellulose column chromatography. The Z-Phe-Arg-MCA cleaving activity of the 26 kDa cysteine protease was eluted as two peaks in the pass-through fraction. In an effort to determine the expression pattern of 26 kDa cysteine protease activity during germination, the change in Z-Phe-Arg-MCA cleaving activity in the unbound DEAE-cellulose fraction was analysed. Cotyledons at 8, 24, 36, 48 and 72 h after imbibition were extracted and fractionated by DEAE-cellulose chromatography as described in 'Experimental procedures' section. The Z-Phe-Arg-MCA cleaving activity of the unbound and bound fractions was assayed in the absence or presence of E-64, a specific inhibitor of papain-like cysteine proteases. Figure 3A shows the time course of Z-Phe-Arg-MCA cleaving activity during germination. Z-Phe-Arg-MCA cleaving activity in the DEAE-cellulose unbound fraction was not detected in seed prior to imbibition. E-64-sensitive activity in

the unbound fraction increased quickly following germination, reached a maximum at 2 days after imbibition and then decreased rapidly. E-64-insensitive activity was not found in the unbound fractions. On the other hand, both E-64-sensitive (CBCP + RD21) and E-64-insensitive (OPB) Z-Phe-Arg-MCA cleaving activity in the DEAE-cellulose bound fraction showed a linear increase after germination. The Z-Phe-Arg-MCA cleaving activity of the unbound fraction was further analysed by Sephacryl S-100 gel filtration. Figure 3B shows the gel filtration profiles of Z-Phe-Arg-MCA cleaving activity in the DEAE-cellulose unbound fraction from cotyledons at 24, 36, 48 and 72 h after imbibition. The activity in cotyledons at 24 and 36 h after imbibition was eluted as a single peak. However, a small shoulder of activity was found in tube numbers 52–53. The activity at 48 h after imbibition was eluted as two peaks. First and second peaks corresponded to the major and shoulder

Fig. 3 Change in E-64-sensitive and E-64-insensitive Z-Phe-Arg-MCA cleaving activity in cotyledons during the germination of embryo. (A) Cotyledon (20 g) extracts prepared from germinating embryo at 8, 24, 36, 48, 72 and 144 h after imbibition were fractionated by DEAE-cellulose chromatography as described in 'Experimental procedures' section. Enzyme activity of unbound (circles) and bound (squares) fractions was assayed in the absence (opened circles, opened squares) or presence (closed circles, closed squares) of E-64. (B) Sephacryl S-100 gel filtration of Z-Phe-Arg-MCA cleaving activity in the DEAE-cellulose unbound fraction. Cotyledon (20 g) extracts prepared from germinating embryo at 24, 36, 48 and 72 h after imbibition were fractionated by DEAE-cellulose chromatography and the unbound fraction was applied to a Sephacryl S-100 column as described in 'Experimental procedures' section. (C) Expression profile of DRCP26 transcript during germination. Total RNA was isolated from daikon cotyledons at 0, 1, 2, 3, 4, 5, 6 and 8 days after imbibition and analysed by RT-PCR using DRCP26-specific primers as described in 'Experimental procedures' section. Actin-2 transcript was amplified as an internal control.

of the cystatin/protease complex. Furthermore, although cDNA analysis indicated that DRCP26 is highly homologous to *Arabidopsis* GCP1 and *Brassica* COT44, both of these cysteine proteases have not been identified at the protein and enzyme levels. Therefore, we attempted to purify active DRCP26 to delineate its enzymatic properties. It is likely that DRCP26 activity reached a maximum at 2 days after imbibition (Fig. 2). Therefore, cotyledons at 2 days after imbibition were used for enzyme purification. An extract of cotyledons (300 g) collected at 2 days after imbibition was applied to a DEAE-cellulose column. Two active peaks (fractions I and II) in the unbound fraction (Fig. 1A) were pooled, concentrated and then subjected to phenyl-Sepharose chromatography. The active fraction was eluted using a linear gradient of ammonium sulfate (1–0 M) and then subjected to Sephacryl S-100 gel filtration as described in 'Experimental procedures' section. Following gel filtration, the specific activity of fraction II (2.0 U/mg) was markedly higher than that of fraction I (0.08 U/mg). Therefore, DRCP26 in fraction II was further purified by Mono-Q column chromatography. Figure 5A and B shows the elution profile of DRCP26 from the Mono-Q column. As shown in Fig. 5C, the final preparation showed a single 26 kDa band on SDS-PAGE. The specific activity of the final preparation was 2.30 U/mg. Purified DRCP26 was completely inhibited by 1 μ M E-64, leupeptin and antipain. EDTA and phenylsulfonylfluoride had no effect.

The cleavage specificity of DRCP26 was examined using synthetic substrate and model peptides. As shown in Table I, the enzyme showed strict cleavage specificity towards synthetic substrates possessing an arginine residue at the P1 position. The best synthetic substrate for DRCP26 was Z-Phe-Arg-MCA. The cleavage of tripeptidyl- and tetrapeptidyl-MCA substrates by the enzyme was negligible. On the other hand, the enzyme showed broad cleavage specificity towards model peptides (Fig. 6). Glucagon, ACTH (1–24) and somatostatin-28 were digested, and the cleavage sites were identified. Five glucagon-derived

Table I. Cleavage specificity of DRCP26 towards synthetic substrates.

Substrate	Relative activity (%)
Z-Phe-Arg-MCA	100
Z-Arg-Arg-MCA	17.3 $\pm$ 1.4
Boc-Leu-Arg-Arg-MCA	2.1 $\pm$ 0.67
Boc-Leu-Lys-Arg-MCA	1.2 $\pm$ 0.31
Boc-Val-Pro-Arg-MCA	0.68 $\pm$ 0.74
Boc-Phe-Ser-Arg-MCA	0.25 $\pm$ 0.10
Boc-Gln-Arg-Arg-MCA	0.68 $\pm$ 0.12
Boc-Glu-Lys-Lys-MCA	0.20 $\pm$ 0.12
Pyr-Arg-Thr-Lys-Arg-MCA	1.4 $\pm$ 0.17
Bz-Arg-MCA	0.41 $\pm$ 0.31

Enzyme activity was determined in 0.1 M acetate buffer (pH 5.5) using various substrates at a concentration of 50 μ M.

Fig. 5 Purification of DRCP26 by Mono-Q column chromatography. DRCP26 was finally purified to homogeneity by Mono-Q column chromatography as described in 'Experimental procedures' section. Elution profile of protein (A) and Z-Phe-Arg-MCA cleaving activity (B). The fraction with activity indicated by a horizontal bar in (A) was subjected to SDS-PAGE (12% gel) and stained with CBB R-250 (C).

Fig. 6 Cleavage of glucagon, ACTH (1–24) and somatostatin-28 by DRCP26. Glucagon, ACTH (1–24) and somatostatin-28 were digested with DRCP26 and digests were analysed as described in ‘Experimental procedures’ section. Cleavage products and major cleavage sites are indicated. Major and minor products obtained following digestion with DRCP26 are indicated by thick and thin lines, respectively. As a reference, cleavage products and cleavage sites of CBCP (13) and RD21 (14) are also shown.

peptides (Phe⁶-Thr⁷, Ser⁸-Lys¹², Lys¹²-Asp¹⁵, Trp²⁵-Met²⁷ and Met²⁷-Thr²⁹), eight ACTH (1–24)-derived peptides (Ser¹-Ser³, Met⁴-Arg⁸, Trp⁹-Gly¹⁰, Lys¹¹-Lys¹⁵, Lys¹¹-Gly¹⁴, Lys¹⁵-Lys²¹, Arg¹⁷-Lys²¹, Arg¹⁸-Lys²¹) and seven somatostatin-28-derived peptides (Ser¹-Met⁸, Ser⁴-Met⁸, Ala⁹-Lys¹⁴, Ala¹⁵-Lys¹⁸, Asn¹⁹-Phe²¹, Asn¹⁹-Trp²², Asn¹⁹-Lys²³) were identified. Glucagon peptides His¹-Thr⁵ and Ser¹⁶-Gln²⁴, ACTH peptide Val²²-Pro²⁴ and somatostatin-28 peptide Thr²⁴-Cys²⁸ were not identified. The glucagon-derived peptide Ser¹⁶-Gln²⁴ was not found when glucagon was digested with RD21. It is likely that these peptides were further digested into smaller peptides or amino acids by DRCP26. As shown in Fig. 6, the following cleavage sites were identified following the digestion of model peptides with DRCP26: glucagon Thr⁵-Phe⁶, Thr⁷-Ser⁸, Ser¹¹-Lys¹², Lys¹²-Tyr¹³, Asp¹⁵-Ser¹⁶, Gln²⁴-Trp²⁵, Leu²⁶-Met²⁷ and Met²⁷-Thr²⁹; ACTH (1–24) Ser³-Met⁴, Arg⁸-Trp⁹, Gly¹⁰-Lys¹¹, Gly¹⁴-Lys¹⁵, Lys¹⁵-Lys¹⁶, Lys¹⁶-Arg¹⁷, Arg¹⁷-Arg¹⁸ and Lys²¹-Val²²; somatostatin-28

Asn³-Ser⁴, Met⁸-Ala⁹, Lys¹⁴-Ala¹⁵, Lys¹⁸-Asn¹⁹, Phe²¹-Trp²², Trp²²-Lys²³ and Lys²³-The²⁴. These results indicated that the cleavage specificities of DRCP26, CBCP and RD21 differed. Compared with the cleavage profile of glucagon and ACTH by RD21, shorter peptides were generated following digestion with DRCP26. It is likely that DRCP26 has broader cleavage specificity than RD21.

Digestion of total proteins with DRCP26

In an effort to delineate the role for DRCP26 during germination, total proteins in cotyledons of germinating embryo (14, 40, 52 and 72 h after imbibition) were analysed by SDS-PAGE (Fig. 7A). In total, 31 kDa protein was detected as a major protein of cotyledons at 14 h after imbibition. This band decreased rapidly between 14 and 40 h although amount of other proteins were almost constant. To know whether DRCP26 can digest the 31 kDa protein, the homogenate of 14 h cotyledon was incubated with DRCP26. As shown in Fig. 7B, 31 kDa protein was not digested with

Fig. 7 Digestion of storage proteins by DRCP26. (A) Homogenate of daikon radish cotyledon at 14, 40, 52 and 72 h after imbibition was subjected to SDS-PAGE (10% gel) and stained with CBB R-250. The protein band (31 kDa) decreased during germination was indicated by arrow. (B) Digestion of storage proteins by DRCP26. Homogenate of daikon radish cotyledon at 14 h after imbibition was incubated with DRCP26 at 37°C for 24 h in the absence and presence of E-64 as described in 'Experimental procedures' section. After centrifugation, the reaction mixture (12 µg protein) was subjected to SDS-PAGE (12% gel) and stained with CBB R-250. Two protein bands (27 and 11 kDa) disappeared by the incubation with DRCP26 were indicated by arrows.

DRCP26, whereas 27 and 11 kDa proteins were digested with DRCP26. Digestion of these proteins with DRCP26 was completely inhibited by papain-like cysteine protease inhibitor, E-64.

Discussion

Recent genetic studies have shown the involvement of plant cysteine proteases in many physiologically important processes such as programmed cell death and disease resistance, in addition to germination and senescence (1, 2, 4-6). Genome analysis of *A. thaliana* has indicated the presence of 34 cysteine protease genes belonging to the Clan CA, C1 family. However, the enzymatic properties of most of these enzymes have yet to be clarified given the difficulties associated with purifying the enzymes and subsequent expression of active recombinant enzymes.

In this study, two fractions (I and II) possessing Z-Phe-Arg-MCA cleaving activity were identified in the DEAE-cellulose unbound fraction from cotyledons at 24 h after imbibition. This activity was transiently expressed during the initial stage of germination. Using cystatin-Sepharose, the cysteine proteases responsible for the cleavage of Z-Phe-Arg-MCA in the two fractions were isolated. DRCP26 was obtained from both fractions, and the amino-terminal sequence (20 residues) of both proteins completely matched. Gel filtration analysis of the Z-Phe-Arg-MCA cleaving activity in growing cotyledons indicated that molecular size of DRCP26 is changed during germination (Fig. 3). Processing of C-terminal region or oligosaccharide of the enzyme might affect behaviour of the enzyme on DEAE-cellulose and Sephacryl S-100. Further analysis is necessary to clarify structural difference of two forms of DRCP26 in fraction I and II. Cloning of a

cDNA encoding the catalytic domain of DRCP26 indicated that DRCP26 is a daikon orthologue of *Arabidopsis* GCP1. Although DRCP26 in fraction I (DRCP26-I) and fraction II (DRCP26-II) showed the same mobility on SDS-PAGE, DRCP26-I eluted faster than DRCP26-II on Sephacryl S-100. DRCP26-I might be associated noncovalently with a C-terminal small peptide. Although our data suggested that DRCP26-I is converted to DRCP26-II during the course of germination, further analysis is necessary. DRCP26 is an extremely unstable enzyme. The activity of the purified enzyme decreased to 50% following overnight storage in an icebath. SDS-PAGE showed that the enzyme is degraded by autolysis. Furthermore, storage of the enzyme at -30°C for 1 month resulted in almost complete loss of activity, and the absence of a 26 kDa band on SDS-PAGE. This instability and transient expression of the enzyme could account for the enzyme failing to be identified at the protein level, even by proteomics (27) or highly sensitive activity profiling analysis of cysteine proteases (15) of *A. thaliana*. Except for Brassicaceae, DRCP26 is highly homologous with putative cysteine proteases from grapes (*Vitis vinifera*, UniProt ID: F6H4F8, amino acid identity: 73%), kidney bean (*Phaseolus vulgaris*, UniProt ID: O24321, 70%), soybean (*Glycine Max*, UniProt ID: C6TFM1, 69%), upland cotton (*Gossypium hirsutum*, UniProt ID: G8H1L9, 69%), vetch (*Vicia sativa*, UniProt ID: Q41696, 68%) (28) and chickpea (*Cicer arietinum*, UniProt ID: Q39452, 66%) (29). These cysteine protease precursors comprise 325–364 amino acids, and do not possess a granulin domain at the C-terminus like GCP-1. Although transcript of the chickpea cysteine protease (cac, Q39452) gene was not detected in cotyledons of dry seed, transcript levels accumulated 24 h after imbibition and then decreased 96 h after imbibition (29). Similarly, analysis of vetch cysteine protease (UniProt ID: Q41696) transcript also revealed a developmentally regulated expression profile in germinating cotyledons (28).

On the other hand, transient expression of a cysteine protease at 4–10 days after imbibition (referred to as proteinase A) was purified from cotyledons of kidney bean (30, 31) and vetch (32). The molecular mass of purified proteinase A enzymes was estimated to be 29–30 kDa by SDS-PAGE, and these enzymes were found to degrade storage proteins such as vicilin, legumin and phaseolin (30–32). The amino-terminal sequence of proteinase A from kidney bean was determined to be LPLHVVDXXD, and this sequence does not match the predicted sequences from cDNAs encoding cysteine proteases (UniProt ID: O24321) homologous with DRCP26. Becker *et al.* purified proteinase A (UniProt ID: Q41697) from cotyledons of vetch seedlings and its cDNA was cloned (32). The amino acid identity of proteinase A to vetch cysteine protease (UniProt ID: Q41696), homologous with DRCP26 (identity: 68%), was 59%. Unlike the case with GCP-1, proteinase A possesses a KDEL (endoplasmic reticulum-retention signal) sequence in its C-terminus. The sequence of proteinase A is most similar to that of cysteine protease SH-EP from germinating mung bean

(33, 34), and includes the presence of a KDEL sequence at the C-terminus of its precursor. These results clearly indicate DRCP26 is not a proteinase A-related enzyme.

In conclusion, the enzyme purified in this study, DRCP26, is a novel member of the papain-like cysteine protease family. Based on the broad cleavage specificity and transient expression profile of DRCP26 immediately after imbibition, it is highly likely that DRCP26 plays an important role in initiating the mobilization of storage proteins. We showed that DRCP26 digested 27 and 11 kDa proteins in cotyledons of germinating embryo at 14 h after imbibition. Future work will focus on identification of substrate proteins for DRCP26.

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Conflict of Interest

None declared.

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