Characterization and cloning of cysteine protease that is induced in green leaves of barley

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ABSTRACT

A sodium dodecyl sulfate (SDS)-dependent cysteine protease was investigated in green leaves of barley (*Hordeum vulgare* L.) by measuring the release of 7-amino-4-methyl-coumarin (AMC) from a synthetic substrate, *N*-succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide (Suc-LLVY-MCA). The enzyme named *Hv*CP3 (*Horedeum vulgare* cysteine protease activated by 0.1% (w/v) SDS) increased well with the development of leaves, but it decreased drastically during senescence. *Hv*CP3 was purified 146-fold with a yield of 7.74% from the crude extracts by four steps of chromatography. The enzyme showed a broad pH optimum at pH 7 to 8 and the enzyme activity was activated about 10-fold by 0.1% (w/v) SDS. The molecular weight of the native enzyme was estimated to be approximately 50 k. SDS-polyacrylamide gel electrophoresis of the protease suggested that the protein was a complex that consists of 33 k and 18 k subunits. The enzyme activity was specifically inhibited by cysteine protease inhibitors such as 10 μM trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64) and 100 μM leupeptin to 5% and 4%, respectively. A full-length cDNA of *Hv*CP3 was cloned and its sequence displayed high similarity to other plant cysteine proteases of the papain family (C1A). The functional role of this protease as a maintainer in cytosol is also discussed.

Keywords: barley; cysteine protease; cysteine protease inhibitor complex; green leaves.

Abbreviations: AMC, 7-amino-4-methylcoumarin; *Hv*CP, *Horedeum vulgare* cysteine protease; PCR, polymerase chain reaction; Suc-LLVY-MCA, *N*-succinyl-Leu-Leu-Val-Tyr-4-methyl-coumaryl-7-amide.

The nucleotide sequence of *Hv*CP3-33 reported in this article has been submitted to DDBJ data libraries under accession number **AB377533**.

1. Introduction

The developmental transitions in plant leaves, for example, greening and senescence, are supported by precise regulation of protein synthesis and degradation. A number of proteases play a role in those processes as regulator, maintainer, or chaperon. The aspartic proteinases (APs) occur in various tissues in plants and have been isolated from several plants such as *Arabidopsis, Oryza,* and *Brassica*. The physiological functions of the protease have been investigated in vitro, and it is considered that APs regulate the conditions in plant cells by cleavage of various protein precursors [1-4]. Also, it was predicted that one of the APs from soybean (soyAP1) is involved in programmed cell death during the autolysis of immature tracheary elements and sieve tube cells [5-7]. Caseinolytic protease is known as a regulator of the development of plastids in dark and light conditions [8], and it serves to remove abnormal proteins [9-11]. In the mature leaves, the 26S proteasome complex is concerned with embryogenesis, hormone signaling, and senescence by degradation of short-lived and damaged proteins by the ubiquitin pathway [12, 13]. Similarly,

filamentation temperature-sensitive H (FtsH) protease is responsible for the turnover of short-lived protein in photosystem II, D1 protein [14, 15]. Furthermore, several proteases such as chloroplast nucleoid DNA-binding protein 41 (CND41) [16, 17] and senescence-associated gene 12 (SAG12) [18] are upregulated during senescence and degrade many proteins for recycling of nutrients.

Recent studies on proteases from plants have mostly concerned analyses of genes based on the sequencing of genomes of many plants. In addition to the amino acid sequence analyses and phenotype observations using several molecular biological techniques, more investigation on the protein level is necessary to further elucidate and better understand the functional roles of the enzymes, especially regulation at post-translational levels. In fact, despite a major cereal grain of barley in the world, little information is available concerning the properties and functions of proteases of barley, particularly on the protein level except for purification and characterization of gibberellic acid-induced cysteine endoproteases in aleurone layers [19-21], although at present more than 20 cDNAs of barley cysteine protease are registered according to web analyses. The study on protein level may be merely confirmation of results accomplished before in other plant system. However, it is significant and challenging topic from their elusive properties of the proteases such as native substrate and activation and also considering a major and useful crop of barley.

In this study, we describe the purification, biochemical characterization, and molecular cloning of the protease that is activated by 0.1% SDS. We operationally named the enzyme using a prefix of the species name to distinguish the sources together with CP (cysteine protease, as

determined by an inhibitor study and specific catalytic site, as shown in the Results), i.e., *Hv*CP3 (*Horedeum vulgare* cysteine protease activated by 0.1% (w/v) SDS, type 3). The suffix number is designated according to the requirement for SDS as described below.

2. Materials and methods

2.1. Plant materials

Barley (*Hordeum vulgare* L., cv. Shunrai) seeds were purchased from Iwakura Shubyou (Shizuoka, Japan). Barley seeds were planted in moist absorbent cotton and grown in a chamber under constant conditions at 23ºC, 74% humidity, and a photoperiod of 14-h light and 10-h dark or in the dark for etiolated seedlings. After incubation for seven days, leaves were detached and immediately processed. To induce senescence, shoots of barley seedlings were excised and placed in 500-ml beakers containing 100 ml distilled water. The seedlings were kept at 23ºC and 74% humidity for five days in complete darkness.

2.2. Chemicals

Chemicals and reagents were obtained from either Wako (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan), unless otherwise noted. *N*-Succinyl-Leu-Leu-Val-Tyr-4-methyl-coumaryl-7-amide (Suc-LLVY-MCA) and 7-amino-4-methylcoumarin (AMC) were obtained from The Peptide Institute (Osaka, Japan). Molecular weight markers were purchased from Bio-Rad and Sigma (Tokyo, Japan). DE52 was obtained from Whatman (Kent, England). HiLoad Superdex 200 gel filtration, Superdex 200 HR 10/30, and Q Sepharose Fast Flow columns and polyvinylidene difluoride (PVDF) membranes were purchased from GE Healthcare (Buckinghamshire, England).

2.3. Protease assay

Protease was assayed by quantifying the AMC released from the synthetic fluorogenic peptide Suc-LLVY-MCA by measuring the fluorescence intensity. The reaction mixture (total volume, 100 μl) consisted of 100 μM Suc-LLVY-MCA, 50 mM Tris (hydroxymethyl) aminomethane-HCl (Tris-HCl) buffer (pH 8.0), and 0.1% (w/v) SDS, unless otherwise noted. The reaction was then started by adding 20 μl enzyme solution and incubated at 37ºC for 30 min. After incubation, the reaction was stopped by adding 2 ml 1% (w/v) SDS in 0.1 M borate-NaOH buffer (pH 9.0). The hydrolysis activity was monitored by measuring fluorescence emission at 460 nm with excitation at 380 nm using a Hitachi fluorescence spectrophotometer, model F-2500 (Tokyo, Japan). AMC concentrations were estimated by comparison of their fluorescence intensities with that of authentic AMC.

2.4. Purification of protease

All steps were carried out at 4ºC, unless otherwise noted. Barley leaves (7-day-old, 500 g fresh weight) were homogenized with 250 ml of TGM buffer (50 mM Tris-HCl buffer (pH 7.5) containing 20% (v/v) glycerol and 2 mM β-mercaptoethanol). The homogenate was filtered through 6 layers of gauze, and the resulting filtrate was centrifuged at 12,000 x g for 10 min to remove cell debris. The supernatant was again centrifuged at 100,000 x g for 1 h, and the resulting supernatant was used as the crude extract. The crude extract was applied to a column (2.5 x 15 cm) of DE52 equilibrated with TGM buffer. The column was washed with three volumes of TGM buffer and eluted with 400 ml of TGM buffer containing a linear gradient of

NaCl (0-0.15 M). The fractions with high protease activity were collected and applied to a column (0.8 x 10 cm) of Q Sepharose Fast Flow equilibrated with TGM buffer containing 0.1 M NaCl. The column was washed with three volumes of the same buffer and eluted with 200 ml of TGM buffer containing a linear gradient of NaCl (0.1-0.3 M) using an ÄKTA Fast protein-liquid chromatography (FPLC) system (GE Healthcare). The fractions with high activity were collected and concentrated with a Centriplus YM-10 (Millipore, Bedford, MA). The concentrated sample was loaded onto a HiLoad Superdex 200 gel filtration column (1.6 x 60 cm) equilibrated with TGM buffer containing 0.15 M NaCl from ÄKTA-*prime* (GE Healthcare). Proteins were eluted with the same buffer. The fractions with high activity were collected, and concentrated with a Centricon YM-10 (Millipore) and characterized. For further purification, after subjecting the HiLoad sample to native-PAGE, the active band was cut out from the gel, and finally the specific band of the protease was obtained.

2.5. Molecular weight determination

The molecular weight of the enzyme was estimated using Superdex 200 HR gel filtration chromatography and SDS-PAGE by comparison with standard proteins. The standard proteins used for gel filtration were alcohol dehydrogenase (M_r 150 k), bovine serum albumin (BSA) (M_r 66 k), and cytochrome *c* (M_r 12.4 k). For SDS-PAGE, myosin (M_r 200 k), β-galactosidase (M_r 116 k), phosphorylase *b* (M_r 97.4 k), BSA (M_r 66 k), ovalbumin (M_r 45 k), carbonic anhydrase (M_r 31 k), and trypsin inhibitor (M_r 21.5 k) were used as standards.

2.6. Electrophoresis

SDS-PAGE was performed using 12% (w/v) polyacrylamide gel with 0.1% (w/v) SDS. Native-PAGE was carried out in 8% (w/v) polyacrylamide gel without SDS. The gels were stained by Coomassie Brilliant Blue (CBB) R-250 or silver-stained.

2.7. Amino acid sequence analysis

The purified enzyme was separated by SDS-PAGE in 12% gels and then cut into two portions of the core protein and 18 k subunit. These proteins were electrophoretically transferred onto a PVDF membrane (GE Healthcare) and subjected to PPSQ-21A protein sequencer (Shimadzu, Kyoto, Japan) to analyze amino acid sequences. To determine the internal amino acid sequence, the protein band was excised and cleaved in gel with a V8 protease (Roche, Penzberg, Germany) before immobilizing as described by Cleaveland et al. (1977) [22]. Database comparisons were performed using BLAST.

2.8. Molecular cloning of HvCP3-33

Total RNAs were isolated from leaves of barley, basically according to the method of Chomczynski and Sacchi (1987) [23]. Total RNA concentrations were determined by UV spectrophotometry. The RNA was used as a template for first strand cDNA synthesis using a PrimeScript Reverse Transcriptase (Takara Bio Inc., Shiga, Japan), following the protocols supplied by the manufacturer. For the next polymerase chain reaction (PCR), reverse transcriptase (RT)-PCR, based on the partial amino acid sequences, degenerate primers were designed as follows: forward primer, 5'-TGYGGRASBTGYTGGGCNTTYTC-3' and reverse primer, 5'-ACRAKCCAGTAGTCYTTSCC-3', where N indicates any, B indicates (C + G + T), K

indicates (G + T), S indicates (C ＋ G), R indicates purines (A + G), and Y indicates pyrimidines (C + T). A partial cDNA (471 bp) was amplified using PrimeSTAR HS DNA Polymerase (Takara Bio) with primers for 30 cycles at 98°C for 10 s, 55°C for 15 s, and 72°C for 30 s. To identify the 3'-end, 3'-rapid amplification of cDNA ends (3'RACE) was carried out. Sequence-specific primers were designed from the results of RT-PCR as follows: primer 1, 5'-AACCTGTGGAACAGCACTTG-3'. A partial cDNA (858 bp) was amplified using PrimeSTAR HS DNA Polymerase with primer 1 and an adapter primer, 5'-GGCCACGCGTCGACTAGTAC-3', for 30 cycles at 98°C for 10 s, 55°C for 15 s, and 72°C for 30 s. To identify the 5'-end, 5'-rapid amplification of cDNA ends (5'RACE) was carried out. Sequence-specific primers were designed as follows: primer 2, 5'-CTCCTCAGAGTCAATACCAC-3' and primer 3, 5'-CAATCTGGTTGATGCCTTCG-3'. Total RNA from leaves was used as a template with primer 3 to produce single-strand cDNA. After a homopolymeric tail had been attached to the 5'-end of the cDNA using TdT and dATP, the synthesized cDNA was purified with a SUPREC-02 system (Takara Bio). Tailed cDNA was amplified using PrimeSTAR HS DNA Polymerase with primer 3 and adapter primer for 30 cycles at 98°C for 10 s, 55°C for 15 s, and 72°C for 30 s. The PCR product was cloned into a pCR Blunt vector and its DNA sequenced.

2.9. Reverse transcription-PCR

Total RNAs were extracted from barley leaves at several stages of development, including senescence, and isolated using TriPure Isolation Reagent (Roche Applied Sciences, Indianapolis,

IN) according to the manufacturer's protocol. For the first-stand cDNA synthesis, 2 μg of RNA was reverse transcribed in a total volume of 35 μl containing 2 μl of 2.5 μM oligo dT primer, 8 μl of 2.5 mM dNTP mixture, and 100 U of Rever Tra Ace (Toyobo, Osaka, Japan) in a 5x reaction buffer. The condition was 40°C for 1 h, followed by 95°C for 5 min to inactivate the reverse transcriptase. cDNA synthesized from approximately 20 ng of total RNA was used as PCR templates. PCR was performed in a 25 μl containing a 0.3 μl aliquot of a cDNA reaction, 2.5 μM gene-specific primers, 2.5 mM dNTP mixture, 0.75 U of PrimeSTAR HS DNA Polymerase, and the reaction buffer. The PCR program was 98°C for 2 min, followed by 35 cycles of denaturation (98°C for 10 s), annealing (55°C for 15 s), and extension (72°C for 30 s), and a single extension at 72°C for 10 min. The primers used were: *Hv*CP3 forward primer, 5'-GGATCCATGAGGACCTCCATG-3'; *Hv*CP3 reverse primer, 5'-GTCGACTGGTACCTGGCGCTG-3'. Actin primers were used as control samples.

2.10. BLAST search

The analogous genomic and amino acidic sequences of *Hv*CP3 were obtained by searching against non-redundant NCBI databases (http://www.ncbi.nlm.nih.gov/BLAST/).

2.11. Protein assay

Protein concentrations were determined by the method of Bradford (1976) [24] with BSA as the standard.

3. Results

3.1. Separation and change of activity with barley development

To search for proteases which involve in the developmental transitions from greening to senescence, we analyzed proteases with a chymotrypsin-like activity using Suc-LLVY-MCA cleaving activity that shares with different types of proteases including proteasome, calpain, and cysteine proteases. Three types of proteases that required SDS were operationally separated from the extracts of mature green leaves (see below) of barley after DE52 chromatography (Fig. 1A). In addition to a non-SDS-requiring type (*Hv*CP1), two peaks were separated that depended on the concentration of SDS used, *Hv*CP2 at 0.02% and *Hv*CP3 at 0.1%. Each activity of these proteases was measured in the same manner as above at four developmental stages, etiolated (7-day grown in the dark), greening (4-day grown under 14-h light and 10-h dark), mature (7-day grown under 14-h light and 10-h dark), and senescent (detached mature + 5 days in the dark) (see *2.1 Plant materials*). *Hv*CP3 increased during the developmental stage and reached a maximum at the mature stage, but decreased to 10% at senescence (Fig. 1B), and it corresponded to about 32% of the total enzyme activity at maturity and 9% at senescence. The decrease in activity of the other proteases was relatively low at senescence and retained more than 50% to 70% of the level at maturity. Thus the protease activated with 0.1% (w/v) SDS showed characteristic feature of a much more drastic disappearance during senescence than other two proteases activated with 0.2% SDS and non-requirement, which had relatively comparable activities in all stages. We hence selected this protease for the following study on the protein level.

3.2. Purification of protease

The protease extracted from green leaves of barley was purified through four steps of successive chromatography using DE52, Q Sepharose Fast Flow anion-exchange, and HiLoad Superdex 200 gel filtration columns. This procedure purified the enzyme to a 146-fold increase in specific degrading activity as estimated by hydrolysis of Suc-LLVY-MCA in the presence of 0.1% SDS from the crude extract, and the overall yield was approximately 7.7% (Table 1). Large amounts of non-specific proteases were effectively removed by DE52, although the specific activity was not much increased. The HiLoad Superdex 200 step was highly effective for purification. The elution profile showed that peaks of activity and proteins were fairly matched and that no other peaks were found. However, the purified protease was not a single band judging from SDS-polyacrylamide gel electrophoresis (PAGE), indicating that the sample was still not homogeneous. To purify it further, Superdex 200 HR 10/30 gel filtration was applied. The enzyme yield was severely lost without further purification, however. Instead, after subjecting the HiLoad sample to native-PAGE, the active band was cut out from the gel, and finally the specific band of the protease was obtained. Analysis of this sample with SDS-PAGE obtained two main bands with molecular weights of approximately 33 k and 18 k.

3.3. Determination of native molecular weight

The molecular weight of the protease in native conditions was determined by Superdex 200 HR gel filtration with FPLC. Linear relationships between the elution volume, V_e and the log molecular weight markers were obtained. The molecular weight of the protease was calculated to be approximately 50 k. This value is in good agreement with the sum of the molecular weight of

the two protein bands determined by SDS-PAGE, indicating that the protease consisted of two different subunits of 33 k and 18 k. Hereafter, we will designate them as *Hv*CP3-33 (33 k protein) and *Hv*CP3-18 (18 k protein), as necessary.

3.4. pH optimum

The pH optimum of the protease activity was examined with a mixture of buffer containing 50 mM 2-[*N*-morpholino]ethanesulfonic acid (MES), 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), and N-[tris(hydroxymethyl)methyl]glycine (tricine) in the pH range from 5.5 to 8.5. The protease had a broad maximum activity from neutral to alkaline pH at a value of 7 to 8.

3.5. SDS-dependency

The protease purified in this study was an SDS-dependent protease similar to the maize protease, cysteine protease of protease-inhibitor complex (CPPIC), reported by Yamada et al. [25-27], although their pH optimums were clearly different. As shown in Fig. 2, the results of SDS-dependency showed high activity at a concentration of 0.1% (w/v) SDS, but it was inhibited at concentrations higher than 0.1%. This finding suggests that SDS is essential for the activity of the protease in vitro, but that excess amounts of SDS are inhibitory.

3.6. Kinetics analysis

Kinetic analysis was applied to the chymotrypsin-like activity of the protease to elucidate the action mechanism. A Lineweaver-Burk plot of triplicate data of nine concentrations of Suc-LLVY-MCA from 10 μM to 1,000 μM gave a linear line, and the K_m value was calculated to be

128 μM.

3.7. Effect of inhibitors

The catalytic mechanisms of the protease were investigated by examining the effects of several types of inhibitors (Table 2). The protease activity was remarkably inhibited by cysteine protease inhibitors such as trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64) (95% inhibition) and leupeptin (93% inhibition) at 10 μM, although *N*-ethylmareimide (NEM) at 100 μM had moderate effect (49% inhibition) which was similar to the stress-induced cysteine protease from *Chlamydomonas* [28]. The serine protease inhibitor phenylmethylsulfonic fluoride (PMSF) inhibited it by 21% at 100 μM, but rather activated it at the higher concentration of 1 mM (109%). Addition of metals such as Mg and Zn and ethylenediaminetetraacetic acid (EDTA) had nearly no effect. These results indicate that this protease is a member of the cysteine proteases.

3.8. Thermal stability

The thermal stability of the enzyme was investigated at temperatures from 30ºC to 80ºC. Residual activity of the enzyme was assayed in the standard conditions after incubation at the indicated temperature for 10 min in the absence of substrate. The temperature at which the activity of the enzyme was decreased by half was 55ºC.

3.9. Determination of N-terminal sequence and cloning of cDNA for HvCP3-33

After being subjected to SDS-PAGE, the active enzyme band was transferred onto a PVDF membrane, and N-terminus amino acid sequence of the enzyme was analyzed using a PPSQ-21A protein sequencer (Shimadzu). Ten amino acid residues of the N-terminus of the enzyme (33 k)

were determined (see Fig. 3). A homology search was performed using the amino acid sequence obtained and the NCBI BLAST protein search program. Homologous sequences were found in proteins of several plants including monocotyledons such as wheat, rice, and maize.

From the RT-PCR and 3'RACE results, the partial cDNA of *Hv*CP3-33 that corresponds to the 1193 bp containing the untranslated region was sequenced. In order to obtain the full sequence-coding region of *Hv*CP3-33, 5'-rapid amplification of cDNA ends (5'RACE) was carried out. Finally, the 1675 bp cDNA fragment from the initiation codon, ATG, to the termination codon, TAA, was cloned by a combination of PCRs with the template synthesized from *H. vulgare* RNA. We named this gene *HvCP3-33* and the nucleotide sequence reported in this article has been submitted to the DNA Data Bank of Japan (DDBJ) with accession number AB377533.

The amino acid sequence deduced from the nucleic acid sequence was 462 amino acid residues excluding a propeptide corresponding to a molecular weight of 35.6 k, which is fairly close to the value obtained from SDS-PAGE. The deduced polypeptide has a Cys-His-Asn residue (Fig. 3B, asterisk), which is presumed to be an active site of cysteine proteases, and peptidase and C-terminus extension sequences including a Pro-rich region and a granulin domain that has high homology to animal proteins of the epithelin/granulin family [29]. The primary structure of this enzyme including domains and motif is schematically illustrated in Fig. 3A

The preliminary results of amino acid sequence analysis of 18 k protein subunit (*Hv*CP3-18) indicated that internal sequences contained LQELARFAV. These sequences are well matched to those of highly concerved unique sequence of cysteine protease inhibitor cystatins (in preparation).

This protein is thus assumed to be cystatin and further this protease is a complex of a cysteine protease and cystatin. Similar complexes are found in several higher plants, including one of the SDS-dependent cysteine proteases from maize, CPPIC [25-27], which has similar characteristics to this barley enzyme.

3.10. Comparison of amino acid sequence of HvCP3-33 with other plant cysteine proteases

Homology searching against the deduced amino acid sequence of *Hv*CP3-33 was performed with BLAST programs using the NCBI databases GenomeNet BLAST2. *Hv*CP3-33 shared obviously high homology with other plant cysteine proteases belonging to the papain (C1A) family, as shown in Fig. 3B. *Hv*CP3-33 showed particularly high homology to the genes such as wheat triticain α (97%) (NCBL accession number BAF02546), rice orizain α (93%) [30], maize Mir3 (87%) [31], and *Arabidopsis thaliana* RD21 (83%) induced by drought and salt stress [32]. Besides the above, the protease showed substantial homology to maize CPPIC (91%) [26] and carrot DcCysP1 (80%) [33]. These proteases characteristically had amino acid residues, Cys-His-Asn (asterisks in Fig. 3B), constituting a catalytic triad that contributes to the activity of cysteine proteases [34]. The unrooted phylogenetic tree among the amino acid sequences from the other identified plant/animal cysteine proteases showed that *Hv*CP3 is closely related to the evolutionary process of monocotyledonous papain C1A type cysteine proteases including rice oryzain α.

4. Discussion

In the present study, an SDS-dependent protease with Suc-LLVY-MCA degradation activity

was highly purified from green leaves of barley using four steps of successive column chromatography. The protease was finally purified 146-fold with a yield of 7.74%. This protease is classified as a member of the cysteine protease family as judged by an inhibitor study and from its catalytic site. Similar to some cysteine proteases [25, 26 and references therein], SDS was essential for the activation of this enzyme in vitro (Fig. 2). Interestingly, the barley enzyme had a broad pH optimum from neutral to pH 8 unlike the other known cysteine proteases, which have mainly acidic pH optima [25]. In addition, the activity of the barley protease was more plentiful in mature green leaves than in senescent yellow leaves (Fig. 1B) in consistent to the results of semi-quantitative RT-PCR analysis (data not shown). This enzyme was a heterogeneous complex composed of two different molecular weights of 33 k core protein and probably 18 k cystatin from the analyses of the native molecular weight by Superdex 200 HR gel filtration and SDS-PAGE analysis.

Although there are many reports on the cysteine proteases, there are few available data on the enzymatic characteristics of cysteine proteases on the protein level. Except for the pH optimum and molecular mass, most of the properties of the barley enzyme were similar to those of the maize SDS-dependent cysteine protease, CPPIC, that exists as a complex with a specific inhibitor(s), cystatin(s) [25-27]. According to CPPIC activation mechanism elucidated by Yamada et al. [27], activation of the enzyme is not explained by a release of inhibitor protein, cystatin, but by a conformational change of the protein with SDS. In this case, to change to the active form, an SDS-like substance must cause a conformational change of the enzyme protein. The question is

what cause the conformational change of the protein in vivo instead of SDS. This question still retained to be answered until now [35]. In barley enzyme, whether an activation mechanism similar to that of the maize enzyme functions or not awaits further studies.

We determined the cDNA sequence of this protease gene and deduced amino acid sequence. The results of a BLAST search for the N-terminus revealed several cysteine protease homologues, and the sequence obtained was highly conserved among them (Fig. 3B). Amino acid sequence analyses thus provided information not only about the alignment of amino acids of the enzyme itself, but also the molecular structure and evolutionary relatedness by comparison with other alignments. These analyses are instantaneous and extremely useful, but sometimes provide little information about the physiological functions of the proteins in plant cells. For instance, *Hv*CP3 and maize CPPIC belong to the same cysteine protease family and have substantially similar alignments, as mentioned above. In fact, the enzymatic properties of these enzymes are very similar to each other, except for the pH optima and molecular masses. CPPIC is a trimer of about 55 kDa subunit and most active at acidic conditions of pH 5 and seems to locate in the vacuole [25, 26], while the barley enzyme has a single protein of 50 kDa with a broad pH optimum at pH 7 to 8 and probably locates in the cytosol. On the intracellular localization of *Hv*CP3, our result showed that enzyme activity was recovered mostly in a cytosolic fraction (100,000 x g, supernatant). This does not contradict the pH optimum of the enzyme as neutral to alkaline pH. Amino acid sequence data also provide some information on the localization of the enzyme. The results of WoLF PSORT analysis predicted that the precursor of this enzyme was transported to the

chloroplast, while no signal sequence to the chloroplasts was found from the analysis of SignalP 3.0 [36]. In addition, this protease was detected abundantly in mature green leaves, but markedly decreased in senescent leaves. Taken together, these results indicate that the barley protease is likely to be a maintainer of degrading, denatured, or short-lived proteins, in cytosol in green leaves. On the other hand, CPPIC had also no correlation with senescence and from their ubiquitous existence of the enzyme at a latent state, it is suggested that CPPIC is relation to some emergent events such as biotic or abiotic stresses [37]. It is obvious that the physiological roles and localization of these enzymes are clearly different.

In the present study, we purified and characterized SDS-dependent cysteine protease with chymotrypsin-like activity from barley leaves and obtained new information concerning the characteristics of the enzyme on the protein level, including the cDNA sequence.

Acknowledgements – The authors are grateful for financial support from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 12640631).

References

[1] K. D' Hondt, D. Bosch, J. Van Damme, M. Goethals, J. Vandekerckhove, E. Krebbers, An aspartic proteinase present in seeds cleaves *Arabidopsis* 2 S albumin precursors in vitro. J Biol Chem 268 (1993) 20884-20891.

[2] A. Mutlu, J.E. Pfeil, S. Gal, A probarley lectin processing enzyme purified from *Arabidopsis thaliana* seeds. Phytochemistry 47 (1998) 1453-1459.

[3] T. Asakura, I. Matsumoto, J. Funaki, S. Arai, K. Abe, The plant aspartic proteinase-specific polypeptide insert is not directly related to the activity of oryzanin 1. Eur J Biochem 267 (2000) 5115-5122.

[4] P. Runeberg-Roos, J. Kervinen, V. Kovaleva, N.B. Raikhel, S. Gal, The aspartic proteinase of barley is a vacuolar enzyme that processes probarley lectin in vitro. Plant Physiol 105 (1994) 321-329.

[5] E.P. Beers, T.B. Freeman, Proteinase activity during tracheary element differentiation in *Zinnia* mesophyll cultures. Plant Physiol 113 (1997) 873-880.

[6] H. Fukuda, Tracheary element differentiation. Plant Cell 9 (1997) 1147-1156.

[7] H. Fukuda, Programmed cell death of tracheary elements as a paradigm in plants. Plant Mol Biol 44 (2000) 245-253.

[8] T. Shikanai, K. Shimizu, K. Ueda, Y. Nishimura, T. Kuroiwa, T. Hashimoto, The chloroplast *clpP* gene, encoding a proteolytic subunit of ATP-dependent protease, is indispensable for chloroplast development in tobacco. Plant Cell Physiol 42 (2001) 264-273.

[9] E.C. Schirmer, J.R. Glover, M.A. Singer, S. Lindquist, HSP100/Clp proteins: a common mechanism explains diverse functions. Trends Biochem Sci 21 (1996) 289-296.

[10] C.K. Suzuki, M. Rep, J.M. Van Dijl, K. Suda, L.A. Grivell, G. Schatz, ATP-dependent proteases that also chaperone protein biogenesis. Trends Biochem Sci 22 (1997) 118-123.

[11] S. Gottesman, M.R. Maurizi, S. Wickner, Regulatory subunits of energy-dependent proteases. Cell 91 (1997) 435-438.

[12] R.D. Vierstra, Protein degradation in plants. Ann Rev Plant Physiol Plant Mol Biol 44 (1993) 385-410.

[13] W. Seufer, S. Jentsch, In vivo function of the proteasome in the ubiquitin pathway. EMBO J 11 (1992) 3077-3080.

[14] A. Zaltsman, A. Feder, Z. Adam, Developmental and light effects on the accumulation of FtsH protease in Arabidopsis chloroplasts-implications for thylakoid formation and photosystem II maintenance. Plant J 42 (2005) 609-617.

[15] W. Sakamoto, Protein degradation machineries in plastids. Ann Rev Plant Biol 57 (2006) 599-621.

[16] Y. Kato, S. Murakami, Y. Yamamoto, H. Chatani, Y. Kondo, T. Nakano, A. Yokota, F. Sato, The DNA-binding protease, CND41, and the degradation of ribulose-1,5-bisphosphate carboxylase/oxygenase in senescent leaves of tobacco. Planta 220 (2004) 97-104.

[17] Y. Kato, Y. Yamamoto, S. Murakami, F. Sato, Post-translational regulation of CND41 protease activity in senescent tobacco leaves. Planta 222 (2005) 643-651.

[18] J.D. Miller, R.N. Arteca, E.J. Pell, Senescence-associated gene expression during ozone-induced leaf senescence in Arabidopsis. Plant Physiol 120 (1999) 1015-1024.

[19] S. Koehler, T-H.D. Ho, Purification and characterization of gibberellic acid-induced cysteine endoproteases in barley aleurone layers. Plant Physiol 87 (1988) 87: 95-103

[20] S.M. Koehler, T-H.D. Ho, A major gibberellic acid-induced barley aleurone cysteine proteinase which digests hordein. Plant Physiol 94 (1990) 94: 251-258

[21] M.T. Bethune, P. Strop, Y. Tang, L.M. Sollid, C. Khosla, Heterologous expression, purification, refolding, and structural-functional characterization of EP-B2, a self-activating barley cysteine endoprotease. Chem Biol 13 (2006) 637-647

[22] D.W. Cleaveland, S.G. Fischer, M.W. Kirschner, U.K. Laemmli, Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J Biol Chem 252 (1977) 1102-1106.

[23] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162 (1987) 156-159.

[24] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72 (1976) 248-254.

[25] T. Yamada, H. Ohta, T. Masuda, M. Ikeda, N. Tomita, A. Ozawa, Y. Shio, K. Takamiya, Purification of a novel type of SDS-dependent protease in maize using a monoclonal antibody. Plant Cell Physiol 39 (1998) 106-114.

[26] T. Yamada, H. Ohta, A. Shinohara, A. Iwamatsu, H. Shimada, T. Tsuchiya, T. Masuda, K.

Takamiya, A cysteine protease from maize isolated in a complex with cystatin. Plant Cell Physiol 41 (2000) 185-191.

[27] T. Yamada, A. Kondo, H. Ohta, T. Masuda, H. Shimada, K. Takamiya, Isolation of the protease component of maize cysteine protease-cystatin complex: release of cystatin is not crucial for the activation of the cysteine protease. Plant Cell Physiol 42 (2001) 710-716.

[28] M. Usui, S. Tanaka, H. Miyasaka, Y. Suzuki, Y. Shioi, Characterization of cysteine protease induced by oxidative stress in cells of *Chlamydomonas* sp. strainW80. Physiol Plant 131 (2007) 519-526.

[29] V. Bhandari, R.G. Palfree, A. Bateman, Isolation and sequence of the granulin precursor cDNA from human bone marrow reveals tandem cysteine-rich granulin domains. Proc Natl Acad Sci USA 89 (1992) 1715-1719.

[30] T. Matsumoto, J. Wu, H. Kanamori et al., The map-based sequence of the rice genome. Nature 436 (2005) 793-800.

[31] T. Pechan, B. Jiang, D. Steckler, L. Ye, L. Lin, D.S. Luthe, W.P. Williams, Characterization of three distinct cDNA clones encoding cysteine proteinases from maize (*Zea mays* L.) callus. Plant Mol Biol 40 (1999) 111-119.

[32] M. Koizumi, K. Yamaguchi-Shinozaki, H. Tsuji, K. Shinozaki, Structure and expression of two genes that encode distinct drought-inducible cysteine proteinases in *Arabidopsis thaliana*. Gene 129 (1993)175-182.

[33] W. Mitsuhashi, T. Yamashita, T. Toyomasu, Y. Kashiwagi, T. Konnai, Sequential development

of cysteine proteinase activities and gene expression during somatic embryogenesis in carrot. Biosci Biotechnol Biochem 68 (2004) 705-713.

[34] J.C. Rogers, D. Dean, G.R. Heck, Aleurain: a barley thiol protease closely related to mammalian cathepsin H. Proc Natl Acad Sci USA 82 (1985) 6512-6516.

[35] R. A. L. van der Hoorn, Plant proteases: From phenotypes to molecular mechanisms. Annu Rev Plant Biol 59 (2008) 191-223.

[36] J.D. Bendtsen, H. Nielsen, G. von Heijne, S. Brunak: Improved prediction of signal petides: SignalP 3.0. J Mol Biol 340 (2004) 783-795.

[37] T. Yamada: Biochemical studies on a cysteine protease complex in maize (*Zea mays*) leaves. Doctoral thesis, Tokyo Institute of Technology,1997, p. 85.

Figure legends

Fig. 1. Three types of Suc-LLVY-MCA activities were separated by DE52 with different concentrations of SDS (A): open circle, no addition; open square, 0.02% SDS; solid circle, 0.1% SDS; linear line, absorbance at 280 nm as amounts of protein. The proteases were separated from the extract of green leaves by DE52 with 200 ml of a liner gradient of NaCl (0-0.4 M) and assayed with the indicated concentrations of SDS. Change of protease activity along with the plastid development (B). Separation and assay of protease were done in the same manner as A to remove the later minor peak fraction and integrated amounts of each activity of peak fractions are shown.

Fig. 2. SDS dependency of purified protease. Peptide hydrolytic activity was assayed after incubation for 30 min at 37°C in a medium containing 50 mM Tris-HCl (pH 8.0), 100 μM fluorogenic peptides, and the indicated concentrations of SDS. The results are averages of three experiments, and the SE is shown. The activity is expressed relative to 0.1% SDS as 100% (Control rate: 1.17 nmol AMC liberated/min/mg protein).

Fig. 3. A schematic of the primary structure based on motif and domains (A) and amino acids sequences of purified *Hv*CP3 from barley and proteins with homologous alignments (B). Multiple alignments were done by ClustalW. Identical amino acids are shown in black boxes, and similar amino acids are shown in gray boxes. The dashed-dotted line is shown the peptide sequence determined. An arrow head shows the likely site of cleavage to generate the mature protein. The

catalytic triads (C/H/N) are designated with asterisks (*). The solid circles indicate an ERFNIN motif. The cathepsin inhibitor domain and granulin domain are shown by solid and dotted lines, respectively. The broken line indicates the peptidase domain. Triticain α wheat cysteine protease; CPPIC, maize cysteine protease of protease-inhibitor compex; Orizain α rice cysteine protease; Mir3, maize cysteine protease, and RD21A, *Arabidopsis thaliana* cysteine protease (see text for reference or accession number).

Table 1. Summary of purification of *Hv*CP3 from green leaves of barley

Crude extract was obtained from 500 g (fresh weight) of green leaves of barley. The enzyme activity was assayed as described in Materials and methods. The activity corresponds to the amount of hydrolyzed Suc-LLVY-MCA.

	Total Activity (nmol/min)	Protein (mg)	Specific Activity (nmol/min/mg protein)	Yield (%)	Purification (-fold)
Crude Extract	71.7	366	0.196	100	1
DE52	36.1	102	0.354	50.3	1.81
Q sepharose Fast Flow	17.6	18.1	0.972	24.5	4.96
Hi-Load Superdex 200	5.55	0.194	28.6	7.74	146

Table 2. Effect of Inhibitors on purified *Hv*CP3

Purified enzyme solutions were incubated with the indicated concentrations of reagent for 30 min at 37ºC prior to addition of substrate. The results are averages of three experiments with SE. The activity is expressed relative to no addition as 100%.

Inhibitor	Concentration (μM)	AMC Released (pmol min^{-1})	Relative Activity (%)
None		8.5 ±1.12	100±13
PMSF	100	6.7 ±0.46	79±7
	1000	9.3 ±1.18	109±13
NEM	100	4.3 ±0.25	51±6
	1000	5.0 ±0.02	59±0.4
E-64	1	1.1 ±0.01	13±0.9
	10	0.41±0.01	5±2
Leupeptin	10	0.57±0.02	7±4
	100	0.35±0.01	4±3
EDTA	1000	11.0 ±0.30	129±3

Fig. 1A

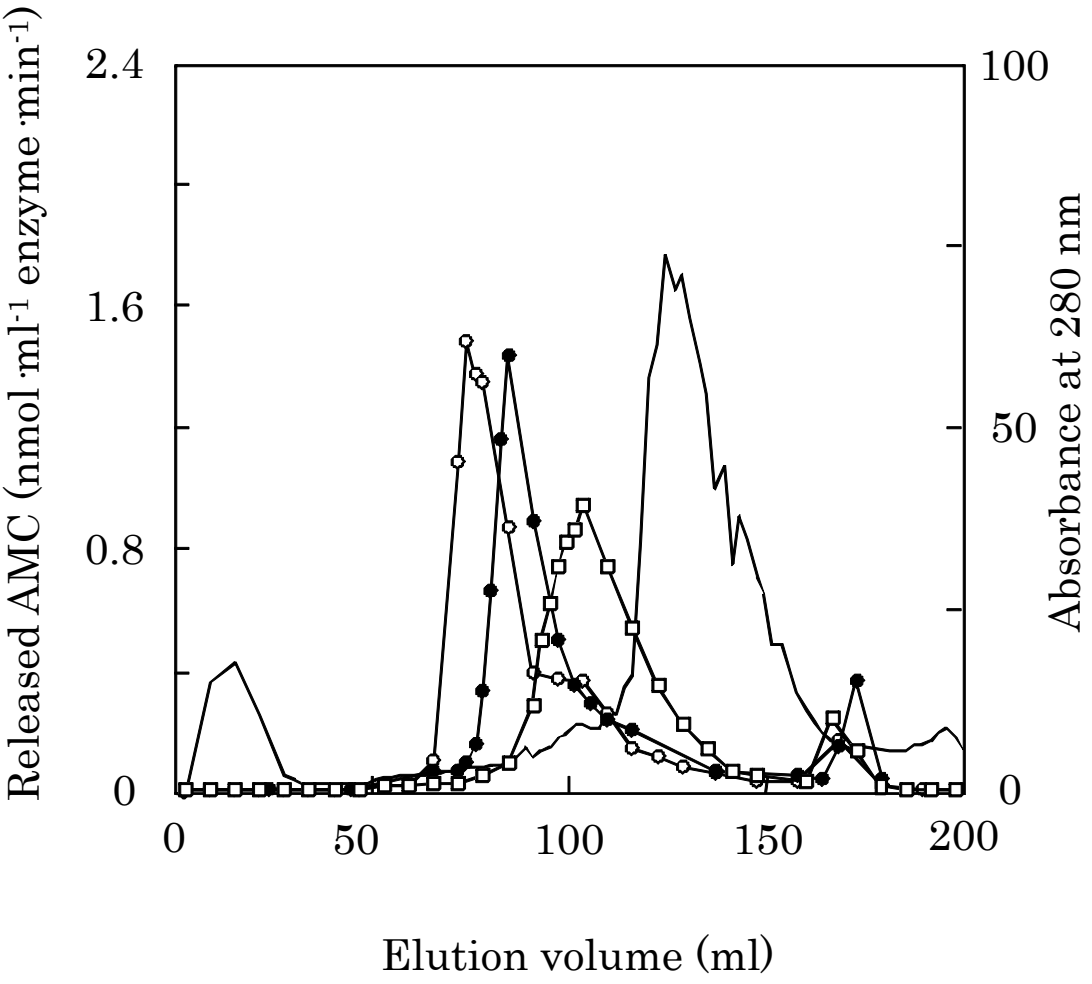

Released AMC (nmol·ml-1 enzyme·min-1)
2.4
1.6
0.8
0
0
50
100
150
200
Elution volume (ml)
100
50
0
Absorbance at 280 nm

Fig. 1B

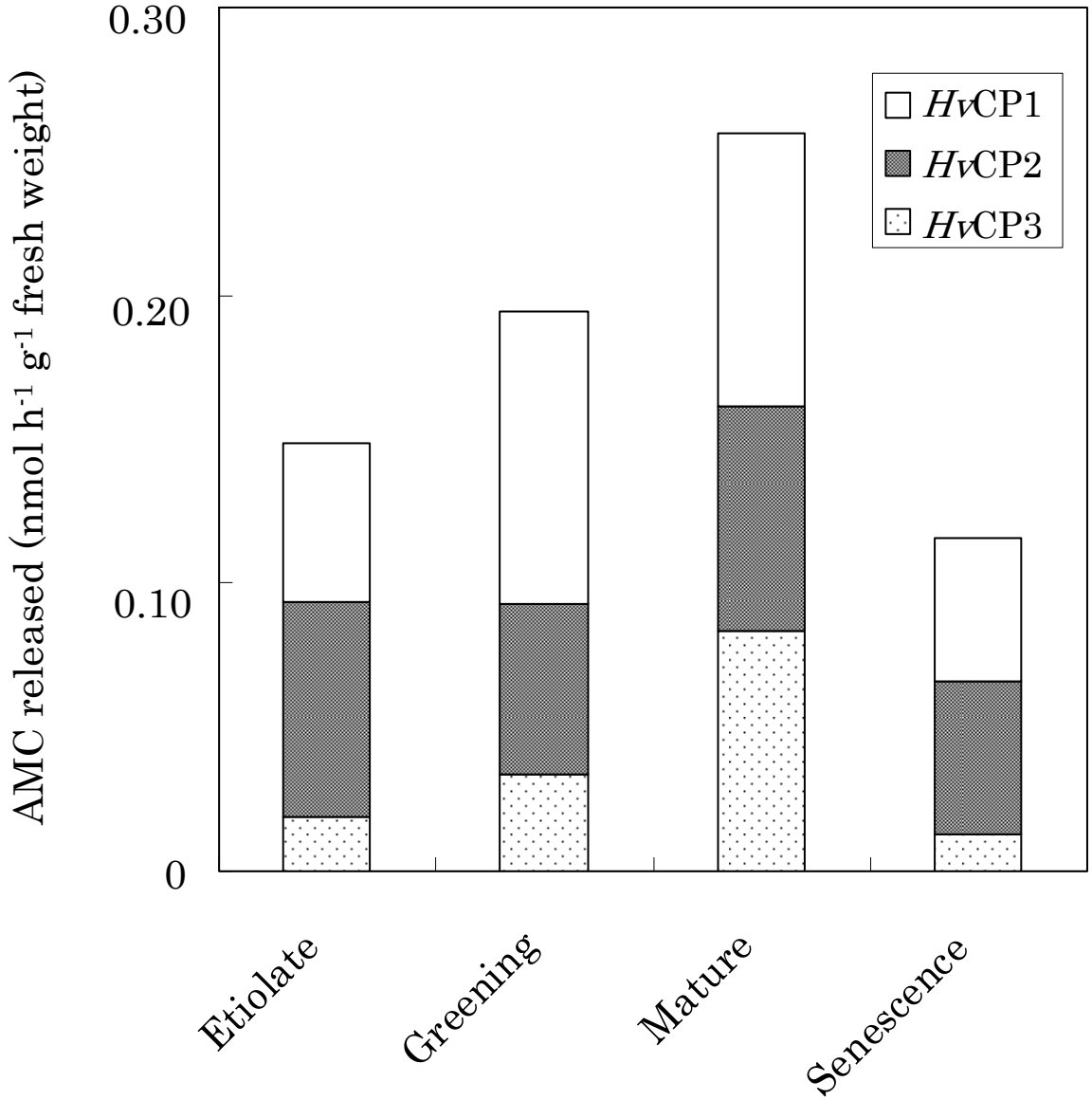

0.30
0.20
0.10
0
AMC released (nmol h-1 g-1 fresh weight)
HvCP1
HvCP2
HvCP3
Etiolate
Greening
Mature
Senescence

Fig. 2

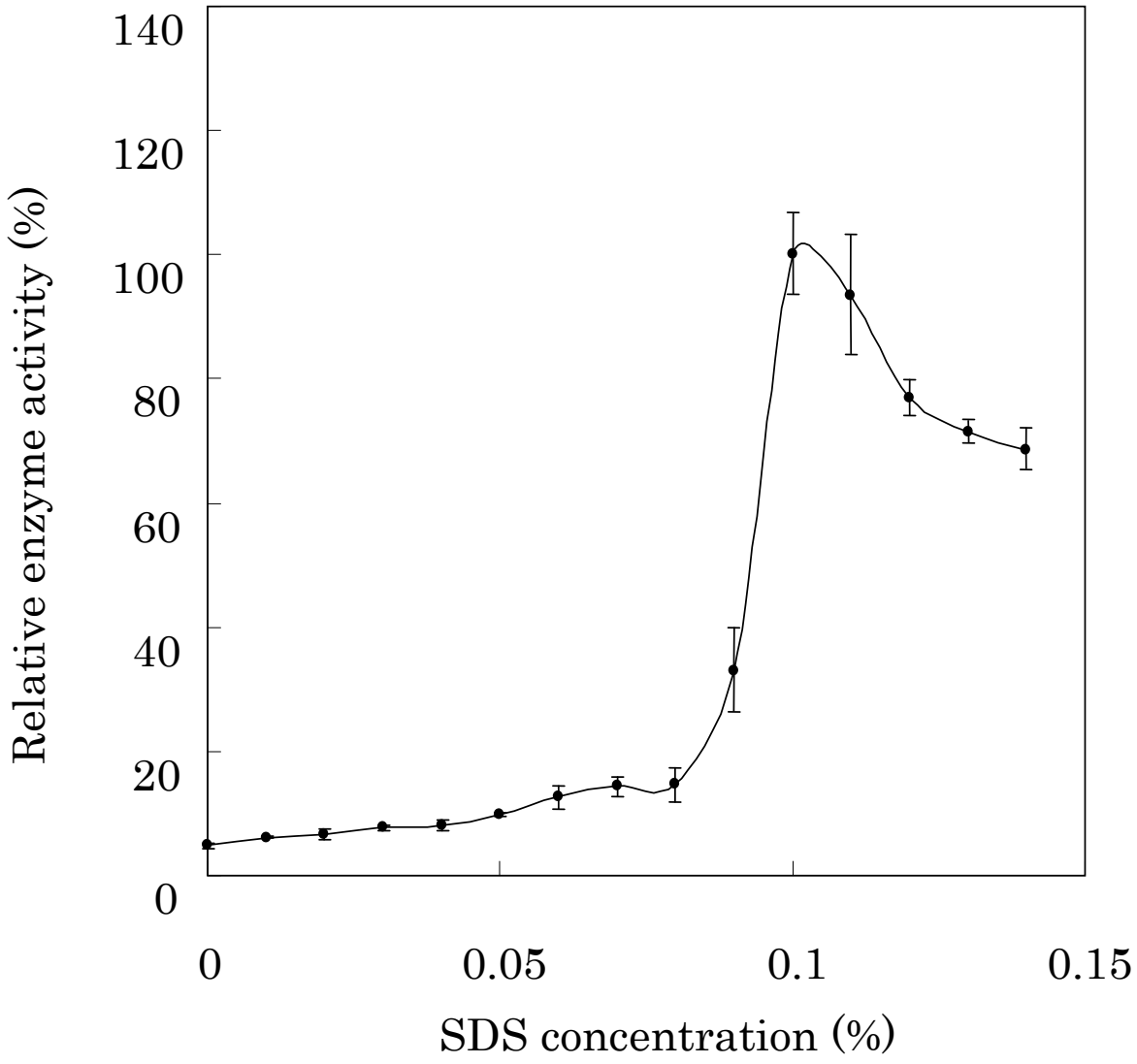
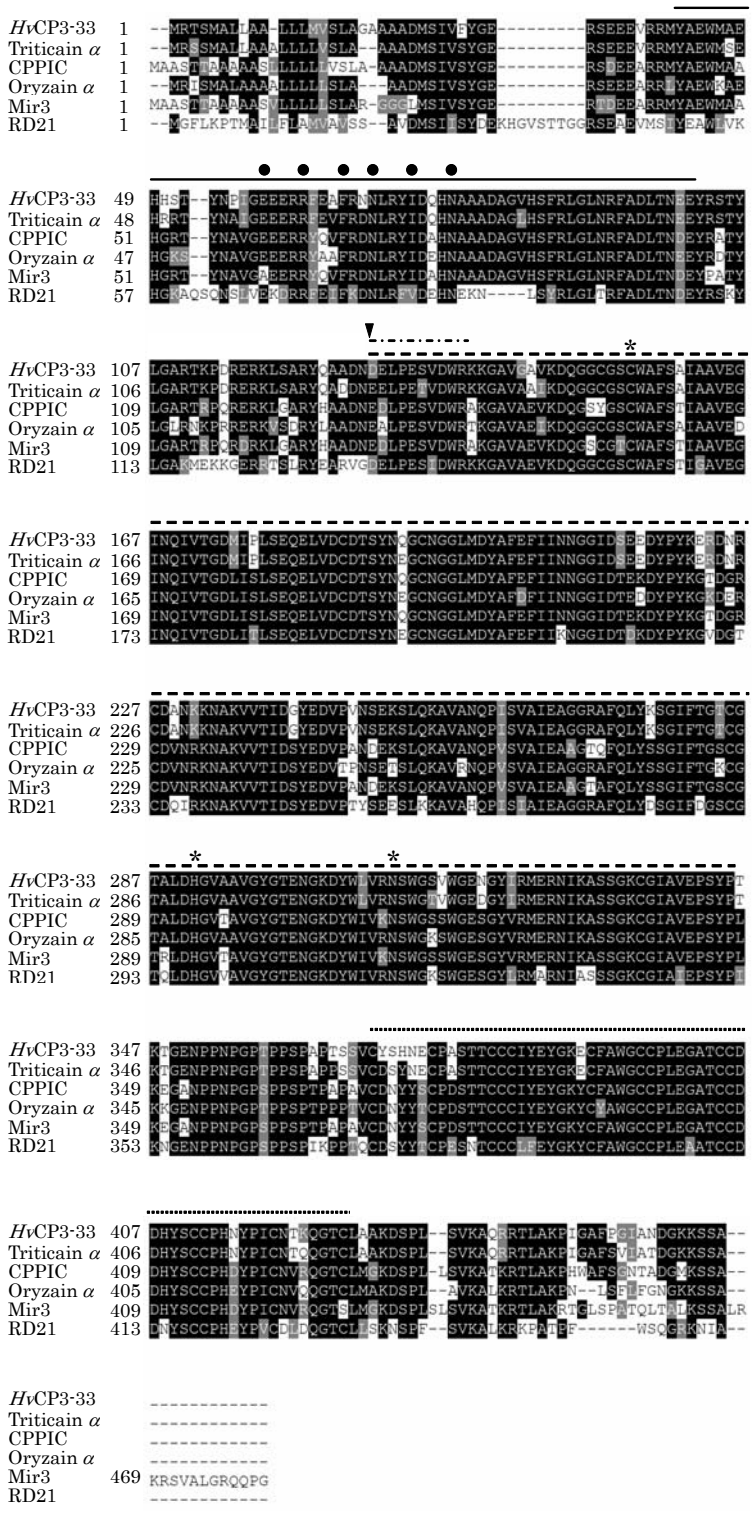

140
120
100
80
60
40
20
0
Relative enzyme activity (%)
0
0.05
0.1
0.15
SDS concentration (%)

Fig. 3

A


Cathepsin propeptide inhibitor domain
Peptidase C1A domain
Pro-rich region
Granulin domain

B


HvCP3-33
Triticain α
CPPIC
Oryzain α
Mir3
RD21