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Biochemical Studies on a Cysteine Protease Complex in Maize (*Zea mays*) Leaves

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CONTENTS

Abbreviations

Ac	acetyl-
AMC	7-amino-4-methylcoumarin
Boc	*tert*-butoxycarbonyl-
BSA	bovine serum albumin
CHAPS	3-[(3-cholamidopropyl)dimethylammonio] propanesulfonic acid
ELISA	enzyme-linked immunosorbent assay
GPC	gel permeation chromatography
GuHCl	guanidine hydrochloride
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
LSU	large subunit of Rubisco
MAb	monoclonal antibody
MCA	4-methylcoumaryl-7-amide
ß-ME	ß-mercaptoethanol
MMP	matrix metalloproteinase
OVA	ovalbumin
PBS	phosphate buffer saline
PMSF	phenylmethanesulfonyl fluoride
PVDF	polyvinylidene difluolide
RP-HPLC	reverse phase-high performance liquid chromatography
Rubisco	ribulose-1,5,-bisphosphate carboxylase/oxygenase
SSU	small subunit of Rubisco
Suc	succinyl-
TLCK	*p*-Tosyl-*L*-lysine chloromethyl ketone
TPCK	*p*-Tosyl-*L*-phenylalanine chloromethyl ketone

Chapter I

General Introduction

Proteolysis is essential for many physiological aspects in living organisms (for reviews see, Vierstra 1993, 1996, Callis 1995) It is responsible for cellular housekeeping and the stress response by removing abnormal and misfolded proteins, for supplying amino acids needed to make new proteins, for assisting in the maturation of zymogens and peptide hormones by limited cleavages, for controlling metabolism, homeosis, and development by reducing the abundance of the key enzymes and regulatory proteins.

In our current understanding of the protein degradation, it involves not only in an important recycling system for amino acids but also in the final step of a complex cascade of regulatory events controlling gene function (Hershko and Ciechanover 1992, Ciechanover 1994, Callis 1995). It has become increasingly obvious that the ability of cells to switch from one developmental state to another or to adapt to new environmental conditions often requires the rapid dismantlement of existing regulatory networks, a process frequently dependent on proteolysis.

In plants, proteases widely localize in all tissues such as seed, root, stem, leaf and flower, and contribute to many physiological events. Protease degrades storage proteins in mature seeds during germination to small peptides or amino acids that are subsequently transported to the growing developing tissues (Preston and Kruger 1986, Wilson 1986, Fincher 1989). Multiple germination-induced proteinase activities have also been described in rice (Watanabe et al. 1991) and maize (de Barros and Larkins 1990, Mitsuhashi and Oaks 1994). Two senescence-induced cDNAs

encoding cysteine protease have been isolated from senescencing *Arabidopsis* leaves (Hensel et al. 1993, Lohman et al. 1994). Transcript levels of one of them, SAG12, are maximally 100-fold higher in senescent leaves with 50% chlorophyll loss than in mature leaves with no visible chlorophyll loss (Lohman et al. 1994). mRNA for the other, SAG2, is present at two- to four-fold higher levels in senescent leaves than in mature green leaves (Hensel et al. 1993).

When tissues are exposed to different environmental stresses, several mRNAs that encoded cysteine proteinase have been shown to accumulate. In plants, almost all proteases which are related to environmental stresses are cysteine proteases. mRNAs encoding for two different cysteine proteinases accumulate in drought- or salt-stressed *Arabidopsis* plants (Koizumi et al. 1993), and in drought-stressed pea (Guerrero et al. 1990). Levels of a mRNA encoding a cysteine proteinase in tomato fruit increase after cold treatment (Schaffer and Fischer 1988). However, little is understood concerning the physiological role of these changes in mRNA levels of proteinase. Indeed, it remains to be clarified whether corresponding changes occur in protein levels of proteinases. In addition, intracellular locations and *in vivo* substrates are needed to be identified. Synthesis of the wound-inducible peptide hormone systemin in tomato requires substantial proteolytic processing of a 200 amino acid precursor to produce the active 18-amino acid fragment (McGurl et al. 1992). With relation to programmed cell death, a cDNA encoding cysteine proteinase involved in the differentiation of *Zinnia elegans* mesophyll cells into xylem elements was isolated (Ye and Varner 1993).

Protease is involved in various regulations in physiological events, and the activity of the protease could be regulated by three steps, which are regulation by its own gene expression, by activation of a latent enzyme, and

by specific inhibitors. There is little direct evidence for regulation by a specific inhibitor. However, matrix metalloproteinase (MMP) is regulated by all of the three steps including regulation by a specific inhibitor on the activity (Gross et al. 1984, Frisch et al. 1987, Springman et al. 1990, Umenishi et al. 1990, 1991, Van Wart and Birkedal-Hansen 1990). With respect to the activation of latent enzymes, it is activated autocatalytically among inter- or intra-molecules (Springman et al. 1990, Umenishi et al. 1990, Van Wart and Birkedal-Hansen 1990). Also, the activity is induced by artificial compounds such as SDS, 4-aminophenylmercuric acetate and *N*-ethylmaleimide which derive autocatalysis-like activation (Nagase et al. 1990, Springman et al. 1990, Van Wart and Birkedal-Hansen 1990). Proteasome which has multicatalytic functions with high molecular weight is also activated by SDS, fatty acids and poly-lysine (Dahlmann et al. 1985, Tanaka et al. 1986, 1989, Saitoh et al. 1989). Interestingly, although SDS is a strong denaturant of protein, it is capable of activating these proteases. The action is regarded as due to a conformational change of the enzymes by SDS.

In plants, there are many reports on proteases whose activity are stimulated by SDS. Proteinase from photosystem II membranes degraded proteins of photosystem II in the presence of 0.01% SDS (Kuwabara and Suzuki 1995). Especially with regard to degradation of ribulose-1,5,-bisphosphate carboxylase/oxygenase (Rubisco), such proteases were mainly reported. Many reports on Rubisco degradation have been described in relation to the protein turnover in development. A protease from extract of rice leaves accelerated the degradation in the presence of 0.2-0.5% SDS at pH 5.5 (Yokota et al. 1990). Such proteases were prepared from chloroplast lysate (Mae et al. 1989, Otto and Feierabend 1994) or thylakoids (Nettleton et al. 1985). In addition, the proteases were

prepared from vacuole (Thayer and Huffaker 1984, Bhalla and Dalling 1986) or protoplast (Dalling et al. 1983). In spite of many investigations, the mechanism for the stimulation is still unknown, although one of the reasons for the stimulation of the activity is considered to be due to increase in the susceptibility of the denatured substrate protein to protease attacks (Mae et al. 1989, Yokota et al. 1990).

Proteinaceous protease inhibitors are generally categorized according to the class of protease that they inhibit. Four types of protease have been identified as serine, cysteine, aspartic and metallo-proteinase based on the active amino acids in the reaction center. The proteinase inhibitors are generally small proteins having molecular weights under 50 kDa and more commonly below 20 kDa. Serine protease inhibitors are the most extensively characterized class of plant protease inhibitors, and assumed to function in defense against herbivores. Plant cysteine protease inhibitors are typified by the phytocystatins which inhibit proteases of the papain superfamily (Ryan 1990). Inhibitors of aspartic and metalloproteinase, which are able to inhibit cathepsin D and carboxypeptidase A, respectively, have also been identified in plants. It is regarded that protease inhibitors have important role as regulatory factors in controlling endogenous proteolytic activity, and as protective factors directed against exogenous proteases such as insect or microbial proteases. With regard to inhibitors against endogenous proteases, cystatins are considered as inhibitors targeting cysteine proteases which are related to protein breakdown during germination (Abe et al. 1987, 1992, 1995, Domoto et al. 1995, Kondo et al, 1990). On the other hand, with inhibitors against exogenous protease, they are mainly involved in defense against herbivores. Transgenic plants expressing serine protease inhibitors or cysteine protease inhibitors exhibited enhanced resistance to predation by

these pests, indicating the function of protease inhibitors in plant defense (Johnson et al. 1989, Urwin et al. 1995).

As described above, in respect to the physiological events, it is very interesting for the regulation of the activity that some proteases can be activated by SDS. Then, the understanding of the activation mechanism, and the relationship between expression of such a proteolytic activity and a physiological events could manifest a significance of a regulation of the activation.

In the present study, a SDS-dependent protease was purified as a complex and characterized (in Chapter II), the activation mechanism of the protease complex by SDS was investigated (in Chapter III), and roles of the protease complex was discussed with relation to physiological events in Chapter IV.

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Chapter II

Purification and Characterization of SDS-dependent
Protease from Maize Leaves

Introduction

SDS is an extremely effective denaturant for proteins, so that in the presence of SDS, most proteins lose their own functions completely or partially with a disruption of tertiary and quaternary structure. In plants, the presence of proteases whose activity was stimulated by SDS has been studied on the degradation of Rubisco (Thayer and Huffaker 1984, Nettleton et al. 1985, Bhalla and Dalling 1986, Mae et al. 1989, Otto and Feierabend 1994). Proteolytic breakdown of Rubisco is important in protein turnover, since it is the most abundant protein whose degradation provides sufficient carbon and nitrogen (Peterson et al. 1973, Makino et al. 1984, Vierstra 1993), and therefore, protease which is involved in the turnover of Rubisco is of interest. In addition to this SDS-stimulated Rubisco degradation, a few peptidases constituting a proteasome isolated from spinach leaves (Ozaki et al. 1992, Watanabe and Yamada 1996) and matrix metalloproteinase (MMPs) (Graham et al. 1991, McGeehan et al. 1992), a zinc enzyme from soybean leaves, also showed stimulation of the activities by SDS.

Since SDS is a strong denaturant, it is interesting to know how such proteases have proteolytic activities in the presence of SDS. The mechanism for the stimulation of the proteolytic activity by SDS has not been elucidated except for MMPs (Springman et al. 1990) or proteasome (Tanaka et al. 1989). It is, however, usually presumed that the

stimulation is caused by a conformational change of the substrate proteins, resulting in the increase of susceptibility of substrate proteins to proteases under the denaturing conditions (Thayer and Huffaker 1984, Bhalla and Dalling 1986, Mae et al. 1989, Otto and Feierabend 1994). In these reports, it appeared that SDS is just an accelerator for the basal activity but not indispensable for the expression of the proteolytic activity *in vitro* because a low but significant activity exists even in the absence of SDS. It is, however, difficult to determine whether the effect of SDS is for denaturation of substrate or for the activation of the protease, because these proteases have not been completely purified.

This chapter shows the purification of a novel type of SDS-dependent protease from maize leaves to homogeneity. The protease was activated only by SDS *in vitro*. Other properties of the protease such as the sensitivity to inhibitors, pH optimum, and in particular, effect of SDS on the proteolytic activity are described. The content in this chapter will be published in Plant Cell Physiology (Yamada et al. 1998).

Materials and Methods

Plant materials

Maize plants (*Zea mays* L. cv. Honey bantam) were cultivated in the field of our Institute, and mature leaves were harvested just before flowering. The leaves were stored at -80°C until use.

Preparation of crude extract

The frozen leaves were broken into small pieces and homogenized in a cold 50 mM HEPES-NaOH (pH 7.3) containing 2 mM $MgCl_2$ (3.4 volumes to the fresh weight of leaves) with a Waring blender. The homogenate was squeezed through eight layers of gauze, and the filtrate was centrifuged at

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187,000 x g for 1 h. The supernatant was used as a crude extract.

DEAE-Toyopearl chromatography

The crude extract was dialyzed against 50 mM Tris-HCl (pH 7.8), and the dialyzed enzyme was applied to a DEAE-Toyopearl column (Tosoh Co., Japan) (5.0 x 11 cm) equilibrated with the same buffer. The column was washed with 50 mM Tris-HCl (pH 7.8) containing 100 mM NaCl until the absorbance at 280 nm returned to the base line, and the adsorbed proteins were eluted with 1,000 ml of a linear gradient of 100-500 mM NaCl in 50 mM Tris-HCl (pH 7.8). The active fractions were collected.

Ultrafiltration

The combined active fraction from the DEAE-Toyopearl chromatography was ultrafiltrated through XM50 (Amicon Co., USA), to remove proteins which had molecular mass lower than 50 kDa, and to concentrate an active fraction.

Gel permeation chromatography (GPC)

The active fraction (15 ml) from the ultrafiltration was applied to a Toyopearl HW-55 (Tosoh Co., Japan) column (3.2 x 47 cm) equilibrated with 50 mM Tris-HCl (pH 7.8) containing 200 mM NaCl. The elution was carried out with the same buffer, and the active fractions were collected.

Immunoaffinity chromatography with the IgG monoclonals

MAb (IgG$_1$) (described below) was fully adsorbed to Protein G PLUS/Protein A-Agarose suspension (600 ml) (Oncogene Science, USA) and coupled on the Agarose with 600 mM pimelidic acid dimethyl ester dihydrochloride (3 ml) (Tokyokasei-kougyo, Japan) (Davies and Kaplan 1972, Schneider et al. 1972). The excess of the cross-linking reagent was removed by washing with 0.2 M 2-aminoethanol and the residual active functional groups were inactivated by incubation with 2-aminoethanol for 2 h. The fraction of GPC was applied to the MAb-Protein G PLUS/Protein A-

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Agarose column equilibrated with phosphate buffer saline (PBS) containing 0.1% Tween 20, and the column was washed with the same buffer of 100-fold column volume, subsequently with 130 mM NaCl in 50 mM Tris-HCl (pH 7.8) containing 12.5% glycerol of 50-fold column volume. The washed resin was incubated with 100 mM leupeptin, and subsequently the protease was eluted by boiling with SDS/sample buffer (ß-mercaptoehanol minus) (4.5% (w/v) SDS, 10% (v/v) glycerol in 0.1 M Tris-HCl (pH 6.8)) (Ausubel et al. 1994) for 5 min after the assay of the proteolytic activity. The supernatant eluted was analyzed on SDS-PAGE after boiling with 10% (v/v) ß-ME.

Preparation of the antigen for immunization

The active fraction from GPC was concentrated by ultrafiltration as described above. PAGE of the concentrated enzyme was carried out in 4.2-20% acrylamide slab gels at pH 8.3 by the method of Davis (1964). The enzyme was electrophoresed in two adjacent lanes of the gel. One lane was used for silver-staining and the other for the preparation of the protease. In the preliminary experiments, the location of a protease band was determined directly by checking the proteolytic activity of the proteins separated on the native gradient PAGE (see Results). A single band of protease was cut out and homogenized, and the enzyme was extracted with 50 mM Tris-HCl (pH 7.8), which was used as an antigen for immunization to mice.

Monoclonal antibodies (MAbs)

The preparation of hybridomas producing MAbs was performed according to the standard techniques (Ozawa et al. 1992, Malik and Lillehoj 1994). A mouse was immunized with the protease (50 mg, total amount per mouse) purified by native gradient PAGE. Female BALB/c mice (4 weeks old) (Nihon Seibutu Zairyou Center Co., Japan) were used.

Each mouse was given four intravenous injections of the protease (10 mg) purified by native gradient PAGE at 3-d intervals, and at 10-d later, last injection of the same amount of the protease was performed. The spleen cells were obtained after 3 d from the last injection and fused with a mouse myeloma cell line (PAI). PAI (BALB/c mouse-derived myeloma) was provided by the Tokyo Metropolitan Institute of Medical Science, Japan.

The hybridoma fusions were screened by the antibody-trapped proteolytic activity. Thus, the existence of antibody in the supernatant of hybridoma cells was detected by the SDS-dependent proteolytic activity immunosorbed to the antibodies on a multiple well plate (Corning, USA). The positive hybrid was cloned by limiting dilution. The isotype of antibody produced by hybridoma was determined by a mouse monoclonal antibody kit (Amersham, UK).

Proteolytic activity

Proteolytic activity was assayed either by measurement of the degradation of Rubisco large subunit (LSU) or the hydrolysis of the synthesized oligopeptides. In the assay of the measurement of the LSU degradation, the assay mixture (total volume, 45 µl) containing the enzyme solution (12.5 µl), 0.5% SDS, 100 mM sodium citrate (pH 5.0), 6% (w/v) sucrose, and 9.6 µg of Rubisco (from spinach leaves, Sigma, USA), was incubated at 35°C for 60 min. In some cases, proteinous substrates, such as Rubisco and BSA, were boiled for 5 min in 4.3 % Softes (LION Co., Tokyo, Japan) before assay (see Table 2). The reaction was stopped by boiling for 5 min after the addition of SDS to 2% and ß-ME to 5%. An aliquot was subjected to SDS-PAGE (Laemmli 1970) and the gels were stained with Coomassie Brilliant Blue R-250. Control experiments were carried out without the enzyme. The amount of LSU on stained gels were quantified by a densitometer (device: The Discovery Series™, pdi, USA; software:

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Quantity One Ver.2.4, pdi, USA). The activity was defined as the percentage of the degradation of LSU.

The hydrolysis of the synthesized oligopeptides, *tert*-butoxycarbonyl-Val-Leu-Lys-4-methylcoumaryl-7-amide (Boc-Val-Leu-Lys-MCA), succinyl-Ala-Glu-MCA (Suc-Ala-Glu-MCA) and Suc-Ala-Pro-Ala-MCA was evaluated by measuring the amount of liberated 7-amino-4-methylcoumarin (AMC). The standard assay was carried out by the incubation of a reaction mixture (450 μl) containing enzyme solution (10 μl), 200 μM oligopeptides, 0.1% SDS, 100 mM citrate buffer (pH 5.0) at 35°C for 60 min. The liberated AMC was determined fluorometrically (excitation at 380 nm and emission at 460 nm) after adding 10 mM leupeptin to stop the reaction. One unit of activity was defined as the amount of enzyme that liberates 1 μmol of AMC per min.

Protein assay

Protein concentrations were determined by the method of Bradford (1976) with the dye reagent (Bio-Rad Laboratories, USA) using bovine serum albumin as the standard.

Immunodot blot analysis

The protease (~0.5 μg) purified by native gradient PAGE was boiled in the same volume of SDS/sample buffer (4.5% SDS, 10% ß-ME, 10% glycerol in 0.1 M Tris-HCl, pH 6.8) (SDS-denatured) or not treated (native). The protease was blotted onto a nitrocellulose membrane (Schleicher & Schuell, Germany). The protein was detected using peroxidase-conjugated anti-mouse IgG as a secondary antibody and immunostain kit of Konica HRP-1000 (Konica, Japan).

Results

Purification of SDS-dependent protease from maize leaves

The protease which was activated by SDS was purified from mature maize leaves by several chromatographic procedures including an immunoaffinity chromatography prepared by a monoclonal antibody (MAb), as summarized in Table II-1. According to this procedure, the purification led to 1,400-fold increase by the immunoaffinity chromatography prepared by MAb raised against the purified protease by native gradient PAGE (Fig. 2-1, see below). The recovery of the activity was 0.157% after the immunoaffinity chromatography. From 2.5 kg of maize leaves, about 10 mg of the purified protease was obtained.

On the native gradient PAGE for the preparation of the antigen, the 179-kDa band in Fig. 2-1 solely showed SDS-dependent proteolytic activity for Rubisco and an oligopeptide (Boc-Val-Leu-Lys-MCA) by direct measurement after extraction from the gels. However, it was quite difficult to identify the band of the protease on SDS-PAGE, because several bands appeared with a highly smear background on SDS-PAGE, even when samples pre-treated with various protease inhibitors was subjected to SDS-PAGE to inhibit hydrolysis of proteins including the protease (data not shown, see Discussion). Therefore, the protease was further purified by means of immunoaffinity chromatography using MAb prepared as follows; Mice were immunized with the protease isolated by the native gradient PAGE (Fig. 2-1, right lane), hybridomas were prepared and cell lines producing MAb by which SDS-dependent proteolytic activity was arrested were screened. Two positive hybridomas were obtained from 2,000 hybridomas and one hybridoma clone was established. Then, using the MAb, an immunoaffinity column was prepared. The partially purified protease by GPC was applied to the immunoaffinity column. Even after exhaustive washing, the resin itself retained the proteolytic activity for

TABLE II-1 **Purification of SDS-dependent protease from maize leaves.**
Aliquots of the various fractions obtained during purification were assayed for activity
and for protein content as described under Materials and Methods. One activity unit
corresponds to 1 μmol of oligopeptide hydrolyzed per min.

Step	Protein (mg)	Total activity (Units)	Yield (%)	Specific activity ($x10^{-3}$ units/mg protein)	Purification (-fold)
DEAE-Toyopearl	2600	24.0	100	9.2	1
Toyopearl HW-55	510	5.65	23.5	11	1.2
Immunoaffinity [a]	0.010[b]	0.139 [c]	0.577	14,000	1,400

[a] A quarter of the activity obtained by GPC was applied.

[b] The amount of the protein was estimated by comparison with that of marker proteins
on SDS-PAGE.

[c] Since the protein was eluted by boiling with SDS/sample buffer, the activity retained
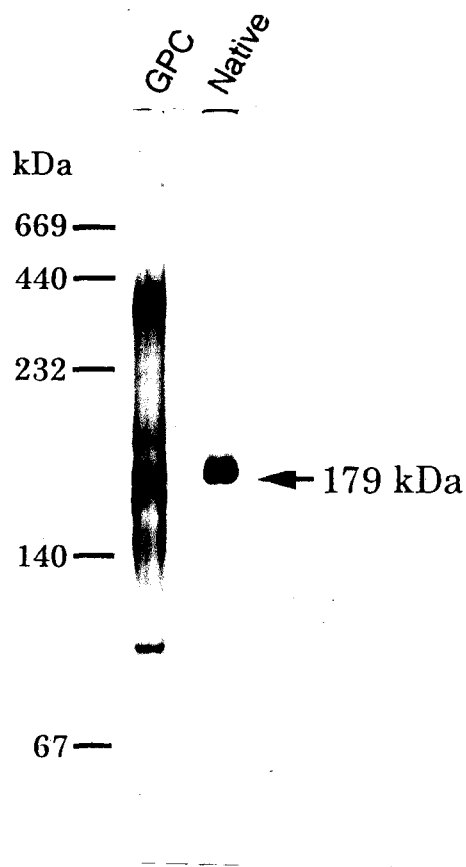on immunoaffinity column just before elution was indicated.

Fig. 2-1 Native gradient PAGE of partially purified protease used as an antigen. The protease was electrophoresed in 4.2-20% acrylamide linear gradient large slab gel under nondenaturing conditions at 20 mA for 7 h at 8°C. Shown are the protease after gel permeation chromatography (GPC) and the protease purified by native gradient PAGE (Native). The molecular marker proteins used were thyroglobulin (669 kDa, hog thyroid), ferritin (440 kDa, horse spleen), catalase (232 kDa, beef liver), lactate dehydrogenase (140 kDa, beef heart), albumin (67 kDa, bovine serum).

synthesized oligopeptide, indicating that the protease was trapped on the resin. The protease was eluted from the resin (300 μl) by boiling with SDS/sample buffer (ß-ME minus) after the treatment with 100 mM leupeptin to inhibit autolysis of the protease. Since the binding of the activity was very strong, other methods for elution (salts, detergent, etc.) were not successful. As shown in Fig. 2-2, when the protease eluted and boiled with 10% ß-ME was applied to SDS-PAGE, three bands, at 40, 15 and 13 kDa correspond to polypeptides of the protease, were observed on SDS-PAGE. When the protease was eluted by guanidine hydrochloride (GuHCl), the eluted fraction showed no activity. However, the eluted protease restored the activity towards Rubisco as well as the oligopeptide effectively, after renaturation by dialysis against the buffer without GuHCl. The recovery of the activity by the renaturation was 26% of the adsorbed activity on the resin. The GuHCl-eluted protease also showed the same band pattern on SDS-PAGE as that eluted by SDS/boiling.

Properties of monoclonal antibody (MAb) against the SDS-dependent protease

We established one hybridoma clone producing a MAb specific for the SDS-dependent protease. The oligopeptide-digesting activity was trapped as sediments of MAb-protease complexes on Protein G PLUS /Protein A-Agarose (Fig. 2-3A) and the complex had the digesting activity. This experiment was performed as follows: Protein G PLUS/Protein A-agarose (100 μl) was incubated with 500 μl of anti-mouse IgG goat antibody (~25 μg) for 2h at room temperature, exhaustively washed with PBS containing 0.1% Tween 20 and incubated with a culture medium (Control), or the medium containing the IgG monoclonals over night at 8°C and exhaustively washed with PBS containing 0.1% Tween 20 (+MAb).
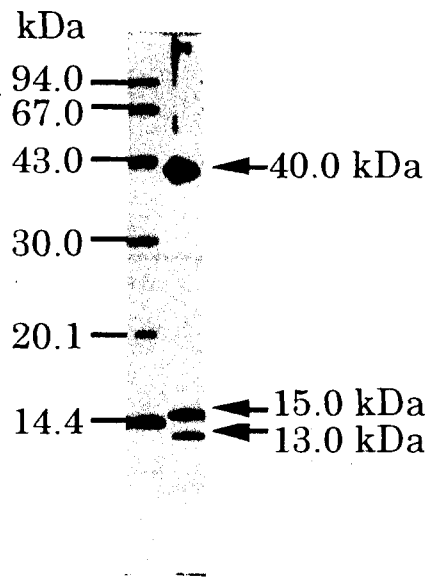
2 1

Fig. 2-2 SDS-PAGE of purified protease. The enzyme bound to the resin of immunoaffinity column was eluted by boiling with SDS-PAGE sample buffer without ß-mercaptoethanol and the eluate was subsequently boiled with 10% ß-mercaptoethanol as described in Materials and Methods. The prepared enzyme was electrophoresed in a 14% acrylamide gel under denaturing conditions by the method of Laemmli (1970) and stained with Coomassie Brilliant Blue R-250. Shown is the enzyme purified by immunoaffinity chromatography with monoclonal antibody.

A
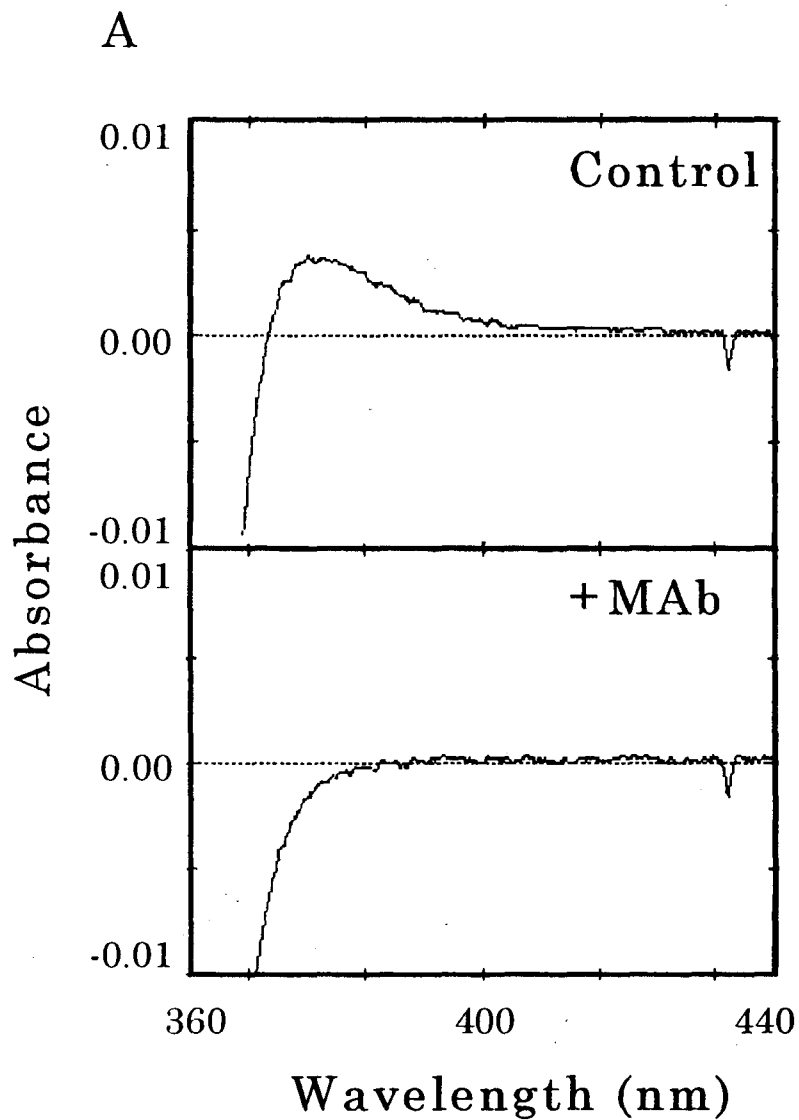
B

Control Native Denatured

Fig. 2-3 Specific binding of monoclonal antibody to native
SDS-dependent protease. A, Immunoprecipitation of native
SDS-dependent protease with monoclonal antibody assayed by
spectroscopic measurement. B, Recognition of monoclonal antibody
to native protease on immunodot blot analysis. Detailed procedure
was described in the text.

2 3

Respective resins were incubated with the enzyme (150 ng, 100 μl) purified by native gradient PAGE at 8°C for 2.5 h. The supernatants were obtained by centrifugation of the incubation mixtures at 100 x g for 2 min, and the activity of the supernatants (10 μl) was assayed by incubation with 190 μl of Boc-Val-Leu-Lys-MCA (200 μM) in the presence of 0.1% SDS for 1 h as described in Materials and Methods. Shown are the absorption spectra of the liberated AMC by the supernatant obtained after the incubation with the mouse IgG resin complex (+MAb) or the control resin (Control). Therefore, the MAb recognized an epitope other than the active site of the SDS-dependent protease. The MAb-purified protease also had a high activity towards Rubisco, supporting that these two activities were due to the same enzyme. The MAb bound to the native form but not to the denatured form of the protease (Fig. 2-3B). This experiment was performed as follows: the protease (~0.5 μg) purified by native gradient PAGE was boiled with the same volume of SDS/sample buffer (Denatured) or not treated (Native) and the proteins were blotted onto nitrocellulose membrane (Schleicher and Schuell, Germany). As a control, SDS/sample buffer was blotted (Control). MAb was of $IgG_1(k)$ isotype.

Effect of SDS and ß-ME on the proteolytic activity

Figure 2-4 shows that the protease had no activity in the absence of SDS. The proteolytic activity towards Rubisco appeared and markedly increased with an increase in the concentration of SDS, reached a maximum at 0.4%, and decreased gradually above 0.4%. As in the case of Rubisco, the protease hydrolyzed the oligopeptide only in the presence of SDS (Fig. 2-5). These results show that the protease itself requires SDS for the activation but not for the denaturation of substrates.

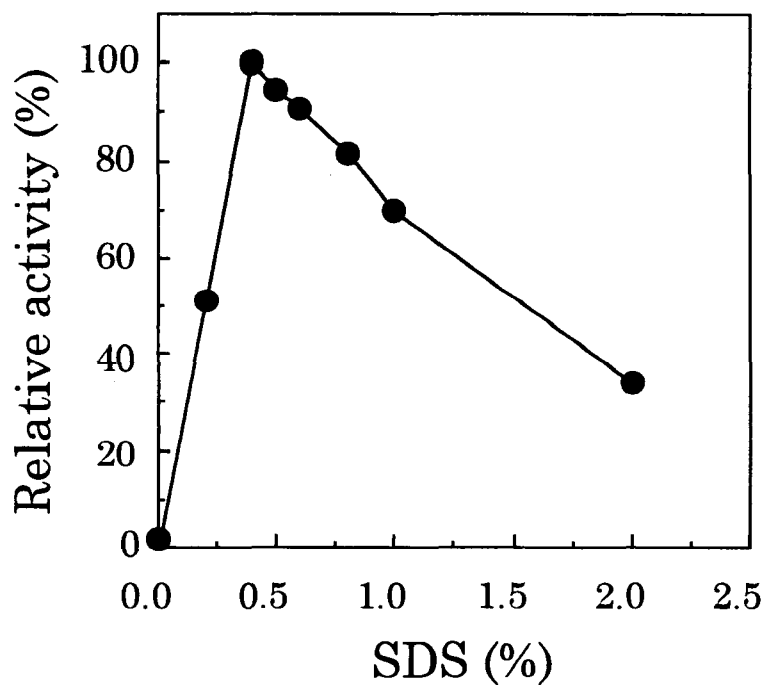The proteolytic activity was enhanced by ß-ME in the presence of

Fig. 2-4 Effect of SDS on the hydrolysis of Rubisco. The reaction was carried out using 150 ng of the enzyme purified by native gradient PAGE. The substrate (9.6 mg of Rubisco) was incubated with the enzyme at various concentrations of SDS, and degraded substrates were analyzed by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue R-250. LSU and SSU represent large and small subunit of Rubisco, respectively. Values are shown as percentages of the maximal activity.
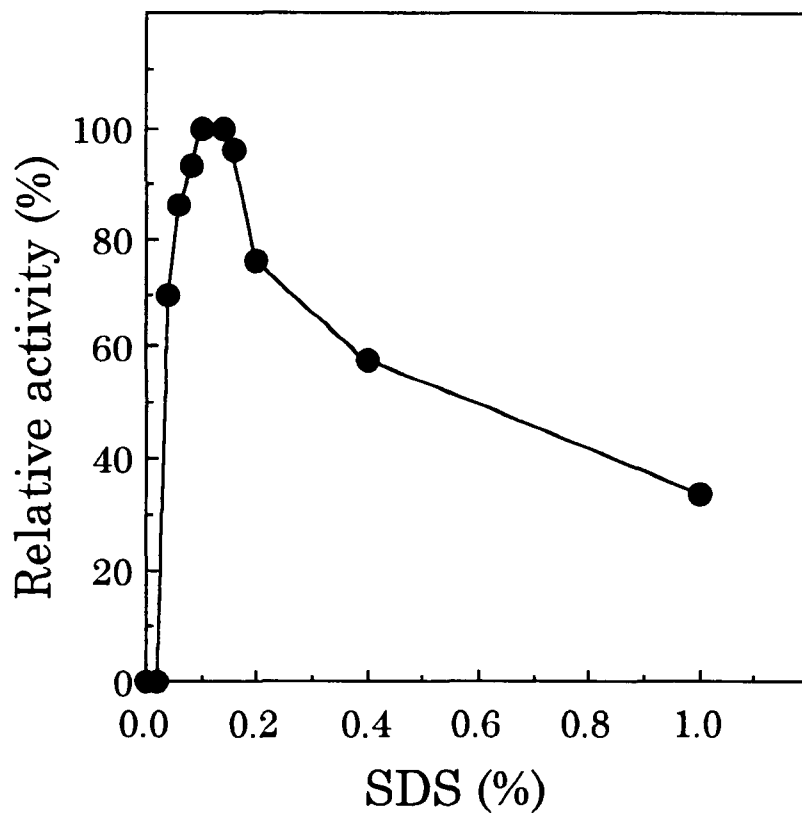
Fig. 2-5 Effect of SDS on hydrolysis of Boc-Val-Leu-Lys-MCA. The reaction was carried out using 150 ng of the purified enzyme in the assay mixture containing 200 mM of Boc-Val-Leu-Lys-MCA at various concentrations of SDS as described in Materials and Methods. Values are shown as percentages of the maximal activity.

0.5% SDS but not in the absence of SDS (Fig. 2-6). Thus, SDS could not be replaced by ß-ME. The optimal concentration was 1% in the presence of SDS. The activity was 1.5-fold enhanced by 1% ß-ME at 0.5% SDS.

Substrate specificity and pH optimum

Table II-2 shows that Rubisco was more efficiently hydrolyzed than other plant or animal proteins, indicating that Rubisco was a good substrate for the protease. Large subunit of Rubisco (LSU) was initially degraded to 43-, 33- and 27-kDa polypeptides and then these bands disappeared, although small subunit of Rubisco (SSU) was not sensitive to this protease (Fig. 2-7). The results indicate that multiple susceptible sites are present in the peptide of LSU. The proteolytic activity towards synthesized oligopeptides was investigated (Table II-3). The protease effectively digested the C-terminus of lysine in Boc-Val-Leu-Lys-MCA. At a slower reaction rate, the protease digested C-terminus of tyrosine. The result showed that the protease has a high specificity for the digestion site. Since properties of side chains of lysine and tyrosine residues are quite different, it is possible that the protease recognizes residues other than lysine and tyrosine. Figure 2-8 shows that the optimal pH was around 4.9 with a narrow active range for Boc-Val-Leu-Lys-MCA as well as Rubisco.

Effects of detergents and compounds on the proteolytic activity

Effects of various detergents and compounds on the proteolytic activity are shown in Table II-4 and II-5, respectively. It is indicated that SDS is specifically effective for activation of the protease. Although sodium 1-decanesulfonate showed 6% activity of SDS, none of other ionic and nonionic detergents tested could exhibit such high activation capacity of SDS. Furthermore, fatty acids, poly-L-lysine and ATP had no effect on

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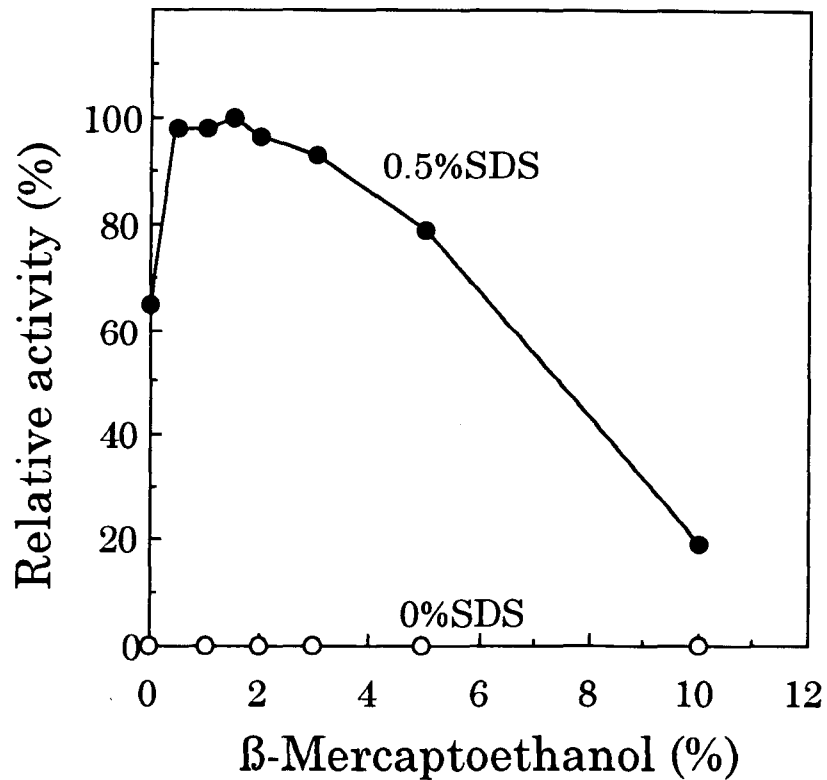Fig. 2-6   Effect of ß-mercaptoethanol on the proteolytic activity for Rubisco in the presence or absence of SDS. The reaction was carried out using 150 ng of the purified enzyme as described in the legend of Fig. 2-4. Open circles, in the absence of SDS; closed circles, in the presence of 0.5% SDS at various concentrations of ß-mercaptoethanol.   Values are shown as percentages of the maximal activity.

TABLE II-2 **Substrate specificity of SDS-dependent protease for several proteins.** Assay was carried out using the protease of 625 ng as described in Materials and Methods. Ferredoxin, γ-globulin, and bovine serum albumin (BSA) were present at 5.0 μg, 8.8 μg and 6.0 μg in the assay mixture, respectively. Each protein was boiled in 4.3% Softes (LION Co., Tokyo, Japan) before assay. The degradation of light harvesting Chl a/b protein (LHCP) was estimated on SDS-PAGE by the decrease of the corresponding protein band of thylakoid fraction prepared from spinach leaves (24 μg protein in the assay mixture).

Substrate	Hydrolysis rate
	nmol/h
Rubisco	2.5
Ferredoxin	0.8
LHCP	0.3
γ-globulin	1.6
BSA	0.3

S E EB EI EN

(kDa)
94.0 ——
67.0 ——
43.0 ——
⬛ LSU
⬛ 43 kDa

30.0 ——
⬛ 33 kDa
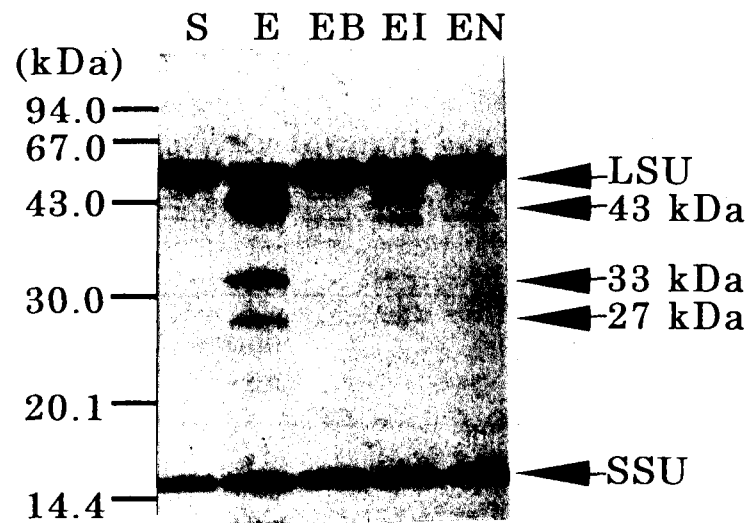⬛ 27 kDa

20.1 ——

14.4 ——
⬛ SSU

Fig. 2-7 **Proteolytic activity of SDS-dependent protease for Rubisco.** The enzyme purified by native gradient PAGE was used in this experiment. The reaction was carried out using 150 ng of the purified enzyme as described in Fig.2-4. The substrate (9.6 mg of Rubisco) was incubated in the presence of SDS (0.5%) with no enzyme (S, control), the purified enzyme (E), the enzyme treated by boiling for 3 min (EB), the enzyme treated with 100 nM E-64 (EI), the enzyme in the absence of SDS (EN), and degraded substrates were analyzed by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue R-250. LSU and SSU represent large and small subunit of Rubisco, respectively.

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TABLE II-3 **Proteolytic activity towards synthesized oligopeptides.**
The assay was carried out in the presence of 0.1% SDS.

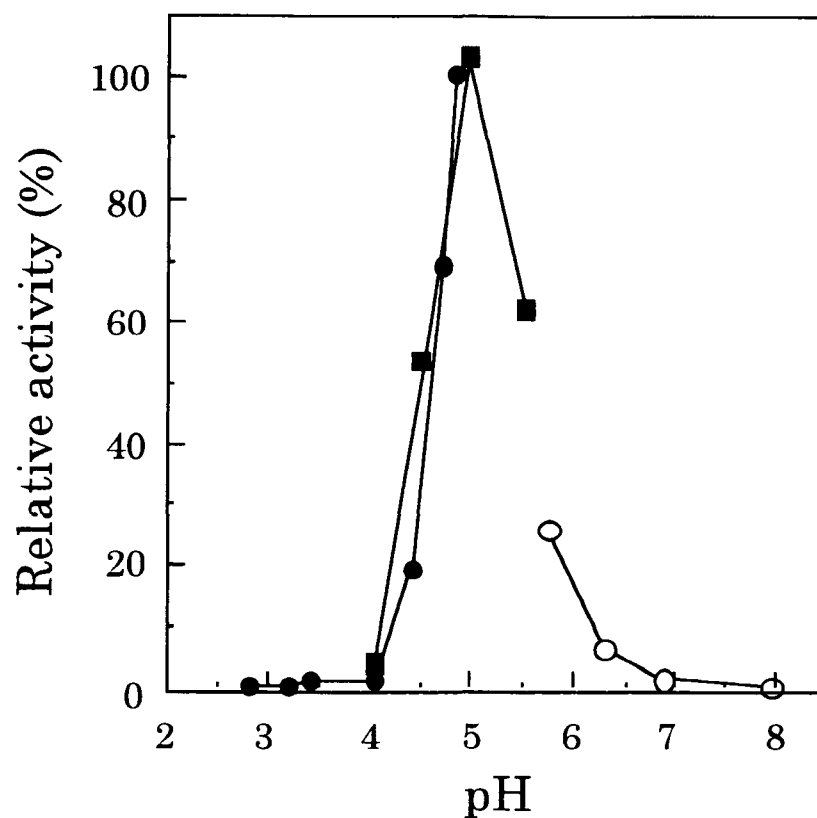| Synthesized peptide	Proteolysis AMC pmol/h
Boc-Val-Leu-Lys-MCA	6109
Boc-Glu-Lys-Lys-MCA	9
Lys-MCA	3
Boc-Leu-Gly-Arg-MCA	4
Boc-Leu-Lys-Arg-MCA	5
Boc-Leu-Arg-Arg-MCA	4
Boc-Phe-Ser-Arg-MCA	4
Arg-MCA	1
Ac-Tyr-Val-Ala-Asp-MCA	7
Suc-Ala-Glu-MCA	0
Suc-Leu-Leu-Val-Tyr-MCA	165
Phe-MCA	3
Suc-Ala-Pro-Ala-MCA	2
Suc(OMe)-Ala-Ala-Pro-Val-MCA	2

Fig. 2-8 pH dependence of the proteolytic activity. The reaction was carried out using 150 ng of the purified enzyme as described in Fig. 2-5 except for the buffer of the reaction mixture. The buffers used (ionic strength = 0.6) were: 0.2 M sodium citrate for pH 3.0 to 5.8 (closed circle); 0.2M sodium acetate for pH 4.0 to 5.52 (closed square); 0.2 M MES for pH 5.8 to 7.0 (open circle). Values are shown as percentages of the maximal activity.

TABLE II-4 **Effects of various detergents and compounds on the proteolytic activity for Boc-Val-Leu-Lys-MCA.** The concentrations of each detergent in the assay mixture is shown in parentheses.

Detergent	Head group /	Tail group		Activation (relative, %)
None				0
SDS	$-SO_4^-$	C_{12}	(0.1w/v%)	100
Sodium 1-decanesulfonate	$-SO_3^-$	C_{10}	(0.1w/v%)	2
			(1w/v%)	6
Sodium lauroylsarcosine	$-COO^-$	C_{12}	(0.1w/v%)	3
			(1w/v%)	2
Cetyltrimethylammonium bromide (CTAB)	$-N(CH_3)_3^+$	C_{16}	(0.1w/v%)	1
			(1w/v%)	1
Laurylpyridinium chloride	$-N^+$◯	C_{12}	(0.1w/v%)	1
			(1w/v%)	0
CHAPS	$-N^+(CH_3)_2(CH_2)_3SO_3^-$	Steroid	(0.1w/v%)	1
			(1w/v%)	1
Tween 20		alkyl ethoxylate	(0.1w/v%)	0
			(1w/v%)	0
Triton X-100		alkyl ethoxylate	(0.1w/v%)	0
			(1w/v%)	0

TABLE II-5   Effect of various lipids and compounds on the activation of the protease for Boc-Val-Leu-Lys-MCA. Concentration of each lipid in an assay mixture was 1mM, and of other is shown in parentheses.

Compound		Activation relative, %
Control		0
SDS (0.1 w/v%)		100
Lipids		
Sodium oleate	(18:1)	0
Phosphatidic acid	(18:1/18:1)	0
Phosphatidylglycerol	(18:1/18:1)	0
Diacylglycerol	(18:1/18:1)	0
Lyso-phosphatidic acid	(18:1)	0
Lyso-phosphatidylglycerol	(18:1)	0
Guanidine hydrochloride (3M)		5
ATP-Mg (1mM)		0
Poly-lysine (54 kDa, 100mg/ml)		0

the proteolytic activity. Since it was reported that one of the SDS-activated proteases, proteasome, was stimulated by these compounds (Ozaki et al. 1992, Watanabe and Yamada 1996), the protease in this study was clearly different from the proteasome.

Effect of inhibitors on the proteolytic activity

The proteolytic activity was inhibited by specific inhibitors for SH-protease such as E-64, leupeptin and antipain in the range between 10 and 100 nM (Table II-6). The activity was also inhibited by N-ethylmaleimide or iodoacetamide although they were less effective. On the other hand, phenylmethanesulfonyl fluoride (PMSF), an inhibitor of serine protease, inhibited at much higher concentration than those of E-64, leupeptin and antipain. EDTA, an inhibitor of metalloproteinase, had no inhibitory effect. These results as well as the stimulation by the thiol compound such as ß-ME indicated that the protease was an SH-protease. p-Tosyl-L-lysine chloromethyl ketone (TLCK) also inhibited the proteolytic activity at a low concentration. Since TLCK inhibits trypsin, which is a protease that digests carboxyl-end of basic amino acids in a peptide, it is possible that the protease is similar to lysine-specific protease as the result of the digestion specificity for oligopeptides.

Effect of metal ions on the proteolytic activity

Figure 2-9 shows the effect of divalent metal ion on the proteolytic activity. Hg^{2+} ion strongly inhibited the proteolytic activity at nanomolar levels (100 nM, 57% inhibition). Zinc and copper ions also inhibited the proteolytic activity at millimolar levels, but other bivalent metal ions (Mg^{2+} and Ca^{2+}) had no effect on the proteolytic activity even at 10 mM. The inhibitory effects of Hg^{2+} and Zn^{2+} ions support that the protease is

TABLE II-6 Effect of protease inhibitors on the proteolytic activity for Boc-Val-Leu-Lys-MCA. Inhibition was expressed as the percentage of inhibition.

Inhibitors		Residual activity, %
E-64	10nM	63
Control		100
	100nM	16
Leupeptin	1nM	75
	10nM	24
Antipain	100nM	51
	1μM	10
N-ethylmaleimide	1mM	91
	10mM	56
Iodoacetamide	100μM	80
	1mM	25
PMSF[a]	100μM	36
	1mM	5
TLCK[b]	1μM	41
	10μM	16
TPCK[c]	100μM	36
EDTA	1mM	100

[a]phenylmethylsulfonyl fluoride, [b]p-tosyl-L-Lysine chloromethyl ketone, [c]p-tosyl-L-Phenylalanine chloromethyl ketone
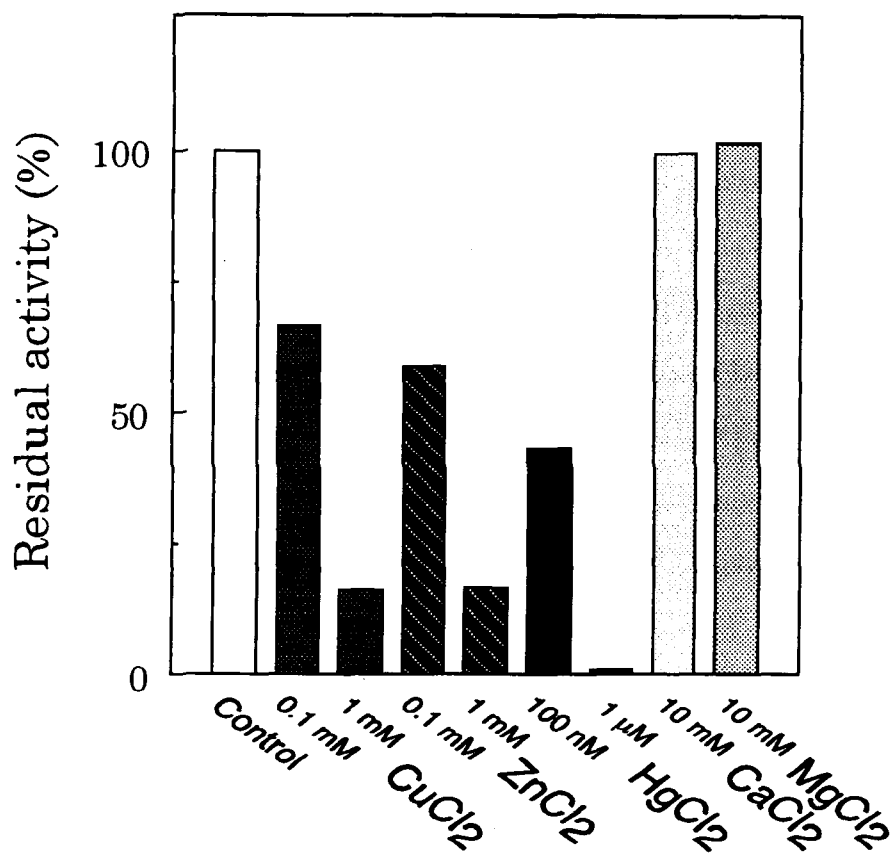
Fig. 2-9 Effect of metal ion on the proteolytic activity for Boc-Val-Leu-Lys-MCA. The assay was carried out as described in Fig. 2-5 but the buffer used 0.2M Acetate (pH 5.0) contained 0.5M NaCl.

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an SH-protease.

Discussion

Purification

In the present study, we purified a novel type of protease which was specifically activated by SDS to a homogeneous state from maize leaves. The specific requirement of SDS is a quite unique feature for this protease. In terms of the specific requirement of SDS, this protease can be distinguished from other SDS-enhanced proteases reported so far (Thayer and Huffaker 1984, Nettleton et al. 1985, Bhalla and Dalling 1986, Mae et al. 1989, Graham et al. 1991, Ozaki et al. 1992, Otto and Feierabend 1994). Most of these proteases, however, have not been completely purified, so that it is possible that the proteolytic activity was assayed as total activity containing the SDS-dependent and SDS-independent activities in the presence of SDS. This study showed that the present protease was composed of three different components. Such a presence of multiple components of SDS-stimulated proteases was not described except for proteasome (Ozaki et al. 1992) and MMPs (Graham et al. 1991). However, in contrast to the proteasome whose active site is considered to be threonine residue in β subunit (Seemüller et al. 1995) and MMPs, this protease is classified to SH-protease as other SDS-stimulated proteases in plants reported so far.

Specific activity was not increased at earlier steps of purification. However, the earlier steps were very effective to remove compounds such as lipids, degraded pigments and polyphenols. It was possible that the proteolytic activity in earlier purification steps reflected the total activity of SDS-dependent, -stimulated or -resistant activity towards Rubisco in

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maize.

Single band showing the SDS-dependent activity could be identified on native gradient PAGE. The band, however, gave several bands with smear background on SDS-PAGE despite the pre-treatment with several protease inhibitors such as leupeptin or E-64 to inhibit autolysis of the protease or despite boiling the enzyme before the electrophoresis. Therefore, at this step, it was difficult to identify which of the bands was the protease. Thus, the following strategy was employed: based on the fact that the single band identified on native gradient PAGE had SDS-dependent activity, MAb against the activity was prepared and subsequently, by means of an immunoaffinity chromatography with this MAb, the protease was purified. Assay system using an oligopeptide instead of Rubisco as a substrate enabled us to use this strategy because of the convenience and high sensitivity of this assay system especially in the screening process of the positive hybridomas. Moreover, since MAb obtained was of IgG_1 isotype not IgM isotype, it was easier to concentrate (or purify) the MAb with Protein A/G. The immunoaffinity chromatography was quite effective in the purification and identification of the SDS-dependent protease. To elute the bound proteolytic activity from the resin, any other conditions except for SDS/boiling or 5M GuHCl were not effective. Furthermore, only by the immunoaffinity chromatography, specific activity of the protease increased more than 1,400-fold. These results indicate that binding of the MAb to the protease is strong and highly specific, and therefore, it can be considered that all of three peptides are components of the protease. However, it is difficult to determine whether all of the peptides are essential to exhibit the proteolytic activity or not. A 55-kDa protein cut out from SDS-PAGE showed the proteolytic activity (Yamada et al. 1995), although the protein band was faint on SDS-

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PAGE. It is possible that the protease previously reported is an intermediate complex composed of two components (40+15 or 40+13 kDa peptide) of this protease complex, including 40-kDa peptide possibly having the proteolytic activity. This is further discussed in Chapter III.

Activation

It is interesting how SDS induces the proteolytic activity *in vitro*. Many detergents tested other than SDS and heat-denaturation of Rubisco showed no effect on the activation. Furthermore, a synthesized tripeptide was also digested only in the presence of SDS. Therefore, it is obvious that denaturation of substrate by SDS is not essential step for the activation. The protease required higher concentration of SDS for the degradation of Rubisco than oligopeptide, presumably because, for Rubisco degradation, higher concentration of SDS is required for the increasing susceptibility of Rubisco to the enzyme. The protease required a high concentration of SDS for the proteolytic activity, at which most proteins are denatured. The result suggests that the active site exists within the folded protease molecule, which becomes accessible to substrates by SDS at weak acidic (optimum) pH region. It is speculated that SDS activation of the latent protease resulted from the conformational change to an active form. Alternatively, SDS activation is the result of dissociation of the inhibitor from the latent complex.

I have investigated with respect to a dissociation of the complex during the activation in detail, and discuss in Chapter III. The protease is similar to proteasome (Tanaka et al. 1986, Ozaki et al. 1992) or MMPs (Birkedal-Hansen and Taylor 1982, Springman et al. 1990) in respect to the SDS-derived activation of the latent form. However, the critical difference between these proteases and the protease in this study is that

the latter absolutely required SDS for activation *in vitro*. MMPs have a zinc atom which is essential for the activity and show maximum activity in the presence of millimolar levels of calcium ion. In contrast, the protease in this study was inhibited by zinc ion, and calcium ion had no effect. Thus, these results show that the regulation of activation of the protease is different from that of MMPs reported as Cysteine Switch (Springman et al. 1990) which was composed of a disruptable zinc-cysteine complex.

ß-ME was not essential for the proteolytic activity, because ß-ME enhanced the proteolytic activity only in the presence of SDS. The result indicates that ß-ME stimulates the activity additionally after conformational change in the protease by SDS. Since endogenous factor(s) in place of SDS probably induces the proteolytic activity *in vivo*, finding of such factor(s) will be an important work to elucidate the mechanism for the activation of the protease.

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Chapter III

Identification of the Components of the Protease Complex and The Mechanism of Activation

Introduction

The most unique property of the SDS-dependent protease is the change from a latent state to an active state, which is probably caused by a specific conformational change of the protease complex by SDS.

The latent state of proteases must be important for preventing autocatalytic destruction of the cells or an inadequate response for mis-signals produced by a proteolytic activity, and thus, it is presumed that there is some mechanism(s) for activating latent proteases *in vivo*.

In some cases, to control a proteolytic activity, the active enzyme is associated with protease inhibitors. This protease-protease inhibitor interaction is probably one mechanism to regulate intracellular protein catabolism (Goldberg et al. 1989, Stetler-Stevenson et al. 1989, Wilhelm et al. 1989, Kleiner et al. 1993). Many kinds of protease inhibitors have been found in plant cells (Ryan 1990). Some of the protease inhibitors are considered as a Protestant against attack of pathogen and insects (Wolfson and Murdock 1990). However, there are a very few cases in which some protease inhibitor was discovered as a true regulatory factor for an endogenous protease in plants. In addition to the regulation by protease inhibitor, a number of other mechanisms have been proposed, for example, an autocatalytic activation or an activation by a different factor (Dubiel et al. 1992, Salamino 1993).

It is, therefore important for understanding the mechanism to

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investigate the changes of enzymatic and molecular properties of the protease complex, namely change of molecular weight or stability between the latent and active states. Furthermore, information of the primary structure of an enzyme protein is useful to identify or characterize the protein.

I have established the purification of the SDS-dependent protease as described in Chapter II. In this chapter, I have prepared again the sample in large scale for analysis of internal amino acid sequences by the established preparation to obtain enough amount of protein. Internal amino acid sequences of the three components of the purified protease was determined, and homology search was performed using available databases. Furthermore, I examined the mechanism for the activation of the latent protease and found that the protease was activated with the change of molecular weight, through a dissociation of the latent complex. Based on these findings, I discuss a possible mechanism for regulation of the activity of the protease *in vivo*.

Materials and Methods

Analysis of internal amino acid sequences

By the established methods in this study described in Chapter II, the SDS-dependent protease was prepared and separated to three different components by SDS-PAGE. The proteins of the three components were electroblotted onto PVDF membrane (Applied Biosystems, USA). Each protein band on the membrane (approximately 10 μg) was cut out. The protein on the membrane was digested by lysylendopeptidase, Achromobacter protease-I to peptides, the digested peptides were separated by RP-HPLC. Then, the amino acid sequences of the digested

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peptide fragments were determined by the method of Iwamatsu (1992). Database comparisons (Genebank, EMBL and PIR) were done using default search parameters of FASTA programs from the GCG software package.

Identification of intermediate complex by GPC

Gel permeation chromatography (GPC) for identification of intermediate complex was performed in the presence of 3M GuHCl by Superdex 200 HR 10/30 column (Pharmacia LKB, Sweden). Other conditions of GPC were the same as described in Chapter II.

ELISA

Antigen (protease fraction from GPC) was adsorbed on a multiple well plate (Corning, USA), and the SDS-dependent protease was detected using anti-SDS-dependent protease MAb with horseradish peroxidase-conjugated anti-mouse IgG as a secondary antibody and O-phenylenediamine (Abs. 492nm) as a substrate.

Results

Determination of internal amino acid sequences of protease complex and their homology with SH-protease and cystatin

Figure 3-1 indicates that amino acid sequences of two fragments from the 40-kDa polypeptide showed a significant homology to those of RD21 (Yamaguchi-Shinozaki et al. 1992) and C14 cysteine proteases (Schaffer and Fischer 1988, 1990). The results strongly suggest that the polypeptide of 40 kDa is a cysteine protease in the protease complex. RD21 and C14 cysteine proteases are known as proteases encoded by the *RD21* gene responding to desiccation in *Arabidopsis thaliana* and by the *C14* gene induced at low and high temperature in tomato, respectively.

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P40-3	KVVTIDSYEDVPANDEK
RD 21	NAKVVTIDSYEDVPTYSEESL
C14	KVVKIDSYEDVPVNNEKAL

P40-7	KCGIAVEPSYPLK
RD 21	SGKCGIAIEPSYPIKNG
C14	SGLCGLAIEPSYPVKTG

Fig.3-1 Alignment of conserved amino acid sequences among 40-kDa polypeptide, RD 21 and C14. P40-3 and P40-7 are fragments derived from the 40-kDa polypeptide. RD 21 and C14 are putative cysteine proteases. Residues in black box indicate conserved amino acid residues among the sequences.

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More interestingly, Fig. 3-2 and 3-3 show that the sequences of internal fragments from 13- and 15-kDa polypeptide basically matched with those of maize cystatin I and II, cysteine protease inhibitors, reported previously (Abe et al. 1992, 1995, Domoto et al. 1995). Cystatin is a inhibitor which specifically inhibits cysteine protease, and is widely distributed in various organs and has multiple functions (Abe et al. 1987, Gruden et al. 1997, Kondo et al. 1990, Misaka et al. 1996, Ojima et al. 1997). A homology search suggests that the 13-kDa protein was a mixture of cystatin I and cystatin II from maize (Abe et al. 1992, 1995, Domoto et al. 1995) (Fig.3-2). The fragments from 15-kDa peptide were highly homologous to sequences in maize cystatin II, but no sequence corresponding to cystatin I was found in fragments from the peptide. Replacement of Glu^{121} of cystatin II to Gly was found in Fragment P15-4.

Dissociation of a latent protease complex in relation to the enzyme activation

In Chapter II, I demonstrated that the protease was slightly activated by guanidine hydrochloride (GuHCl). The optimal concentration of GuHCl for the activation was 3 molar (Fig. 3-4). Under this condition, the protease showed 5% of the maximal activation. The change in the molecular weight between the native form and the active form with 3 M GuHCl was investigated by GPC. GuHCl was more useful than SDS to investigate an active form of the protease, because the property of GuHCl has been little changed by physical parameters such as pH value, temperature and ion strength, differently from that of SDS with the change of critical micellar concentration. Figure 3-5 shows the elution pattern of the native (top) and the 3M-GuHCl treated (bottom) protease. The proteolytic activity of a native form was eluted with a peak of 185 kDa at

Cystatin I

```
  1   MRKHRIVSLVAALLVLLALAAVSSTRSTQK   30

 31   ESVADNAGMLAGGIKDVPANENDLQLQELA    60
                            P13-1

 61   RFAVNEHNQKANAILGFEKLVKAKTQVVAG    90
                            P13-2

 91   TMYYLTIEVKDGEVKKLYEAKVWEKPWENF   120

121   KQLQEFKPVEEGASA135
              P13-3
```

Cystatin II

```
  1   MRKHRIVSLVAALLILLALAVSSNRNAQED   30

 31   SMADNTGTLVGGIQDVPENENDLHLQELAR   60

 61   FAVDEHNKKANAILGFEKLVKAKTQVVAGT   90
                            P13-2

 91   MYYLTIEVKDGEVKKLYEAKVWEKPWEKFK  120

121   ELQEFKPVEEGASA 134
           P13-4
```

Fig.3-2 Identity of internal amino acid sequences derived from 13-kDa polypeptide with maize cystatin I and II. Underbars P13-1 to P13-4 indicate amino acid sequences obtained for the peptides derived from 13-kDa polypeptide. Residues in black box indicate amino acid residues conserved among various cystatins.

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Cystatin II

```
  1   MRKHRIVSLVAALLILLALAVSSNRNAQED   30

 31   SMADNTGTLV██IQDVPE█ENDLHLQE██    60

 61   ███DEHNKKA█AI█GFEKI█KAKT██A█T    90
      ━━━━━━━━━━━━━━━━━━━━━━━
                P 15-1
 91   MYYL█IEVKD█EVK██YEAKVWEK██EK██   120
                    ━━━━━━━━━━━━━━━    ■
                      P 15-2            ■
121   █░QE██PVEEGASA 134
      ━━━━━━━━━━━━━━ P 15-3
      G━━━━━━━━━━━━━ P 15-4
```

Fig.3-3 Identity of internal amino acid sequences derived from 15-kDa polypeptide with maize cystatin II. Underbars P15-1 to P15-4 indicate amino acid sequences obtained for the peptides derived from 15-kDa polypeptide. Residues in black box indicate amino acid residues conserved among various cystatins.
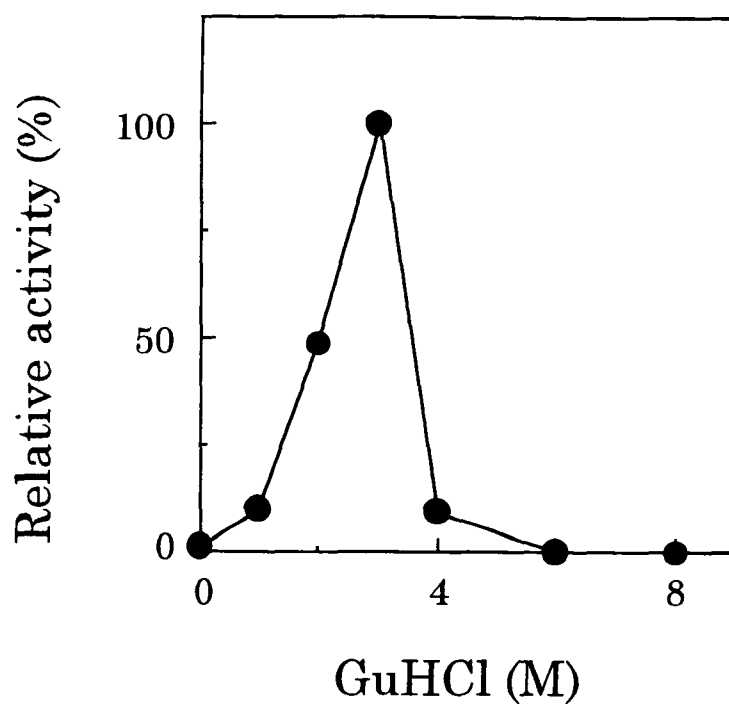
Fig. 3-4   Activation of the protease by guanidine hydrochloride (GuHCl). The protease was incubated at 35°C for 1h in assay mixtures containing various concentrations of GuHCl instead of SDS as described in materials and methods.
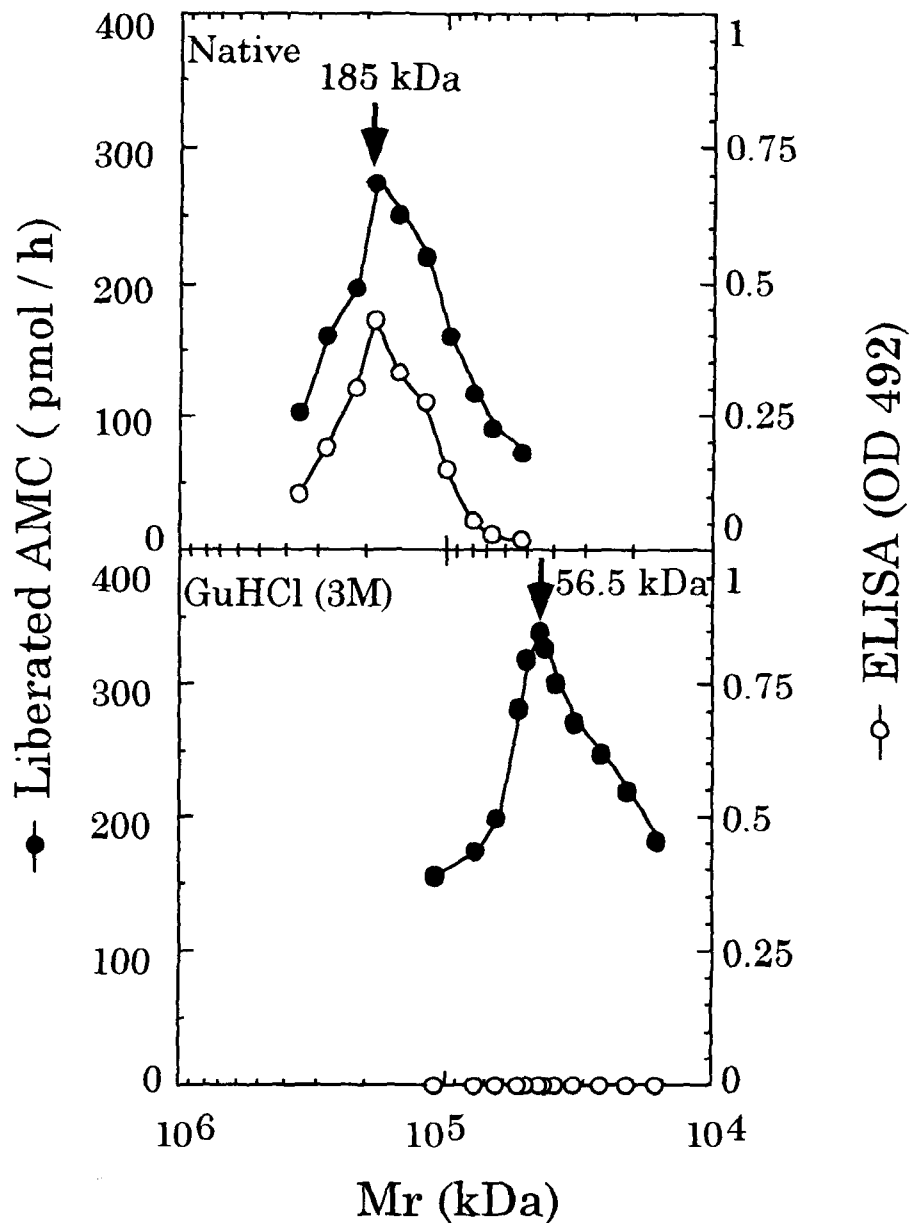
Fig.3-5 Dissociation of the protease complex from a latent to an active form. The protease purified by native gradient PAGE was treated with 3M guanidine chloride (GuHCl) or not (Native), and was separated by Superdex 200 HR 10/30 chromatography under the condition with or without GuHCl. The molecular weight was estimated by the elution volume of marker proteins, alcohol dehydrogenase (141 kDa,), albumin serum bovine (67 kDa), OVA (43 kDa) and cytochrome c (12.4 kDa). Twenty microliter of each fraction was used for the measurements of activity and protein of each fraction were assayed by ELISA described in materials and methods.

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the same pattern of the elution of MAb-recognized protein. However, when the native sample was treated with 3M GuHCl, the peak of its proteolytic activity shifted from 185 to 56.5 kDa but the protein was not detected by MAb, probably because MAb recognized only the 185-kDa complex. The protease in 56.5-kDa fraction also absolutely required SDS for its further activation.

Multiple actions of SDS against the protease

The protease was activated by the addition of SDS in an assay mixture. Then, it was examined whether SDS was required for only a formation step of an activated protease or also maintenance of the active state (Fig. 3-6). Once the protease was pre-incubated in an SDS-containing assay mixture for 5 min, the pre-incubated protease could digest the oligopeptide efficiently even after a dilution of SDS to 0.002% in which concentration no proteolytic activity was expressed without pre-incubation. That is, the action of SDS appears to lead to the formation of a persistently activated state of the enzyme, even after the dilution of SDS. These results indicate that 0.1% SDS is not essential for maintenance of active state of the enzyme once the enzyme was activated.

Stability of the protease under neutral or optimal pH condition with /without SDS was shown in Fig.3-7. The protease treated with 0.1% SDS under neutral pH condition at 35°C were very stable. However, it was inactivated very rapidly under optimal pH condition especially in the presence of SDS. To clarify whether the rapid inactivation was due to autolysis or not, the enzyme was first incubated with 200 μM leupeptin and then the proteolytic activity was assayed after dilution of its inhibitory effect. The results indicate that the inactivation could not be inhibited by the treatment of leupeptin (Fig. 3-8). It is supposed that the inactivation
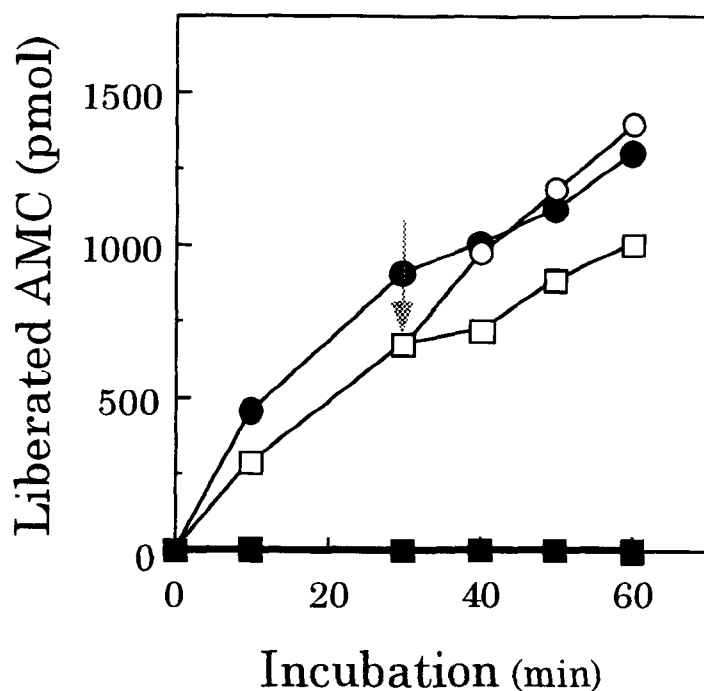
Fig. 3-6 The role of SDS on the formation of the active form. The protease (40 μl) added assay mixture (200 μl) containing 0.1% SDS were activated by pre-incubation at 35°C for 5 min. One-sixth volume of the protease (20 μl) was used for assay of the proteolytic activity in fresh assay mixture (900 μl) with (closed circle) or without (open square) 0.1% SDS. At the time shown by an arrow, 0.1% SDS was added (open circle). Closed square shows that the assay carried out in the absence of SDS using the enzyme without pre-treatment.
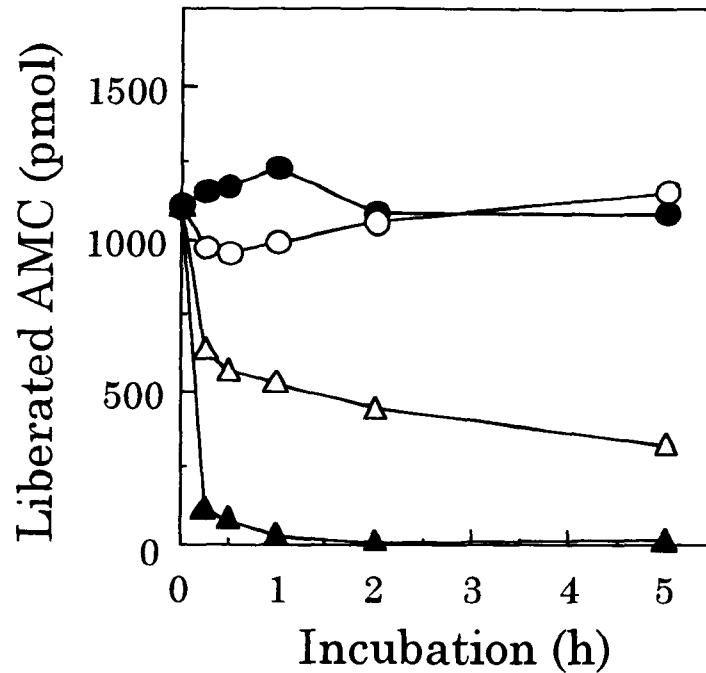
Fig. 3-7 Stability of the protease under neutral or optimal pH condition with /without SDS. The protease (20 μl) mixed with a treatment buffer (80 μl) was incubated at 35°C , an aliquot (10 μl) was taken, and its proteolytic activity was assayed at 35°C for 1h. Treatment buffers used were as follows: 0% (open circles) or 0.1% (closed circles) SDS in 200 mM Tris-HCl with 200 mM NaCl (pH 7.8), or 0% (open triangles) or 0.1% (closed triangles) SDS in 200 mM sodium acetate with 200 mM NaCl (pH 5.0).
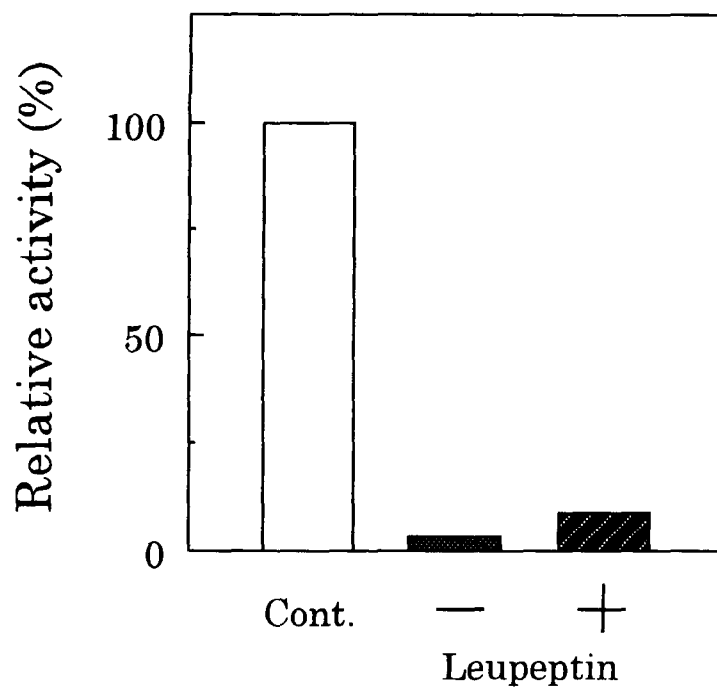
Fig. 3-8   Effect of leupeptin on an inactivation of the protease by the incubation with SDS. The enzyme (10 μl) was incubated in   solutions (40 μl) containing 0.1 % SDS plus/minus 200 μM leupeptin at pH 5.0 for 1h. The assay was carried out after the dilution of the reaction mixture to abolish the inhibitory effect of leupeptin.

was not caused by autolysis but by the structural change of the enzyme itself by SDS, or modification of the active site by its exposure by SDS. These interpretations are reasonable because SDS is, in general, known as a strong denaturant for proteins.

As described above, the enzyme was rapidly inactivated in the presence of SDS at optimal pH region (pH5.0). It is, however, partially protected by the concomitant presence of an oligopeptide substrate (200 μM) during the incubation at 35°C (Fig. 3-9). To confirm the protective effect of the substrate, the dose-dependence was examined on the residual activity after pre-incubation with various concentrations of the oligopeptide. As shown in Fig. 3-10, higher concentration of oligopeptide could protect the enzyme against the inactivation. Since it showed poor solubility in concentrations of more than 200 μM, the maximal protection could not be determined. As described in Chapter II, for the activation, the optimal concentration of SDS is 0.1%. However, the protease is activated by 0.04% SDS to 70% of the optimal activation (see Fig. 2-5). The extent of the protection depended on the concentration of SDS as shown in Fig.3-11. The protease treated with 0.04% SDS alone lost its activity almost completely in 1h-incubation. However, if the enzyme was incubated with the oligopeptide, 80% of the activity remained after incubation for 1h.

Discussion

In this chapter, I demonstrated that the purified protease complex consisted of an cysteine protease and cystatin, cysteine protease inhibitor. It is possible that the slight activation by treatment of 3 M GuHCl is caused by the presence of a small amount of 40-kDa protease produced or by a weak proteolytic activity of a 56.5-kDa intermediate complex itself. The
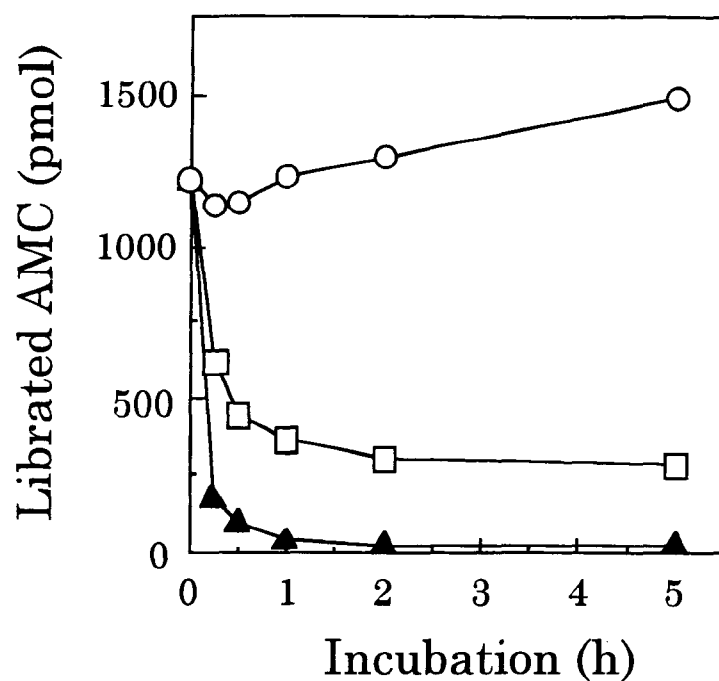
Fig. 3-9 Effect of an oligopeptide on the stability
of the protease in the presence of SDS. The
enzyme was incubated with SDS at pH 7.8 (open circles)
or at pH 5.0 with SDS plus (open squares) /minus (closed
triangle) 200 μM Boc-Val-Leu-Lys-MCA. The assay was
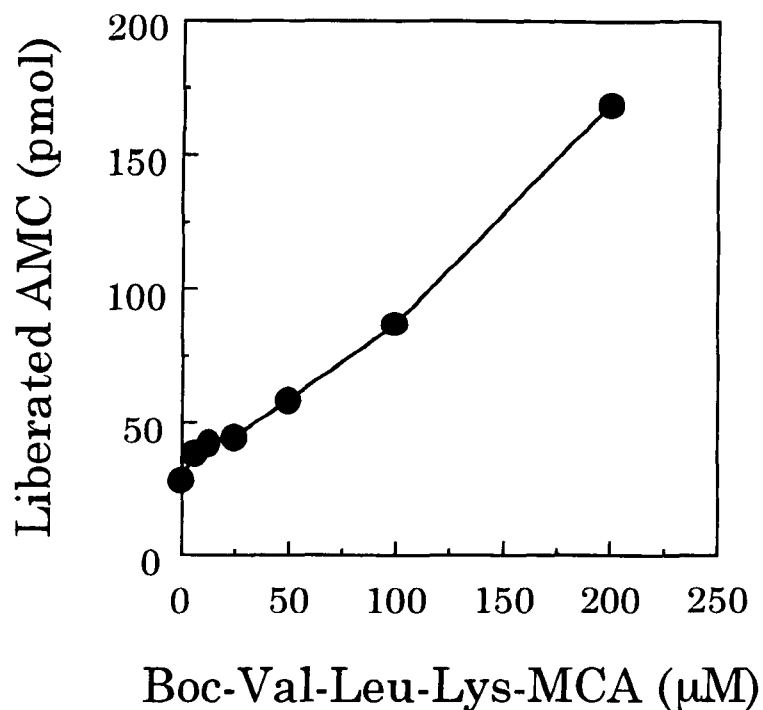carried out as shown in Fig. 3-7.

Fig. 3-10 Dose-dependence of an oligopeptide on the protection of the enzyme against the inactivation. The protease (10 μl) was incubated at 35°C for 1h in solutions (40 μl) of various concentrations of Boc-Val-Leu-Lys-MCA in the presence of 0.1% SDS, and then enzyme activities (10 μl) were assayed at a final concentration of 200 μM.
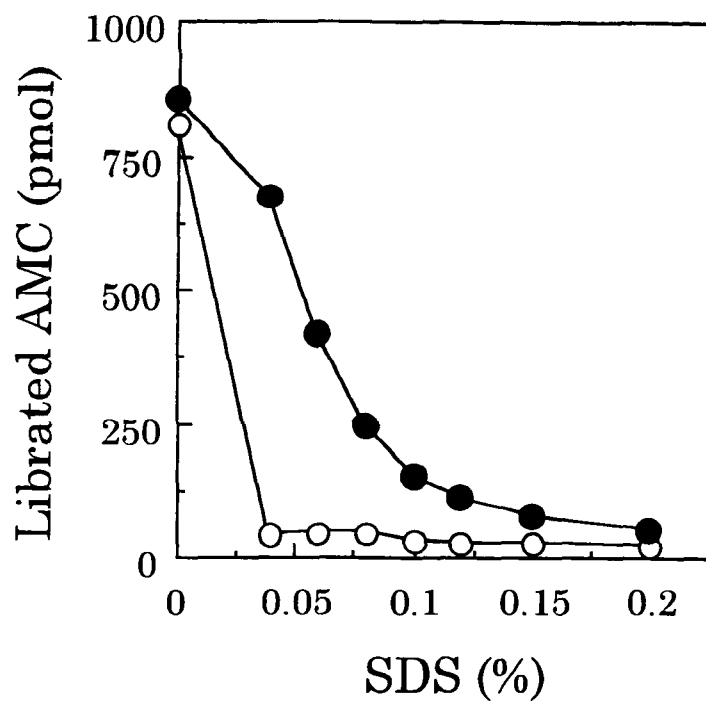
Fig. 3-11 Dose-dependence of SDS on the inactivation of the protease in the presence /absence of the oligopeptide. The enzyme (10 μl) was incubated in solutions (40 μl) of various concentrations of SDS with (closed circles) or without (open circles) 200 μM Boc-Val-Leu-Lys-MCA. The assay was carried out as described in Fig. 3-9.

ratio between the protease (40 kDa) and the total amount of inhibitors (13+15 kDa) on SDS-PAGE seems to be 1:1. These results together with the absolute requirement of SDS for activation of 56.5-kDa intermediate complex suggest that 40-kDa protease basically forms protease-protease inhibitor 1:1 complex (40 kDa +15 kDa or 40 kDa + 13 kDa), and the larger native complex of 185 kDa is made up of three units of these heterogous 'protease-protease inhibitor' complexes. It is interesting that there may be more than one binding site of inhibitor in the protease molecule and the activation may be regulated by multiple inhibitors. However, it is still difficult to clarify the molecular structure of the native complex of 185 kDa.

The presence of two 13 and 15 kDa cystatins in this complex probably reflects heterogeneity of inhibitor protein itself, namely it means that heterogous inhibitors are involved in the formation of native large 'protease-protease inhibitor' complex. From these results, it is very probable that *in vitro* function of SDS on activation of this latent protease is induction of dissociation of the latent complex and release of inhibitor from produced 'protease-protease inhibitor' complex. In addition, the dissociation and the release proceed only in the optimal pH region around 5.0. Proteasome is activated by SDS with no change in molecular weight (Dahlmann et al. 1985), differently from the present protease. These results imply that more than two steps contribute to the activation; the first is the dissociation of the large complex of 185 kDa to 'protease - protease inhibitor' complex of 56.5 kDa and the second is the release of a protease inhibitor from 'protease - protease inhibitor' complex of 56.5 kDa, and a 40-kDa protease produced may need further activation with a slight conformational change after liberation of the inhibitor from the complex. Such multiple steps of regulation of the activity imply a requirement for strict suppression of the protease activity *in vivo*. Moreover, the large

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complex of latent state might contribute to the stability of the complex in addition to the strict regulation of the activity. These suggested that the regulation was closely related to the prevention of expression of the protease activity for the protection of cells from any damage. SDS is an artificial modifier for the conformational change of the proteins *in vitro*, but there must be a similar mechanism for the activation of latent protease *in vivo*.

Once the latent protease is rapidly converted to the active state by SDS, the activated protease does not need SDS for the peptidase activity as shown in Fig. 3-6. The native complex was very stable under the non-activation condition. However, under the active condition, namely at weak acidic pH and the presence of SDS, the protease was rapidly unstabilized probably with a conformational change but not with autolysis. Furthermore, its instability was reduced in the presence of substrate. It is reported that an enzyme in proteasome becomes stable in the presence of SDS but not in the absence (Tanaka 1989). Therefore, it is speculated that the protease is active at just only short period when the protease must play its role *in vivo*, but the protease which has finished its role is rapidly converted to an inactive state. This mechanism could account for the strict regulation of the proteolytic activity and the prevention of inappropriate proteolysis.

Peptide fragments derived from the 40-kDa protein exhibited significant homology to two thiol proteases encoded by stress-inducible genes (Schaffer and Fischer 1988, 1990, Yamaguchi-Shinozaki et al. 1992). These cysteine proteases are generally considered to be regulated in the transcriptional level of the gene expression. However, my finding strongly suggests that post-translational regulation system by protease inhibitor is also important for similar type of protease *in vivo* including stress-

inducible cysteine proteases. Homology search revealed that the 13- and 15-kDa proteins were maize cystatin I or II, a cysteine protease inhibitor of maize (Abe 1992, 1995, Domoto 1995). Difference in the molecular mass between 13- and 15-kDa protease inhibitors was probably attributed to the presence of both precursor and mature type of inhibitors or some modifications of the inhibitors by such as a binding of sugar chain or phosphorylation.

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Chapter IV

The protease activity in organs and various plants

Introduction

Proteolysis contributes to physiological processes such as seed germination, senescence, and intracellular protein turnover. During seed germination, hydrolysis of seed storage proteins provides amino acids for protein synthesis in the growing seedling. Multiple germination-induced protease activities have been described in rice (Watanabe et al. 1991) and maize (de Barros and Larkins 1990, Mitsuhashi and Oaks 1994). During senescence, proteins and chlorophylls are degraded, and produced amino acids and other compounds are recycled (Smart 1994). In particular, proteolytic breakdown of Rubisco is important in a protein turnover (Miller and Huffaker 1982, Mae et al. 1984) during senescence.

Many of SDS-stimulated proteases are considered to be proteases which degrade Rubisco (Thayer and Huffaker 1984, Nettleton et al. 1985, Bhalla and Dalling 1986, Mae et al. 1989, Otto and Feierabend 1994). The degradation takes place particularly during senescence in plants. Therefore, I investigated the relationship between the senescence or aging and protein degradation. It is also important to investigate the localization and the distribution of the protease for understanding of the physiological role of the protease.

The properties of this SDS-dependent protease are useful to examine the localization of the activity. That is, the protease shows requirement of SDS for the activation and has a high specificity for an oligopeptide as described in Chapter II. The protease activity must also be

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regulated at post-traslational levels as discussed in Chapter III. It is, therefore, important to examine the localization of the activity as well as the protease protein itself. It was not successful to examine the localization of the protease protein within crude enzyme fractions by the obtained antibody. It is a probable reason that other proteins prevent the antibody from the recognition for the protease.

In this chapter, I investigated the localization or distribution of the proteolytic activity in the presence and absence of SDS and discussed the role of the protease in the senescence.

Materials and Methods

Plant materials

Plants (maize, wheat, barley, rye, cucumber, horse radish, pea and soybean) were cultivated in boxes filled with vermiculite in the sunlight.

Extraction of the protease

The enzyme was mechanically extracted with a mortar from various organs with a cold 50 mM HEPES-NaOH (pH 7.3) containing 2 mM $MgCl_2$ (10mL/g fresh weight). The extract was centrifuged at 9,810 x g for 15 min and the supernatant was used as enzyme sources.

Protein assay

Protein concentrations were determined by the method of Bensadoun and Weinstein (1976) using bovine serum albumin as the standard.

Results

Tissue specificity of the protease activity in early developmental

stage

The protease shows the dependency on SDS for activation and a high specificity for a digestion of oligopeptides as described in Chapter II. The properties are useful to examine the localization of the protease activity in plants. First, the localization of the SDS-dependent activity of various organs in early developmental stage was examined. The activity was assayed in the presence and absence of SDS (Fig. 4-1). The protease activity was found in every organ and its activity in primary leaves, cotyledons, stems, and root showed high levels in the presence of SDS. Especially, the activities of primary leaves and cotyledons were observed only in the presence of SDS, but not in the absence. This indicated that the protease at latent form dominantly existed in these organs. The activities in dry seed and soaked seed exhibited lower activity with similar levels, suggesting that the protease activity was not related to the break of dormancy which is the first step for germination.

Relationship between senescence and the protease activity

It has been reported that the senescence is induced by the dark treatment of plants like a natural senescence. Dark-treated plants show senescencing with yellowing of leaves caused by a degradation of chlorophylls. The protease activity did not alter during days after dark treatment (Fig. 4-2), indicating that the protease activity was not related to the senescence.

In monocotyledons, the top of primary leaf is older than its bottom connecting to the stem. A primary leaf was divided into 9 sections from bottom to top of the leaf, and the activity in each section was examined (Fig. 4-3). The protease activities showed a relatively constant level. Thus, it is clear that the protease activity has no relation to senescence or leaf age.
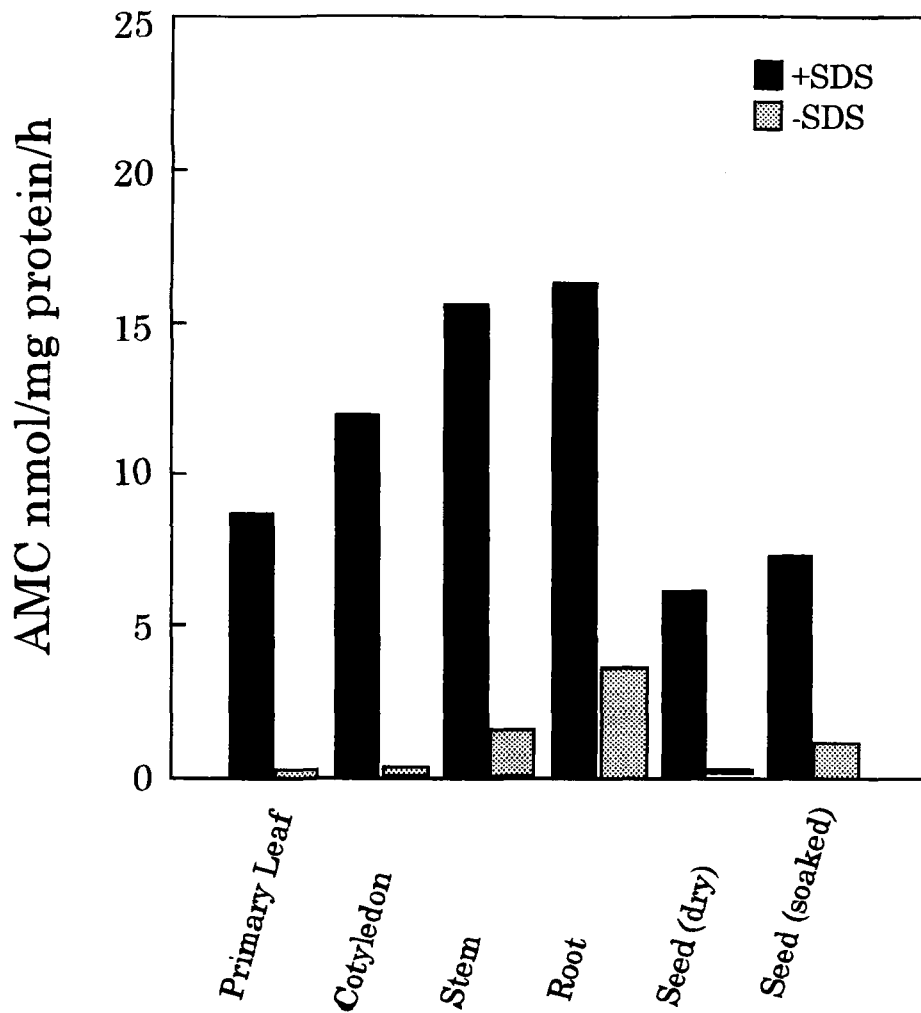
Fig. 4-1 Tissue specificity of the protease activity in early developmental stage of maize. The maize seeds were germinated and seedlings were grown for 13 days in the sunlight. Each organ was collected for assays for the activity. Seed (soaked), seeds treated with water for 2h; Seed (dry), seeds without treatment. The assays were carried out in the presence (0.1%) and the absence of SDS.
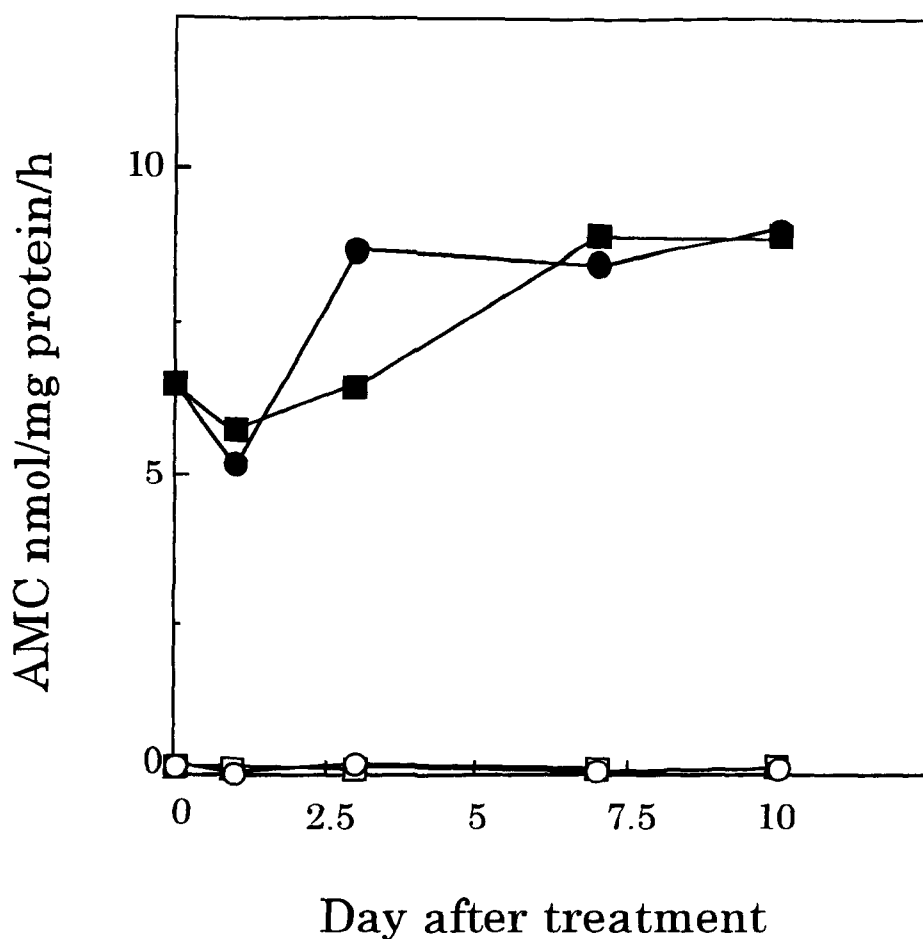
Fig. 4-2 Effect of dark-inducible senescence in a primary leaf on the proteolytic activity. The maize seeds were germinated and seedlings were grown for 13 days in continuous light. Then, some of them (dark-treated) were further grown in darkness and the others (control) were grown in the light. Primary leaves were collected on the day indicated for assays for the activity. The assays were carried out in the presence (0.1%) (closed square, control; closed circle, dark-treatment) and the absence (open square, control; open circle, dark-treatment) of SDS.
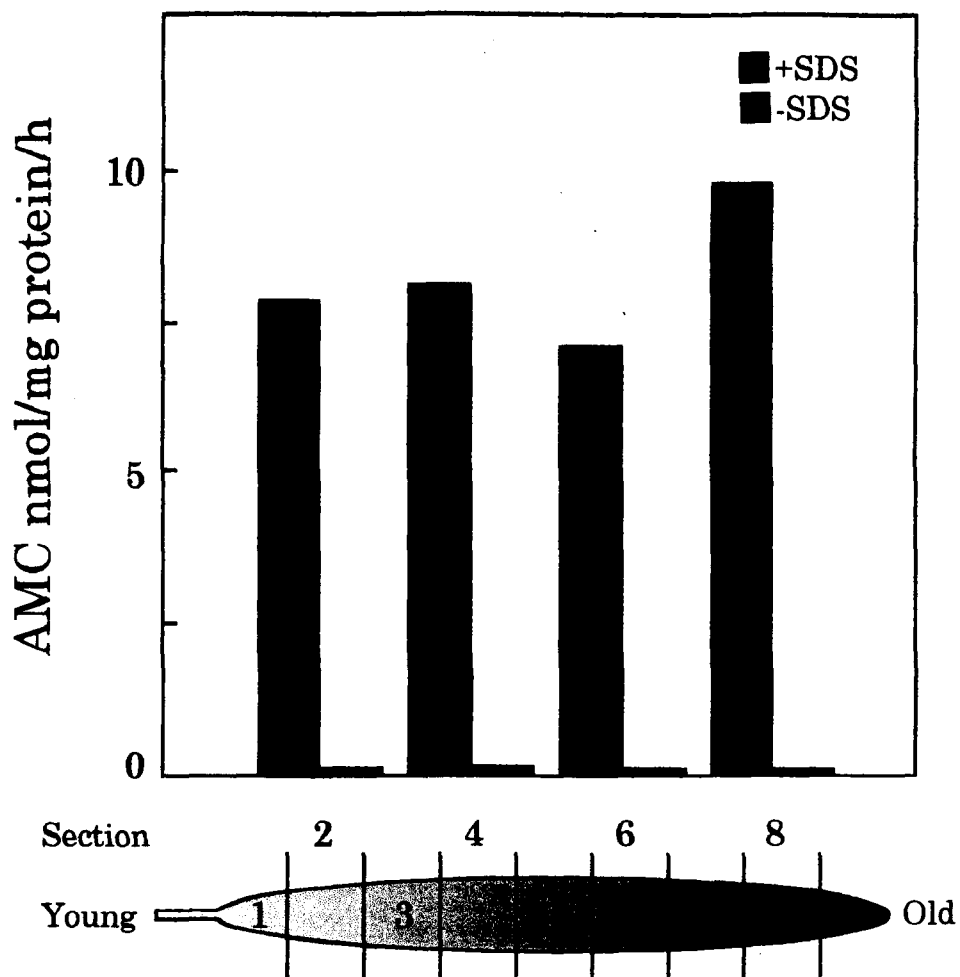
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Fig. 4-3 Effect of aging in a primary leaf on the proteolytic activity. A primary leaf of 13-day-old seedling was devided into 9 sections. The activities of four sections (2,4,6,8) shown in this figure were examined. The assays were carried out in the presence (0.1%) and the absence of SDS.

Distribution of the protease activity in various plants

Distribution of the protease activity was examined in various monocotyledons and dicotyledons (Fig.4-4). The protease activity existed in all the plants tested, suggesting that the protease ubiquitously exists regardless of plant species. Interestingly, in maize and cucumber, the activity was found only in the presence of SDS but not in the absence, indicating that the protease exists only at latent form.

Discussion

In a cotyledon and a primary leaf of maize, the SDS-dependent protease activity could be measured specifically, since SDS-independent activity was trace. At least under this condition, it can be considered that the SDS-dependent activity basically reflects the amount of this protease-protease inhibitor complex. The SDS-dependent activity was widely localized in all of the organs tested of developing maize plants. Although protease activities in many plants are induced by soaking their seeds, SDS-dependent activity remains unaltered by soaking of the seeds. That is, the proteolytic activity has no relation with early germinating step. It is unclear whether the SDS-dependent protease in the seed is identical to that in the developing plant. It is, however, interesting that both SDS-dependent activities show the same magnitude despite the difference between a storage and a vegetative organs.

To examine the relationship between physiological events and the proteolytic activity, I focused on the senescence of plants , because Rubisco degradation is very important during senescence, and SDS-stimulated proteases are often considered to be involved in this degradation. From
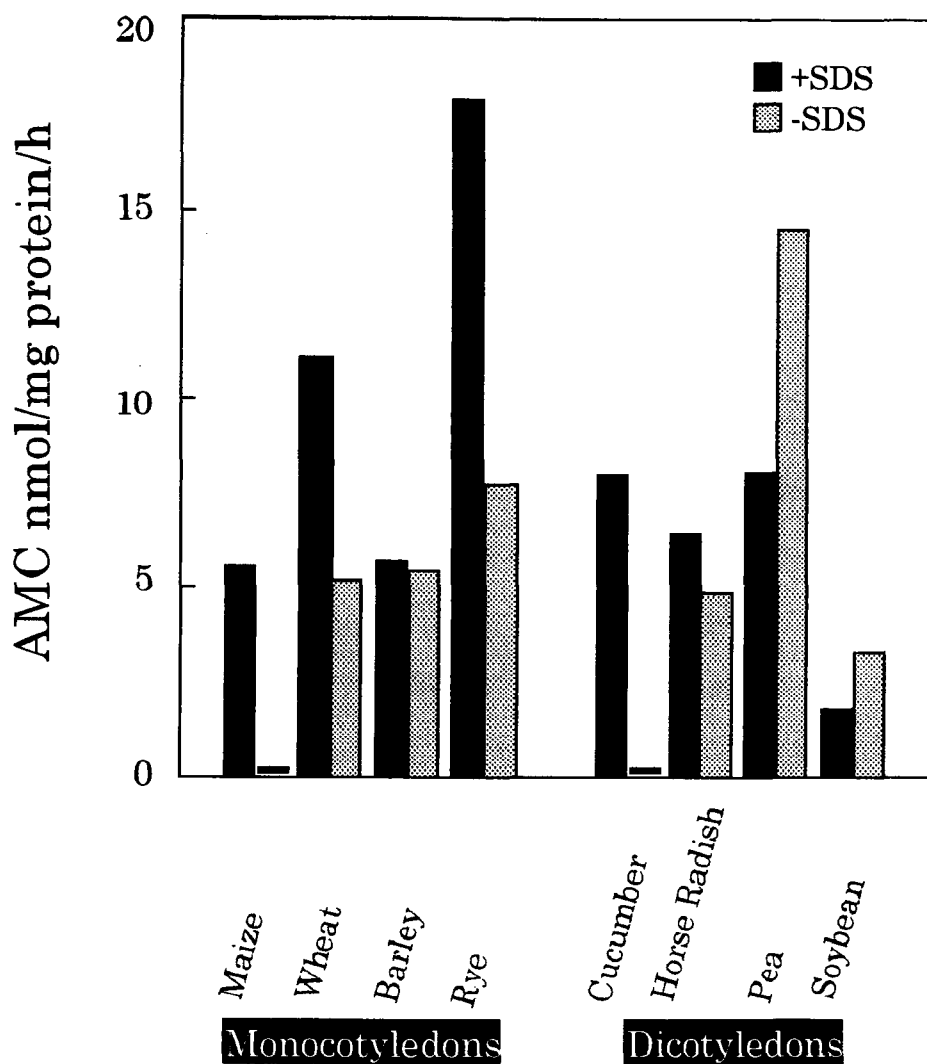
Fig. 4-4 Distribution of the SDS-dependent protease activity in various plants. The enzyme was prepared from primary leaves of various plants. The assay was carried out in the presence (0.1%) and the absence of SDS.

the results using a primary leaf and dark-induced senescent primary leaf, it was indicated that the SDS-dependent activity had no correlation with senescence. Therefore, the protease is different from the SDS-stimulated protease (Dalling et al. 1983, Mae et al. 1989) which degrades Rubisco during the senescence, although the present protease efficiently degrades Rubisco.

With regard to the distribution of the protease in various plants, it seemed that the activity was widely distributed in plants. The activities in the absence of SDS in pea and soybean were higher than those in the presence. The activity without SDS probably indicates the presence of other type(s) of protease which catalyze degradation of this oligopeptide. However, this SDS-independent protease activity may be inactivated in the presence of SDS. And thus, the activities in the presence of SDS in Leguminosae may reflect the presence of this 'protease-protease inhibitor' complex. Interestingly, the extract from cucumber showed only the SDS-dependent activity similar to maize.

Thus, it is suggested that the SDS-dependent protease, namely 'protease-protease inhibitor' complex, ubiquitously exists in different organs in plants, and distributes in various plants, and is not related with senescence in maize. The protease, therefore, probably is not related to the turnover of proteins. On the other hand, ubiquitous existence of the present protease at a latent state suggests its relation to some emergent events such as biotic or abiotic stresses.

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Chapter V

General Discussion and Conclusion

The present study shows that the SDS-dependent protease is purified as a latent complex with high molecular weight containing 'protease-protease inhibitor' complexes, and therefore, the activation by SDS is probably caused by a dissociation of the inhibitor from the complex. The purification by means of MAb affinity chromatography enables us to understand the activation mechanism. That is, the purification is efficient to obtain the protease retaining the state *in vivo*, resulting in catching the 'protease-protease inhibitor' complex. To my knowledge, in plants, this is the first example to purify a protease as a complex with protease inhibitors, although it has been reported in mammalian (Kleiner et al. 1993). Therefore, it is important to investigate the role of the protease in association with the regulation of the activation. With regard to SDS-stimulated proteases reported so far (Thayer and Huffaker 1984, Bhalla and Dalling 1986, Mae et al. 1989, Yokota et al. 1990, Otto and Feierabend 1994), the mechanism of the action of SDS is obscure although it is presumed that SDS raises the susceptibility of substrate proteins to proteases. However, ubiquitous distribution of this SDS-dependent protease, namely 'cysteine protease-cystatin' complex strongly suggests that some of the SDS-stimulated Rubisco degrading activity are attributed to this type of 'protease-protease inhibitor' complex. In plants, main investigations with regard to regulatory mechanism of an proteolytic activity focussed on regulations of transcription such as responses of gene expressions by hormone (Watanabe et al. 1992, Skriver et al. 1991). However, regulations of a proteolytic activity are carried out at various

stages, namely transcriptional and translational stages of the gene, and an activational stage of the protein. The post-traslational regulation is the last step on the expression of function, and is very important as the others. The present study could contribute to the elucidation of the regulation at the activational stage through the understanding the action by SDS.

On the basis of the results described above, a possible mechanism for the activation of the SDS-dependent protease is proposed (Fig. 5-1). The large latent protease complex is very stable and rigid, so that it is unable to be activated without a modifier which can derive a conformational change such as SDS and GuHCl. The large latent protease (185 kDa) is composed of trimer of a protease (40 kDa) -protease inhibitor (13 or 15 kDa) complex (P-I complex). The large latent complex (P-I)$_3$ is dissociated to P-I complex by 3M GuHCl, although a small amount of the protease is activated. The P-I complex is further dissociated to the protease and the inhibitor by 0.1% SDS, resulting in the activated protease. In this step, the protease free from the inhibitor may further require a little more conformational change for the activation. The large latent protease complex is, however, fully activated only by addition of SDS. SDS is capable of inducing the dissociation of the latent complex, the production of P-I complex, the dissociation of P-I complex and consequently activation of the protease itself. The structure of the active protease is unstable, resulting in conversion to an inactive form.

Furthermore, the role of the protease *in vivo* is proposed from the present data. The SDS-dependent protease is ubiquitously present in all organs and distributed in various plants as the latent form. The protease exists as a large complex with latency. It is probably important in strict suppression of the activity in the complex and compartmentation. It is speculated that the protease functions at an emergent event such as a
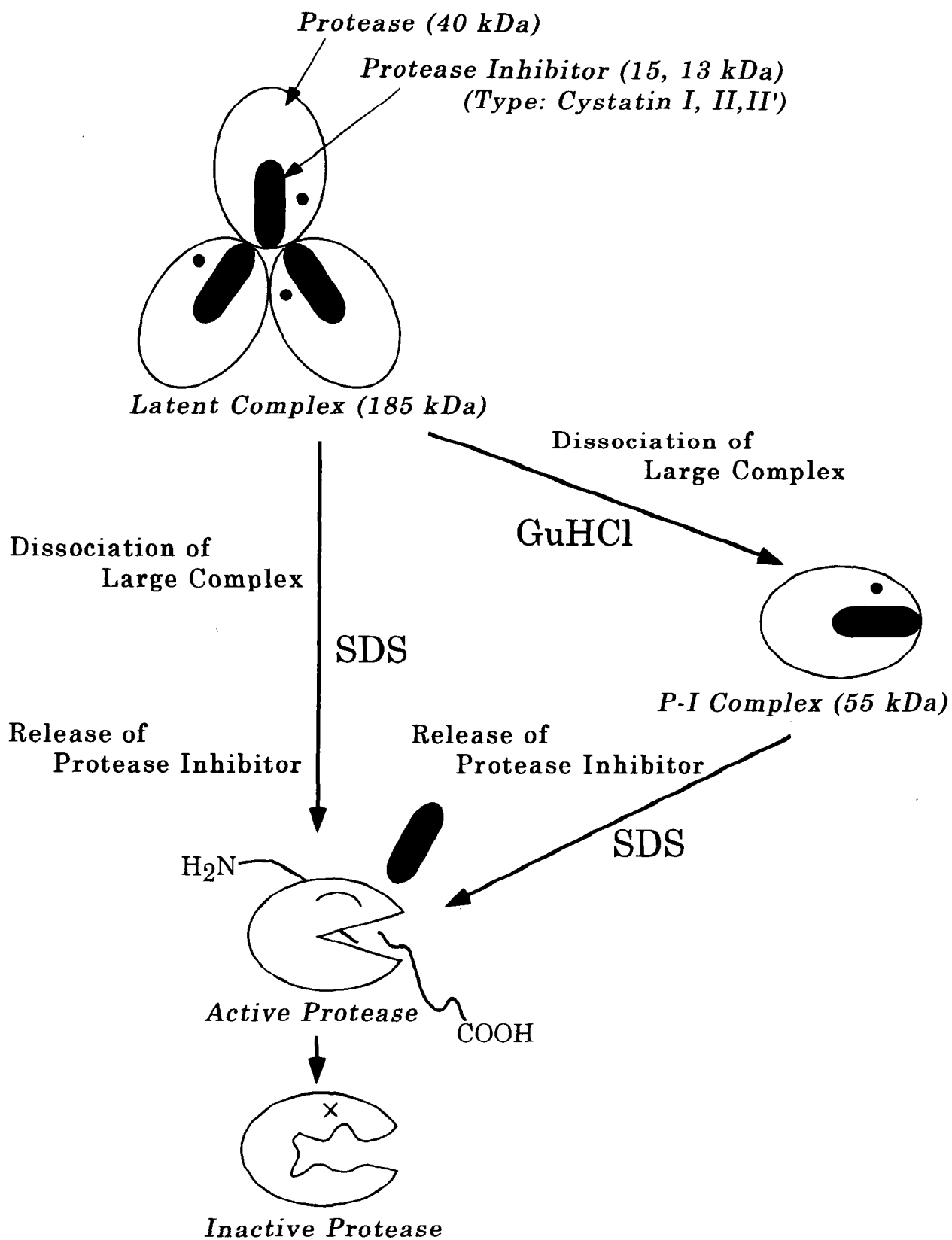
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Fig. 5-1 A proposed mechanism for the activation
of the cysteine protease complex.

drought, or low and high temperature stress. To response to the emergent events, the enzyme is activated by some factors in the optimal pH region. If the large latent protease complex is stored in a compartment at a neutral pH such as cytosol, it may be transported to an appropriate area to express the activity. When the substrate is consumed, the active protease is immediately inactivated. That is, the protease which has finished the role is inactivated quickly to prevent from mis-digesting important proteins.

With regard to cystatin in plants, the most of cystatins investigated are those from seeds (Abe et al 1992, 1995, Domoto et al 1995, Misaka et al 1996, Ojima et al 1997). It is considered that two types of cystatins are related to distinct roles in seeds, namely a maturation and germination of seeds (Kondo et al 1990). However, since a cystatin from rice inhibits exogenous proteases such as insects proteases, it is also considered that cystatins are related to defense systems (Liang et al 1991). Potato cysteine protease inhibitors (PCPIs) are recently found in potato tuber, and they belong a new type of cysteine protease inhibitor superfamilies (Gruden et al 1997). Accumulation of PCPIs is observed in vacuoles of stems after treatment with jasmonic acid (JA) using immunocytochemical localization. Since JA has been shown to be a messenger molecule in the signal transduction pathway of plant defense response to wounding (Farmer and Ryan 1992), it implys that PCPIs are part of a potato defense mechanism against insects and pathogens. As described above, the present protease binding the cystatin shows a significant homology to stress-inducible cysteine protease such as drought-responding and high and low temperature-inducible proteases. Also, the protease is widely localized and distributed in all tissues of maize and various plants. These findings of the protease binding the cystatin could imply that cystatins are involved in various roles in plants, although cystatins of seeds have been mainly

investigated in association with maturation and germination of seeds or defense mechanism in plants.

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