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Induction and Characterization of An Excreted Proteinase from Rice Suspension Cultures

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ABSTRACT

Induction of an extracellular proteinase in rice suspension cultures by salt, fungal elicitor, gibberellic acid or by replacing inorganic nitrogen compounds with casein were investigated. Sodium chloride with concentration of 2 mM stimulated cell growth and increased proteinase excretion. When inorganic nitrogen in the culture medium was replaced with casein, a sharp increase of enzyme activity was observed after one day. Inclusion of both gibberellic acid and casein in the medium showed an additive effect on proteinase induction. The result of RNA blot analysis indicated that proteinase transcripts was enhanced by treatment of gibberellic acid, fungal elicitor or sodium chloride. The partially purified proteinase had an optimum pH of 3.5 at 30℃. The enzyme was stable at 45℃ for at least one hour and inhibited by leupeptin, p-hydroxymercuribenzoic acid or N-ethylmaleimide, but not by phenylmethylsulfonylfluoride. Result of inhibitor test indicates that the excreted proteinase is likely a sulfhydryl proteinase.

Key words: Induction, Excreted proteinase, Rice suspension culture, Enzyme properties

Introduction

Proteinases are widely distributed in unicellular and multicellular organisms (Rogers *et al.*, 1985; Bond and Butler, 1987; Hoober and Hughes, 1992; Griffin *et al.*, 1992). Proteolysis in a plant is involved in many physiological processes, such as storage protein breakdown, senescence and intracellular protein turnover. Mobilization of storage protein by proteinases during the early events of germination is well studied in cereals, in which the expression of the endoproteinase gene is induced effectively by gibberellic acid (Nolan and Ho, 1988; Watanabe *et al.*, 1991). In cotyledons of leguminous plants the sulfhydryl proteinases are characterized and the mRNA of the enzyme is accumulated during germination (Mitsuhashi *et al.*, 1986; Mitsuhashi and Minamikawa, 1989; Tanaka *et al.*, 1993), but it is unclear whether plant growth regulators are involved in the regulation of proteinase gene expression in the cotyledon. In plant growth and development proteinases are shown to involve in xylogenesis (Ye and Varner, 1996) and leaf expansion (Huangpu and Graham,

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1995). Recent evidences demonstrate that proteinase gene expression are induced in tomato fruit by treatment of thermal stress (Schaffer and Fischer, 1988; 1990), as well as in pea by salt and dehydration (Jones and Mullet, 1995). Although variant responses to exogenous stimulus between differentiated and undifferentiated cells may be expected, cultured cells could serve as suitable materials for investigation of stress responses. mRNA of α-amylase accumulated rapidly in cultured cells of rice under sucrose starvation (Yu *et al.*, 1991), but there are limited studies on proteinase from cell suspension culture (Cordeire *et al.*, 1993).

We previously investigated plant hormones and other exogenous stimuli that affect cell growth and metabolic activity (Chou and Tong, 1993). In the present work, we report the effect of salt, fungal elicitor, gibberellic acid or casein on the excretion of endoproteinase from cultured rice cells, and some properties of partially purified proteinase.

Materials and Methods

Cell cultures

Cultured rice cells (*Oryza sativa* cv Taipei 309) were kindly provided to us by Dr. S. M. Yu, Institute of Molecular Biology, Academia Sinica. Cells were grown in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) incubated on a gyratory shaker at 150rpm under the photoperiod with 14 hours illumination at 30℃. Cells were counted by Haemocytometer during their growth. An aliquot of suspension cells was diluted 20-fold before counting. The cell number was determined as the mean of three counts.

Preparation of fungal elicitor

The elicitor was prepared by modifying the method of Kombrink and Hahlbrock (1986). *Rhizoctonia solani*, a rice fungal pathogen, was used in the preparation. A pure culture was obtained by inoculation of mycelium on potato dextrin agar, followed by growing at 25℃ for seven days. The fungal mycelium was then transferred to a V-8 liquid medium and grown at 25℃ on a gyratory shaker at 120 rpm for 14 days. Mycelium was harvested by centrifugation at 8,000xg for 15min and washed twice with doubly distilled water. This water (100ml) was added to the washed pellet and autoclaved for 30 min. The supernatant was collected by centrifugation and sterilized again before storage at 4℃.

Partial purificatin of proteinase

The medium of five-day rice culture treated with 2 mM sodium chloride was used for enzyme purification. Cells were removed by centrification at 8,000 xg for 15 min. Ammonium sulfate cut was performed as described by King (1972). Powdered ammonium sulfate was added slowly to the supernatant with stirring until 90% saturation was reached. Celite (diatomaceous earth, Sigma, USA) was then added to the solution with vigorous stirring, the suspension was carefully packed onto the column (2×35cm) and the

proteinase was eluted by 10mM Tris-HCl buffer, pH 7.0, with a flow rate 4ml/h, 1.2ml per fraction. The fractions with comparable activity were pooled, dialyzed and loaded onto a DEAE-Sephacel column (2×50cm), which was previously equlibrated with 10mM Tris-HCl buffer (pH 7.0). A linear gradient of KCl (0-1.0M) containing in 10 mM Tris-HCl buffer (pH 7.0) was applied at a flow rate of 30ml/h and fractions each 1.2ml were collected with a fraction collector (ISCO). Fractions of enzyme activity peak eluted with 0.4M KCl were pooled and dialyzed. The chromatographic steps were performed at 4℃. The enzyme solution was concentrated by lyophilization and stored at -20℃. The purity of concentrated enzyme was analyzed by 10% SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (1970). After electrophoresis the gel was stained with silver nitrate (Wray *et al.*, 1982).

Determination of enzyme activity and protein content

Proteinase activity was determined by the degradation of hemoglobin (Hammerton and Ho, 1986) or azocasein (Mitsuhashi *et al.*, 1986). In the hemoglobin assay, reaction mixture containing 3.75ml of 33mM sodium succinate buffer (pH 4.0) and 0.25ml of 2% hemoglobin. Reaction begain after adding 0.5ml enzyme solution. The mixture was incubated at 30℃ for 2 hours, followed by addition of 0.5ml of 50% TCA and kept on ice for 30 min. After centrifugation at 8,000xg for 30min. 1.0ml of supernatant was withdrawn for ninhydrin test. L-leucine was used as a standard. One unit of enzyme activity was defined as mmol of free amino groups detected per hours. In the azocasein assay, the reaction mixture containing 0.15ml of 2% azocasein solution made by dissolving 0.2g azocasein in 10ml of 33mM sodium succinate buffer, pH 4.0. After 0.15ml enzyme solution was added, incubation proceeded at 30 ℃ for 5 hours. After incubation 0.7ml of 5% TCA was added and the mixture was centrifugated at 5,000xg for 20min. The supernatant was decanted and absorbance at 366nm was measured. The variation of 0.01 absorbance per hour was denoted as one unit of enzyme activity. Protein content was determined by coomassie brilliant blue method (Bradford, 1976).

RNA slot blots

Cells were groung to powder in a mortar with a pestle in the presence of liquid nitrogen. Total RNA was extracted according to the procedure of Maniatis *et al.*, (1982). The powder (0.1g) was extracted with 1.0 ml of 25mM sodium citrate buffer (pH 7.0) containing 6M guanidine isothiocyanate, 0.5% lauroyl sarcosine and 0.1M 2-mercaptoethanol. Barley proteinase cDNA,pHVEP 4 (Koehler and Ho, 1990) donated by Dr. T. H. D. Ho(Washington university, USA), served as probe. The cDNA probe was labelled by the random primer method (Feiberg and Vogelstein, 1983). RNA (12μg per lane) was dropped in a slot on a nylon membrane and the filter was baked at 80℃ for 2 hours. Hybridization was performed at 45℃ overnight and the filter was

washed with 0.1 x ese solution containing 0.1% SDS at 55°C. The filter was then exposed to x-ray film (Kodak x-omat) for autoradiography.

Results

Induction of excreted proteinase

No cell growth was observed during the first days when inorganic nitrogen compounds in the medium were replaced with 0.002% solublized casein. Gibberellic acid treatment promoted cell growth during this period of incubation (Fig. 1). The rapidly increased proteinase activity corresponded to a greater rate of cell growth during the third to fifth day. Gibberellic acid treatment induced proteinase activity increased in both MS and casein media. Inclusion of sodium chloride in the medium at a concentration of 2mM stimulated two-fold increase of cell growth on day three (Fig. 2). Activity of extracellular proteinase was stimulated by addition of 20μM sodium chloride. A greater enzyme activity was observed when the concentration of sodium chloride was elevated to 2mM, but at 10mM cell growth was inhibited (data not shown).

Both cell growth and proteinase activity were promoted by inclusion of fungal elicitor in the medium (Fig. 3). After day five, however, cell growth slowed down and enzyme acivity decreased. The induction of proteinase in cultured rice cells by environmental stress might reflect the increase of either de novo synthesis of excretable enzymes or excretion of pre-existing intracellular enzymes. To determine whether de novo synthesis is involved in proteinase induction we extracted total RNA from the cells and levels of proteinase transcripts were determined by RNA blot hybridization. Proteinase transcript was detected on the first day of treatment and its concentration increased sharply on the third day (Fig. 4). Gibberellic acid treatment alone induced the increase of proteinase transcript and an additive effect was observed in the casein medium containing gibberellic acid. The results indicate that the induction of excreted proteinase by environmental alternation occurs at the level of either transcription or mRNA stabilization.

Partial purification of the enzyme

As the amount of secreted proteins was less than the amount of intracellular proteins of rice cells in the same culture, two liters of the five-day medium was collected from cultured cells treated with 2mM sodium chloride. After ammonium sulfate fractionation, 2.5-fold purification was achieved with 61.8% recovery. A second step of purification was performed with ion-exchange chromatography on a DEAE-Sephacel column. The maximum proteinase activity was eluted with fractions containing 0.4M KCl (Fig. 5). The efficiency of purification by ion-exchange chromatography was 6.8 fold with 14.4% recovery. The result of the partially purified proteinase was separated by SDS-gel electrophoresis and stained with silver nitrate is shown in Fig. 6. The molecular weight of the enzyme was estimated to be 46-48kDa.

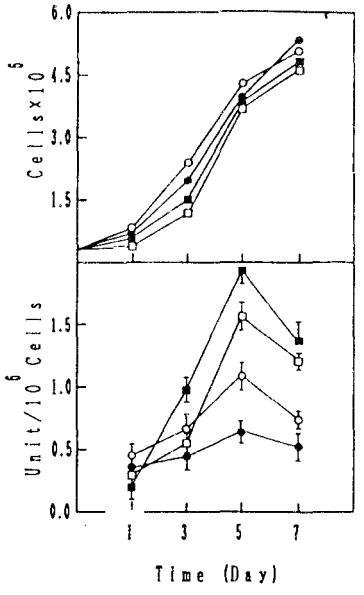

Figure 1. Effect of gibberellic acid and casein on cell growth (upper panel) and extracellular proteinase activity (lower panel). Cells were grown in MS medium (●), MS medium containing 10μM gibberellic acid (○), MS medium without inorganic nitrogen compounds but containing 0.002% casein (□), MS medium containing 0.002% casein and 10μM gibberellic acid without inorganic nitrogen compounds (■).

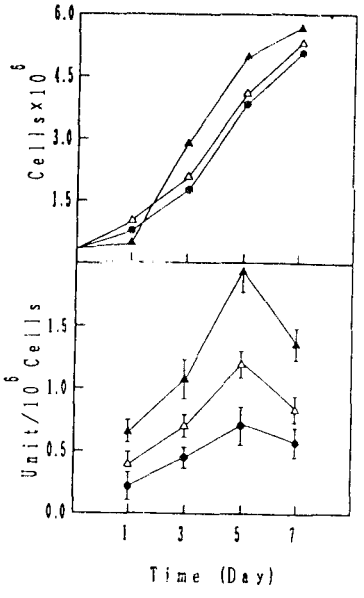

Figure 2. Effect of sodium chloride on cell growth and extracellular proteinase activity. Cells were grown in MS medium (●), MS medium containing 20μM NaCl (△), MS medium containing 2 mM NaCl (▲).

Properties of the enzyme

The enzyme exhibited an optimal pH of 3.5, and the enzymatic activity decreased with the increase of pH (Fig. 7). It indicates that the excreted enzyme is an acidic proteinase. When

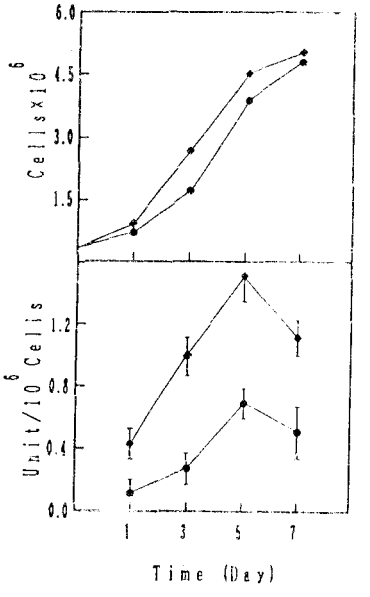

Figure 3. Effect of sodium chloride on cell growth and extracellular proteinase activity. Cells were grown in MS medium (●), MS medium containing fungal elicitor (◆).

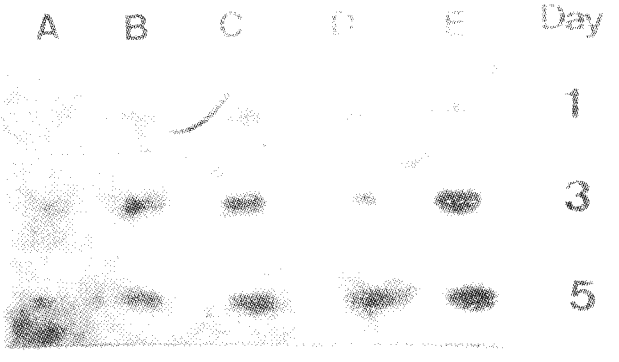

Figure 4. Northern blot hybridization. Total RNA (12μg) extracted from cells was loaded and blotted on nylon filter and hybridized to cDNA probe. Cells were grown in MS medium (Lane A), MA medium containing 10μM gibberellic acid (Lane B), MA medium containing fungal elicitor (Lane C), MS medium containing 2mM NaCl (Lane D), MS medium lacking inorganic nitrogen compounds but containing 0.002% casein and 10μM gibberellic acid (Lane E).

the enzyme was tested at various temperatures for five hours, it showed a maximum activity at 45℃. At a higher temperature the enzyme activity declined, and at 75℃ no enzymatic activity was detected (Fig. 8A). Thermostability of enzyme was assayed by incubation of the enzyme at various temperatures for 60min. The proteinase activity declined rapidly in the first 10min at a temperature 65℃ or higher, whereas only 5% activity was lost at 45℃ even after 60min incubation (Fig. 8B).

Some chemicals known to be proteinase inhibitors were examined (Table 1). Phenylmethylsulfonyl fluoride did not inhibit the enzyme activity even at a concentration of 100μM. Leupeptin, p-hydroxymercuribenzoic acid and N-ethylmaleimide are effective inhibitors for the enzyme at a low concentration (10μM), and about 80% of the enzyme activity was inhibited by preincubation for one hour of these inhibitors at a concentration of 100μM. When the period of preincubation was extended to 10 hours no enzyme activity was detected. Hence the enzyme might be a sulfhydryl proteinase rather than a serine proteinase, as phenylmethylsulfonyl fluoride is not an effective inhibitor.

Discussion

Cultured cells were grown under the cell cycle without characteristic differentiation and yet exhibited hypersensitivity to environmental changes. Gibberellic acid is known to play an inductive role in seed germination of most monocotyledons. Synthesis and excretion of hydrolytic enzymes of aleurone cells were stimulated effectively by exogenous gibberellic acid (Nolan and Ho, 1988). The response of cells under nutrient stress, in which inorganic nitrogen was replaced by casein, showed the time requirement of cells to adapt to the changing environment. The additive enhancement effect of gibberellic acid and casein indicates that both stimulators independently affect cell growth and proteinase excretion (Fig. 1 and 4).

In all eukaryotes, more concentrated salt denatures the enzymes and thus cannot be tolerated by the cells. Osmoregulation is evoked in response to salt stress (Jefferies, 1981). Thus the mild stress of salt (2mM NaCl) became a stimulus to induce both cell growth and proteinase excretion. Since the DNA probe, pHVEP 4 from barley encoded a gibberellic acid-inducible cysteine proteinase, at least part of the accumulated transcripts observed might represent the mRNA of excreted proteinase. Thus,the face that enzyme induction correlated with the enhancement of transcriptional activity indicates that gene expression might be associated with cell treatments with salt, gibberellic acid, and casein (Fig. 1,2 and 4).

Elicitore served to trigger the responses of the plant defense mechanism. Gene expression was involved in the defense mechanism (Somssich et al., 1986). In cultured rice cells we have previously shown that the activity of extracellular amylase as well as intracellular peroxidase and phenylalanine ammonia-lyase are enhanced by fungal elicitor treatment (Chou

and Tong, 1993). Therefore, the excreted proteinase activity increased by fungal elicitor might also be related to the cell defense mechanism.

Although it may not invariably be the case, most endopeptidase of plants have been

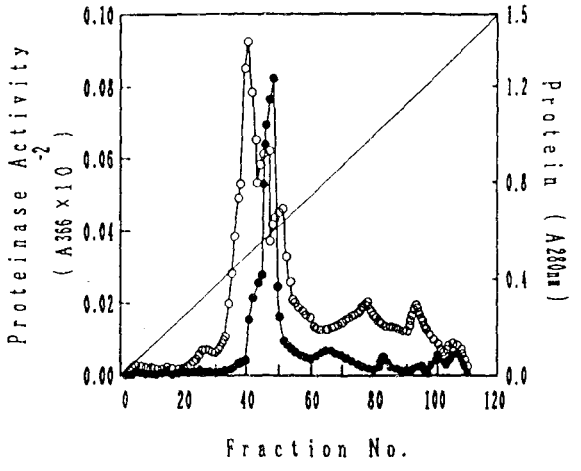

Figure 5. Elution profiles of protein (○) and proteinase activity (●) from DEAE-Sephacel column. The pooled fraction from ammonium sulfate column was applied to a DEAE-Sephacel column (2×50cm). The column was eluted with a linear gradient of KCl (0~1.0M) in 10 mM Tris-HCl buffer, pH 7.0, at a flow rate 30ml/h. Fractions of volume 1.2ml were collected.

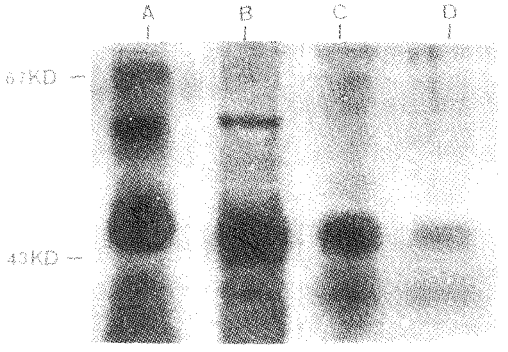

Figure 6. SDS-PAGE analysis of proteins. Crude extracellular proteins (Lane A), proteins from ammonium sulfate fractionation (Lane B), fractions pooled from DEAE-Sephacel column chromatography (Lane C and D). The amount of proteins loaded in C was double that of D.

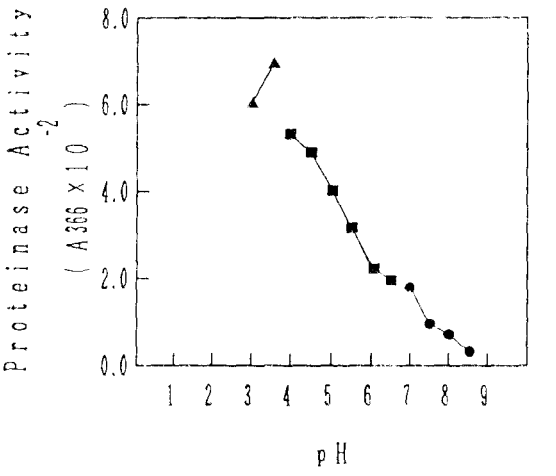

Figure 7. Effect of pH on proteinase activity. Enzyme was incubated in 33 mM of glycine-HCl (▲), sodium succinate (■), or Hepes (●) buffer and kept in ice for 20 hours. Enzyme activity was determined by the azocasein method.

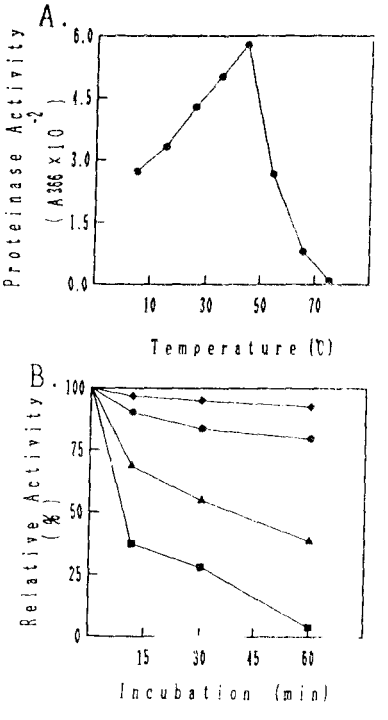

Figure 8. Effect of temperatureon the proteinase activity. A: Enzyme activity was measured after incubation with azocasein at various temperature for five hours. B: Thermostability of the enzyme was determined by incubating the enzyme with sodium succinate buffer (pH 4.0) at 45(◆), 55(●), 65(▲), 75(■)℃ and an aliquot of the enzyme was withdrawn at various times for activity assay.

reported to be sulfhydryl enzymes (Ryan and Walker-Simmons, 1981). Two endo-type proteinases from rice seed have been purified and characterized (Doi *et al.*, 1980; Abe *et al.*, 1987). The endo-type proteinases are involved in degrading the storage protein in vivo. The proteolytic enzyme partially purified from the medium of cultured rice cells showed an optimal pH of 3.5 at 30℃, and the molecular weight of the enzyme was estimated to be 46-48Kda. The property of this enzyme differs from that of the endo-type proteinase of rice seed. The excreted proteinase from cultured cells is likely a sulfhydryl enzyme and not a serine proteinase according to the following observations: first, the proteinase activity was inhibited effectively by 0.1mM of leupeptin, p-hydroxymercuribenzoic acid or N-ethyl-maleimide, but not by phenylmethylsulfonyl fluoride (Table. 1); second, activity of the proteinase was induced with salt and elicitor treatments, consistent with the result of accumulation of transcripts detected by hybridization with a cDNA probe of a sulhydryl proteinase from barley (Fig. 4). In contrast, there has a significant amount of proteinase activity in cells under our testing condictions, and this intracellular proteinase activity failed to response to salt and elicitor treatments. The pH value of the fresh medium was 5.8, but decreased to 4.0 in the five-day culture. The environmental change to lower pH fits well for the activity of excreted proteinase. As the enzyme was induced by casein or elicitor, the physiological role of the enzyme might be to seek proteinoid substances or a protective mechanism shown for pathogen.

Table 1. Effect of various compoundson the proteinase activity.

Additions	Concentration(μM)	Relative Activity (%) * Preincubation 1hr.	Preincubation 10hrs.
None		100	100
Leupeptin	10	48	26
	100	17	0
pHMB	10	59	30
	100	19	2
NEM	10	63	35
	100	22	3
PMSF	10	97	103
	100	102	101

The enzymes were preincubated at 0℃ in 33mM sodium succinate buffer (pH 4.0), containing various compounds. After preincubation, enzyme activity was assayed by azocasein method.

* Activity of a control with no addition was taken as 100%.

Multiple cDNA for cysteine proteinase from rice seeds have been cloned and characterized (Watanabe *et al.*, 1991). A gene family for cysteine proteinase might also exist in the rice genome. We have isolated a genomic clone of putative cysteine proteinase gene by hybridization using pHVEP cDNA insert as probe from rice genomic library. DNA sequencing and transcriptional regulation of the gene are proceeded.

Acknowledgments

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水稻細胞釋出性蛋白分解酶之誘導及性質分析

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摘　要

環境壓力如高鹽、眞菌釋出物、吉貝素及改變氮源等處理水稻細胞，對蛋白分解酶釋出之誘導爲研究要點。培養基添加 2 mM 氯化鈉，能促進細胞之生長，並提高蛋白分解酶之合成及釋出。以酪蛋白替代培養基內的無機含氮成分，則在一天後細胞外酶活性會急速升高。若培養基除添加酪蛋白外，亦含吉貝素則對酶釋出之誘導有加強效果。從核糖核酸之轉漬分析結果顯示，高鹽、眞菌釋出物或吉貝素之處理，都能促使酶基因之轉錄訊息增高。部分純化之酶蛋白，其最適酸鹼值爲 3.5。在 45℃溫度下，酶活性仍相當穩定。根據抑制物之檢測結果可以推論，此釋出性蛋白分解酶應爲硫氫蛋白分解酶。

關鍵詞：誘導、釋出性蛋白分解酶、水稻細胞、酶性質