Note

Evidence for the Presence of Two Types of Acid Proteinases in Germinating Seeds of Rice

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Proteolytic enzymes in plant seeds have been extensively studied in relation to mobilization of storage proteins of seeds.¹⁾ Most endopeptidases detected in seeds are enzymes which are optimally active at acidic pH and may be classified as acid proteinases. Various plant species have been examined for the activity of such endopeptidases, *e.g.*, lotus,²⁾ azuki bean,³⁾ hemp,⁴⁾ mung bean,⁵⁾ corn,^{6,7)} wheat,⁸⁾ barley,⁹⁾ and rice.^{10,11)} Although these enzymes have not always been characterized well, some of them shown to be inhibited by SH-inhibitors and/or activated by reducing reagents,^{5-9,11)} and the others were not affected by any specific inhibitors. No agreement has been obtained for the general properties of acid proteinases in plant seeds.

This communication describes the presence of two types of acid proteinases in rice seeds and the methods to distinguish them.

Enzyme solutions were obtained from endosperms of resting and germinating seeds of rice (*Oryza sativa* var. *Nihonbare*), which were called the R- and G-enzyme solutions, respectively (Table I). Both of the enzyme solutions were optimally active for the hydrolysis of 2% haemoglobin at pH 3 to 3.5. Effects of various inhibitors on their activities are shown in Table I. No inhibition by DFP, STI and EDTA was observed for both the R- and G-enzyme solutions. SH-inhibitors such as PCMB and NEM and leupeptin, a specific inhibitor for trypsin, and SH-proteases such as papain and cathepsin B, inhibited about half of the activity in the G-enzyme solution and inhibited the activity in the R-enzyme solution a little. Pepstatin, a specific inhibitor for pepsin and cathepsin D, inhibited most of the activity in the R-enzyme

Abbreviations used: BSA, bovine serum albumin; DFP, diisopropyl fluorophosphate; NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoic acid; STI, soybean trypsin inhibitor; G- and R- enzyme solutions, the enzyme solutions extracted from germinating and resting seeds, respectively.

TABLE I. EFFECTS OF VARIOUS INHIBITORS
ON RICE SEED PROTEINASES

Endosperms of resting or germinating (5 day-old) seeds of rice (300 grains each), from which had been removed the embryos, plumules, and radicles, were homogenized with 15 ml of 0.01 M Na phosphate buffer, pH 7.5, containing 0.3 M NaCl. The homogenates were centrifuged and the obtained supernatants were used as the R- and G-enzyme solutions. One hundred μ l of the R-enzyme solution or 40 μ l of water and 60 μ l of the G-enzyme solution, were preincubated with 100 μ l of inhibitor solution for 30 min at 37°C. After preincubation, 1 ml of 2% haemoglobin in 0.05 M glycine buffer, pH 3.25, was added to each enzyme solution. The enzyme reaction was carried out for 3 hr at 37°C. The reaction was stopped by the addition of 1 ml of 0.44 M trichloroacetic acid. After centrifugation of the reaction mixture, absorbance of the supernatant solution at 280 nm was measured.

Addition	Concentration*	Residual activity in	
		R-enzyme solution (%)	G-enzyme solution (%)
None		100	100
STI	0.05 %	102	98
DFP	1.0 mM	100	100
EDTA	0.1 mM	100	102
Leupeptin	1.3 mM	99	55
NEM	10 mM	85	50
PCMB	0.3 mM	92	47
Pepstatin	0.02 mM	19	64
PCMB and Pepstatin	0.3 mM	13	7

* Concentrations are those in the preincubation mixture.

solution and about half of the activity in the G-enzyme solution. Co-existence of PCMB and pepstatin inhibited about 90% of the activity in both the R- and G-enzyme solutions. These findings indicate that at least two different types of acid proteinases are present in rice seeds, that is, a pepstatin-sensitive enzyme and an SH-enzyme. The pepstatin-sensitive enzyme was predominant in resting seeds. In germinating seeds, the activities of the pepstatin-sensitive enzyme and the SH-enzyme were almost equal.

Separation of the pepstatin-sensitive enzyme and the SH-enzyme was achieved by gel chromatography on Sephadex G-100, as shown in Fig. 1. The first peak of activity corresponded to that of the pepstatin-sensitive enzyme and the second peak corresponded to that of SH-enzyme. The second peak did not reflect the amount of the original activity in the

FIG. 1. The Separation of Two Types of Acid Proteinases by Gel Chromatography on a Sephadex G-100 Column.

-----, protein; ○—○, activity in the absence of inhibitor; ●—●, activity in the presence of PCMB; ▲—▲, activity in the presence of pepstatin.

Five day-old endosperms of rice seeds (2,000 grains) were extracted with 120 ml of 0.01 M Na phosphate buffer, pH 7.5, containing 0.3 M NaCl as described in Table I. The extract (70 ml) was concentrated to 8 ml by use of polyethylene glycol #20,000. The concentrated extract was applied on a column (2.5×100 cm) of Sephadex G-100 which had been equilibrated with 0.01 M Na phosphate buffer, pH 7.5, containing 0.3 M NaCl. Each 200 μ l of the eluate was preincubated with 100 μ l of water, 100 μ l of 3×10^{-5} M pepstatin or 100 μ l of 6×10^{-4} M PCMB for 30 min at 37°C. After preincubation, the activity was assayed by the addition of 1 ml of 2% haemoglobin solution in 0.05 M glycine buffer, pH 3.5, for 4 hr at 37°C as described in Table I. The elution volumes of marker proteins are indicated at the top of the figure.

G-enzyme solution, because of instability of the SH-enzyme. The molecular weights of the two enzymes were roughly estimated to be 65,000~70,000 for the pepstatin-sensitive enzyme and 20,000~25,000 for the SH-enzyme.

The present results demonstrate the presence of at least two types of acid proteinases in germinating rice seeds. And the presence of these two types of endopeptidases in other plant seeds is suggested. Inhibition by pepstatin has not been reported for acid proteinases in plant seeds. The use of pepstatin and SH-inhibitors facilitates the analysis of the change of proteolytic activity in germinating seeds. Detailed analysis of the proteinases in germinating rice seeds will be published elsewhere.

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