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Protease Activity During Rice Leaf Senescence*

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Abstract. A protease activity was detected in rice (*Oryza sativa* L. cv. Ratna) leaves that hydrolysed hemoglobin more efficiently than bovine serum albumin. The activity was high when the enzyme was extracted and assayed with tris-maleate buffer [tris (hydroxymethyl) methyl amino-maleate] pH 7.0 rather than with water or with citrate-phosphate buffer pH 7.0. The enzyme had a strong dependence on sulfhydryl groups for its activity without which it was inactive. The pH optimum was 7.0 and the temperature optimum was 40 °C. Protease activity expressed per unit leaf fresh weight (absolute activity) increased only little during senescence of detached rice leaves while the same activity expressed per unit soluble protein content (specific activity) increased by a greater factor (about 5 times) than absolute activity. Total and soluble protein content decreased during the senescence of detached leaves. Benzimidazole (10^{-3} M) and kinetin (0.5×10^{-5} M) treatment arrested the increase of the protease activity and the decrease in the protein content during detached leaf senescence. It was indicative that protease protein was more stable than the bulk of other proteins in senescing leaves.

We have previously reported that rice leaves upon detachment show a rapid decline in the levels of chlorophyll and protein and an increase in the level of α-amino nitrogen (KAR and MISHRA 1975, SINGH and MISHRA 1975). The rapid decline in the level of total protein with the concommitant accumulation of α-amino nitrogen suggests that senescing leaves must have higher protease activity than the control. Earlier reports showed that absolute protease activity increases only little in tobacco leaves and leaf disks (ANDERSON and ROWAN 1965, BALZ 1966, KAWASHIMA *et al.* 1967) and corolla of Morning Glory (MATILE and WINKENBACH 1971). SPENCER and TITUS (1972) obtained no correlation between proteolytic activity and protein loss during autumnal senescence of apple leaves. They reported that total protein declined 40% from its maximum before proteolytic activity ensued to increase significantly. BEEVERS (1968) also noted no correlation between proteolytic activity and protein loss in *Nasturtium* leaves during senescence. MARTIN and THIMANN (1972) reported that in oat leaves absolute protease activity measured at pH 7.5 increased only slightly while the activity measured at pH 3.0 increased sharply till the 4th day after detachment and then decreased steeply to the 6th day. The present work was undertaken, as a part of our programme "Enzyme activity during rice leaf senescence", to study (a) some of the properties of the rice leaf protease, and (b) the changes in protease activity during detached rice leaf senescence.

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Material and Methods

Seeds of a high-yielding variety of rice were grown in pots and the leaves from 8-week-old plants were used in the experiments. Seven cm tips from fully expanded and mature leaves were washed in distilled water, randomized and floated in groups of five (weighing *ca.* 200 mg) in 30 ml of distilled water or in solutions of benzimidazole, 10^{-3} M (Fluka, Switzerland) or kinetin 0.5×10^{-5} M (Cambrian Chemicals Ltd., Beddington) in 10 cm Petri dishes in the dark. Samples were taken initially and at intervals for analyses.

Protease Extraction and Assay

The leaf samples weighing about 200 mg were frozen and homogenized with 8 ml of cold water or buffer (tris-maleate or citrate-phosphate buffer, 0.05 M) containing 40 mM cysteine-hydrochloride and 4% sodium chloride and adjusted to pH 7.0 with sodium hydroxide. The homogenate was centrifuged at 17 000 *g* for 20 min in a refrigerated centrifuge at 2 °C. The supernatant was assayed for the protease activity with hemoglobin (Sigma Chemical Co., U.S.A.) or bovine serum albumin (Sigma Chemical Co., U.S.A) as the substrate.

Three ml of the assay mixture for the protease activity contained: 1.5 ml of 0.05 M buffer pH 7.0 (tris-maleate or citrate-phosphate buffer), 0.5 ml of freshly prepared 2% hemoglobin or bovine serum albumin and 1.0 ml of the enzyme extract. The mixture was incubated for 2 h at 40 °C and the reaction was stopped with the addition of 1.0 ml of 20% trichloroacetic acid. A zero time control was run at the same time with the enzyme solution added immediately before trichloroacetic acid was added. Then the assay mixture was aged overnight in a refrigerator, centrifuged at 4 °C for 15 min. The supernatant was adjusted to pH 5.0 with sodium hydroxide, diluted properly and 0.1 ml of the diluted supernatant was taken for α-amino nitrogen determination by the ninhydrin method (Moore and Stein 1948). Protease activity (absolute activity) is expressed as μmoles of α-amino nitrogen released per hour by 1.0 ml enzyme solution as calculated from a standard graph using L-leucine as the reference standard. Specific activity was obtained by dividing the absolute activity with μg of protein present in 1.0 ml of the enzyme solution.

Protein Extraction and Estimation

Total protein and tris-maleate buffer (0.05 M, pH 7.0) extractable protein (soluble protein) was extracted and estimated as reported previously (Kar and Mishra 1976).

Results and Discussion

Substrate Specificity

When assayed at the neutral pH, protease activity was higher when hemoglobin was used as the substrate rather than when bovine serum albumin was used. The absolute protease activity was 1800 and 1500 units when hemoglobin and bovine serum albumin respectively were used as the substrates. Moreover, since hemoglobin is easily soluble over a wide pH range and even in water it was preferred to bovine serum albumin in the routine assay of the protease activity.

Extraction Conditions

The enzyme was extracted with water, citrate-phosphate buffer or with tris-maleate buffer containing 40 mM cysteine-hydrochloride, 4% sodium chloride and adjusted to pH 7.0. The assay mixture contained either citrate-phosphate or tris-maleate buffer pH 7.0. The results are presented in Table 1.

TABLE 1

Activity of rice leaf protease with the use of different medium for the extraction and assay of the enzyme

Extraction medium containing 40 mM cysteine-hydrochloride and 4% sodium chloride and adjusted to pH 7.0	Assay medium (buffer pH 7.0)	Units of absolute protease activity
Water	Citrate-phosphate buffer	440
Water	Tris-maleate buffer	1800
Citrate-phosphate buffer	Citrate-phosphate buffer	400
Tris-maleate buffer	Tris-maleate buffer	1880

Higher protease activity was observed when tris-maleate buffer was used for the extraction as well as for the assay of the enzyme. These results suggest that citrate-phosphate buffer is inhibitory to protease activity and as a matter of fact PIKE and BRIGGS (1972) reported the inhibition of protease activity by potassium-phosphate buffer. It is indicative that the phosphate ion present in the buffer is the actual agent which inhibits the protease activity. Protease activity declined very little when water was used in the extraction medium in place of tris-maleate buffer, other conditions remaining the same. No protease activity was detected when cysteine-hydrochloride was excluded from the extraction medium, suggesting that this enzyme requires sulfhydryl groups for its activity. With the increase of the cysteine-hydrochloride concentration in the extraction medium the absolute protease activity increased, the activity being 200, 540, and 1860 units with 10, 20, and 40 mM cysteine-hydrochloride respectively. PIKE and BRIGGS (1972) during their extensive study on etiolated oat leaf protease also suggested that the enzyme requires sulfhydryl groups for its activity.

Optimum Temperature

The assay mixture for the protease activity was incubated at a different temperature at an interval of 5 °C. The absolute protease activity in units were 1200, 1320, 1860, 1800, and 1440 at the temperatures of 30, 35, 40, 45, and 50 °C respectively. Thus the optimum temperature necessary for the protease activity was 40 °C. However, using hemoglobin as the substrate HARVEY and OAKS (1974) obtained a temperature optimum of 46 °C for the maize endosperm protease.

Optimum pH

Protease activity was assayed between the pH range of 5.0 to 8.0 using tris-maleate buffer. The pH optimum for the degradation of hemoglobin was 7.0. The absolute protease activities in units were 880, 1400, 1800, 1860, 1940,

1860, and 1840 at the pH of 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 respectively. HARVEY and OAKS (1974) obtained the peak activity at 3.8 and when they assayed the enzyme between pH 5.8 and 8.5 a second pH optimum was not observed. MARTIN and THIMANN (1972) using hemoglobin as the substrate obtained two peaks at pH 3.0 (larger peak) and at pH 7.5 (smaller peak) for oat leaf protease. ROBINSON (1956) who assayed the enzyme activity in bean root tips using hemoglobin as the substrate also obtained two peaks at pH 3.0 and at pH 7.0. We have not assayed the enzyme below pH 5.0 and therefore our pH optimum for rice leaf protease may be comparable with the second pH optimum obtained by ROBINSON (1956) and MARTIN and THIMANN (1972).

Activity During Detached Leaf Senescence

When detached rice leaves were floated on water, there was a rapid decline in the level of total protein as well as soluble protein with time (Fig. 1). Benzimidazole (10^{-3} M) and kinetin (0.5×10^{-5} M) treatment arrested the general decline in the level of total and soluble protein. These are the chemicals previously known to delay the senescence of detached rice leaves (KAR and MISHRA 1975).

While there was a rapid decline in the level of total and soluble protein during detached leaf senescence absolute protease activity increased only slightly during the experimental period (Fig. 2). Even at the end of five days of floating the leaves, there was less than a 30% increase in the absolute protease activity over the initial value. On the other hand, specific protease activity increased by a greater factor than the absolute activity. At the end of 5 days of floating, there was about 150% increase of the specific activity over the initial value. Treatment of benzimidazole and kinetin inhibited the increase in the absolute and specific protease activity caused upon detachment of leaves (Fig. 2).

The steady rise of absolute protease activity during senescence and the sharp rise of the specific activity by a greater factor than the absolute activity is similar to the protease activity at pH 7.5 of MARTIN and THIMANN (1972). ANDERSON and ROWAN (1965) have also reported that the absolute protease activity increased only little during senescence of tobacco leaf disks while specific activity increased by a greater factor than the absolute activity. The sharp increase in the specific activity is due to the rapid breakdown of soluble protein during senescence and the results do not give any indication of the large increase in the absolute amount of the protease protein. During senescence the absolute protease activity increases only little but there is a marked decline in the protein content of leaves. It is suggestive that the protease protein is more stable than the bulk of other soluble proteins and that the bulk of other proteins are more exposed to the already existing proteases due to the loss of cellular integrity during senescence (SACHER 1967).

The significance of this report is that rice leaf protease has a pH optimum at 7.0 and that its absolute activity changes very little during senescence whereas specific activity increases markedly thus suggesting that protease protein is more stable than other proteins during leaf senescence.

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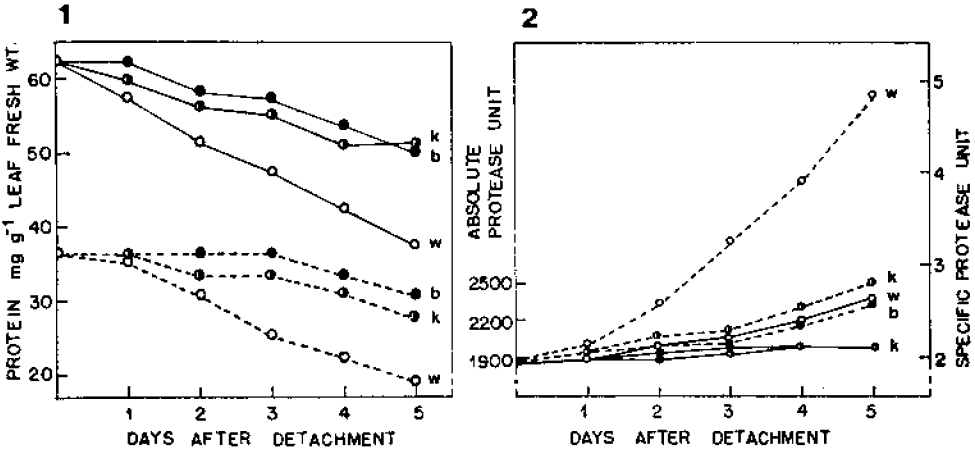

Fig. 1. Changes in the total protein (continuous line) and soluble protein (broken line) content in detached rice leaves with time floated on water (*w*), benzimidazole (*b*) and kinetin (*k*) solution.

Fig. 2. Changes in the absolute (continuous line) and specific (broken line) protease activity in detached rice leaves with time floated on water (*w*), benzimidazole (*b*) and kinetin (*k*) solution.

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