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Peptide Hydrolases in Senescing Ragi Leaves

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Abstract. Two peptide hydrolases — one active at pH 7.0 and the other at pH 3.4 (SH-dependent) were detected in ragi (*Eleusine coracana* GAERTN. cv. PR 202) leaves. The absolute activities of both acid and neutral peptide hydrolases were more or less stable in attached leaves and exhibited a mild rise in excised leaves, whereas the specific activities exhibited a significant rise. Light and growth regulators were ineffective in changing the course of absolute activity whereas cytokinins stimulated specific activities of both acid and neutral peptide hydrolases. Though the rates were different, protein decline was positively correlated with the changes in activities of peptide hydrolases. Cycloheximide retarded chlorophyll and protein decline and also prevented the rise in peptide hydrolases. Peptide hydrolases thus do not have an active role in senescence.

Proteolysis is an important aspect of senescence and the decline in protein content is either due to failure in synthesis or an increased degradation (BEEVERS 1976). Understanding the role of peptide hydrolases in crop plants is important in order to manipulate the yield because senescence and thus proteolysis and proteolytic enzymes are linked with yield in monocarpic plants (FRITH and DALLING 1980). Physiology of senescence in ragi leaves is not well understood and no study exists of the proteases of ragi, an important millet which may contribute to production increase in both developing and developed regions of the world (RACHIE 1975).

The present paper deals with the response of two peptide hydrolases found in ragi leaves to light and growth regulators during senescence of excised leaf.

MATERIALS AND METHODS

Plants

For the selection of material and growth of plants see KUMAR and KHAN (1984). For excised leaf study, leaves (first) from 7 d-old plants were washed with distilled water, randomized and samples of *ca.* 200 mg fresh matter were floated in separate Petri dishes containing 0.04 mM benzyladenine (BA), or 0.84 mM benzimidazole (BZI), or 0.57 mM gibberellic acid (GA_3),

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or 30 μM cycloheximide (CH). Dishes were incubated in the dark at 28 $\pm$ 2 °C or exposed to fluorescent lamps (38 W m^{-2}, Philips TL 40 W/54). Protein content of the leaves was estimated by the method of LOWRY *et al.* (1951).

Extraction and Assay of Peptide Hydrolases

Buffer solutions of pH ranging from 3.0 to 8.6 were tested in the presence or absence of 5 mM dithiothreitol. Enzyme from the fresh first leaf (200 mg)

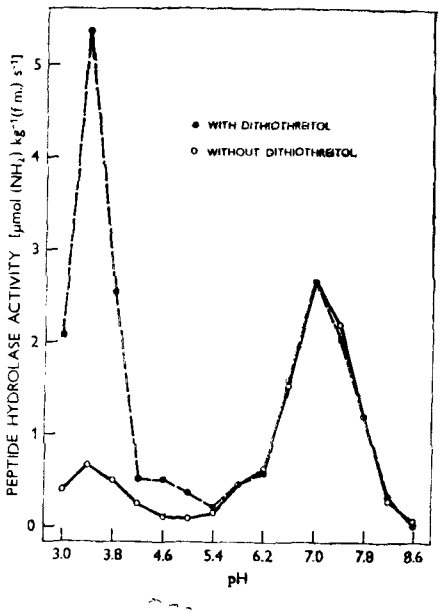

Fig. 1. Effect of pH on peptide hydrolase activity of ragi leaves.

of the 7 d-old plant was extracted with 3 cm^3 of each of the buffer separately by grinding the leaves in a pre-chilled mortar with pestle. The crude enzyme extract was centrifuged at 20 000 $\times g$ for 30 min, and the supernatant was used as the source of peptide hydrolase.

The assay mixture for acid peptide hydrolases which contained 1 cm^3 0.025 M citrate buffer (pH 3.4), 1 cm^3 freshly prepared 2 % bovine serum albumin and 1 cm^3 enzyme extract [pH 3.4 (or other pH in the preliminary tests) in the presence of 5 mM dithiothreitol] was incubated for 2 h at 40 °C, and the reaction was stopped by adding 5 cm^3 20 % (m/v) trichloroacetic acid. A blank was prepared by adding enzyme solution to the reaction mixture immediately before the addition of trichloroacetic acid at the end of 2 h. The assay mixture was then aged overnight in a refrigerator and centrifuged at 10 000 $\times g$ at 4 °C for 20 min. The pH of the collected supernatant was adjusted to 5.0 by adding trisodium citrate. 0.1 cm^3 of the dilute supernatant was taken for the estimation of α-amino nitrogen by a modified method of MOORE and STEIN (1948).

Neutral peptide hydrolase was assayed in a way similar to the acid peptide hydrolase except that the buffer used for extraction and incubation was Tris-maleate buffer (pH 7.0) and did not contain dithiothreitol.

RESULTS

Peptide hydrolase activity exhibited two peaks — one at pH 3.4 and the other at pH 7.0, and it was low at other pH values (Fig. 1). At pH 7.0 the activity of the enzyme was approximately 5times the activity at pH 3.4.

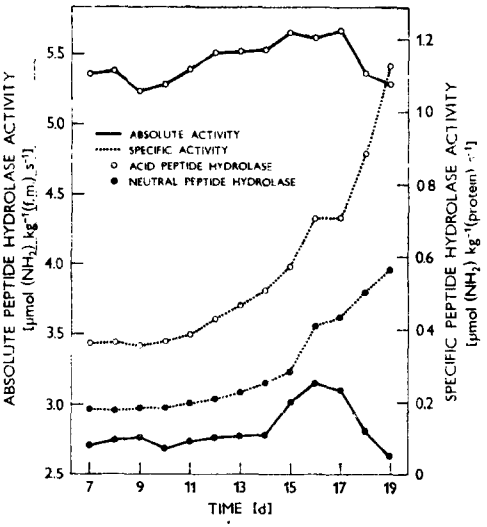

Fig. 2. Changes in acid and neutral peptide hydrolase activities in attached ragi leaves during senescence.

However, there was an 8-fold rise in enzyme activity at pH 3.4 in the presence of dithiothreitol. Dithiothreitol showed its effect on the acid enzyme up to pH 5.5 (Fig. 1).

Absolute activity of acid peptide hydrolase (SH dependent) was relatively stable in attached leaves (Fig. 2) throughout the course of senescence, *i.e.* yellowing of the attached leaf did not affect it. The absolute neutral peptide hydrolase activity was stable till day 14 after which there was a rise followed by a mild decline towards the end of senescence. The specific activities of both acid and neutral peptide hydrolases exhibited a continuous rise in attached leaves (Fig. 2).

There was a rise in the neutral peptide hydrolase activity in excised leaves incubated in both dark and light. The acid peptide hydrolase was more stable throughout the incubation period exhibiting only a mild rise in ia activity. Growth regulators had no significant effect on both acid and neutrts peptide hydrolases (Fig. 3, top).

In contrast to the absolute activity, the specific activity of neutral and acid peptide hydrolases in dark incubated leaves exhibited a significant rise

(Fig. 3, bottom). The rise in both acid and neutral peptide hydrolases was significantly suppressed by BA and BZI, while IAA and GA_3 had no effect. The specific activity of both acid/neutral peptide hydrolases exhibited a mild rise in illuminated excised leaves and growth regulators had no significant effect on it (Fig. 3, bottom).

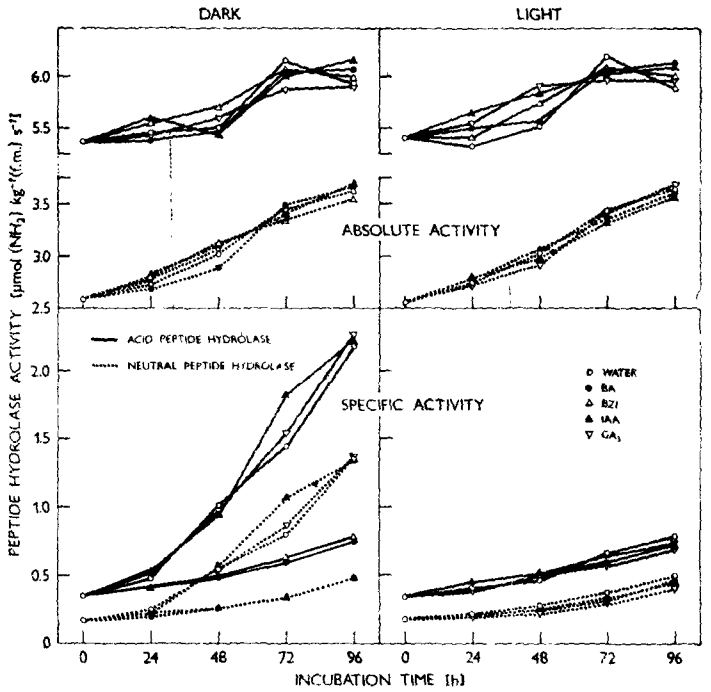

Fig. 3. Effect of growth regulators on the absolute (*top*) and specific (*bottom*) peptide hydrolase activity of excised ragi leaves during senescence in the dark or light.

The decline in protein content of attached leaves was gradual (Fig. 4) but there was a rapid loss of protein in the excised leaves incubated in the dark (Fig. 5). BA and BZI significantly retarded protein decline in excised leaves but GA_3 and IAA had no effect. Protein decline was arrested significantly in illuminated excised leaves. However, the effects of BA and BZI on protein decline were not additive to light effects (Fig. 5).

Cycloheximide significantly retarded chlorophyll and protein contents decline and prevented the mild rise in absolute and specific activities of acid and neutral peptide hydrolases (Fig. 6).

DISCUSSION

In ragi leaves we found two peptide hydrolases, one active at pH 3.4 and dependent on the SH group, and the other active at pH 7.0 and independent of the SH group. In this respect ragi leaves are distinguished from oat, rice and barley leaves where the neutral proteases are SH dependent (Martin and Thimann 1972, Perez *et al.* 1973, Peterson and Huffaker 1975,

VILLAREAL and JULIANO 1976, KAR and MISHRA 1977, DRIVDAHL and THIMANN 1977, 1978).

Earlier studies did not find any correlation between the rate of protein decline and the rise in peptide hydrolases (BEEVERS 1976). However, we observed a negative correlation between the rate of protein decline and

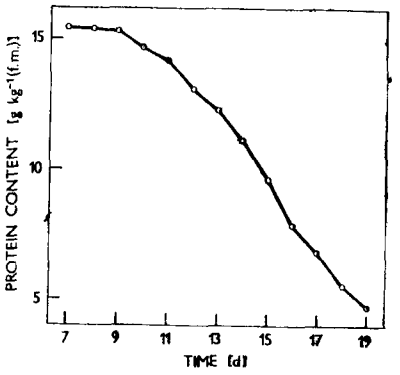

Fig. 4. Changes in protein content of attached ragi leaves during senescence.

changes in absolute and specific peptide hydrolases (Fig. 7) except the acid peptide hydrolase in attached leaves. However, the increase in peptide hydrolases was much lower quantitatively in the attached and detached ragi leaves at a time when there was already a significant decline in protein content. Hence, peptide hydrolase cannot be treated as the sole factor responsible for the decline in protein content.

Kinetin may retard absolute peptide hydrolase activity in the leaves of different plants during senescence (ANDERSON and ROWAN 1965, BEEVERS 1968, MARTIN and THIMANN 1972, PETERSON and HUFFAKER 1975, KAR and MISHRA 1977). However, both acid and neutral absolute peptide hydrolases

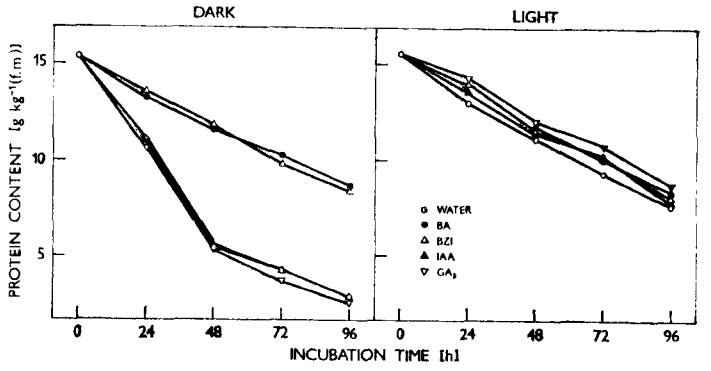

Fig. 5. Effect of growth regulators on protein content of excised ragi leaves during senescence in the dark or light.

of ragi leaves were insensitive not only to cytokinins but also to auxin, gibberellin and light. However, the specific peptide hydrolase at both the pH optima was sensitive to cytokinins. The rise in specific peptide hydrolases may be attributed to the fast decline in protein, and the suppression of specific activity in the presence of cytokinins may be due to the latter's effect on the total leaf protein.

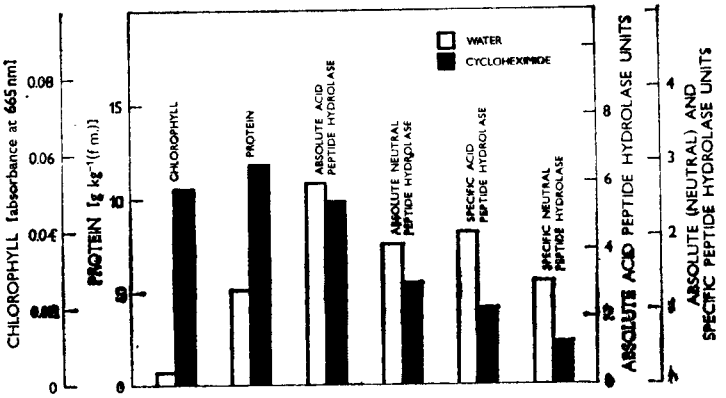

Fig. 6. Peptide hydrolase activitv protein and chlorophyll contents in excised ragi leaves 48 h after cycloheximide (CH) treatment.

The suppression of peptide hydrolases by cycloheximide indicates that the rise is due to new protein synthesis. MARTIN and THIMANN (1972) suggested that cycloheximide controlled senescence in oat leaves by preventing the rise in protease activity. But such a conclusion would not be applicable to our ragi leaves because the rise in peptide hydrolase was small and could not account for the rapid decline in protein. Further, growth regulators which had high antisenescence (checking protein decline) properties, had the least effect on absolute peptide hydrolases. This is contrary to the findings of MAR-

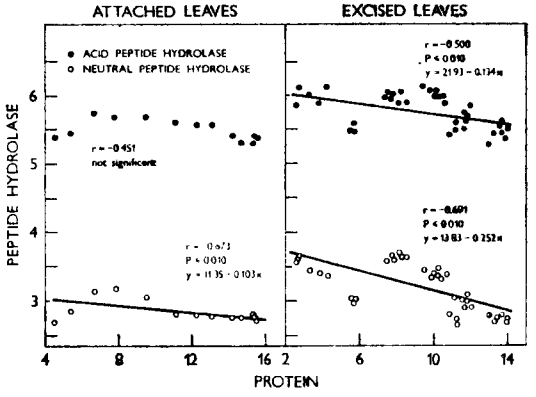

Fig. 7. Correlation between protein content and acid/neutral peptide hydrolase activity in senescing ragi leaves.

TIN and THIMANN (1972) in oat leaves, where cycloheximide and kinetin were equally efficient in preventing chlorophyll decline and rise in peptide hydrolases.

The results of the present investigation do not favour the active involvement of peptide hydrolase in leaf senescence. We cannot but conclude that protein breakdown may not depend on the synthesis of senescence specific peptide hydrolases, even though senescence may often be accompanied by an increase in peptide hydrolases. VANLOON *et al.* (1978) found that the rise in proteinase activity which normally accompanied the yellowing in leaves floated on water, did not occur when the leaves were placed upright, nevertheless, the protein loss occurred to the same extent in both the sets of leaves.

Because of varied characteristics and behaviour of peptide hydrolases in different plants, it may be inferred that the enzyme is species specific and its role in senescence cannot be generalised.

Acknowledgements

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REFERENCES

ANDERSON, J. W., ROWAN, K. S.: Activity of peptidase in tobacco leaf tissue in relation to senescence. — Biochem. J. **97** : 741—746, 1965.

BEEVERS, L.: Growth regulator control of senescence in leaf discs of nasturtium (*Tropaeolum majus*). — In: WIGHTMAN, F., SETTERFIELD, G. (ed.): Biochemistry and Physiology of Plant Growth Substances. Pp. 1417—1436. Runge Press, Ottawa 1968.

BEEVERS, L.: Senescence. — In: BONNER, J., VARNER, J. E. (ed.): Plant Biochemistry. Pp. 771 to 794. Academic Press, New York 1976.

DRIVDAHL, R. H., THIMANN, K. V.: Protease of senescing oat leaves. I Purification and general properties. — Plant Physiol. **59** : 1059—1063, 1977.

DRIVDAHL, R. H., THIMANN, K. V.: Protease of senescing oat leaves. II Reaction to substrates and inhibitors. — Plant Physiol. **61** : 501—505, 1978.

FRITH, G. J. T., DALLING, J.: The role of peptide hydrolases in leaf senescence. — In: THIMANN, K. V. (ed.): Senescence in Plants. Pp. 117—130. C.R.C. Press, Boca Raton 1980.

KAR, M., MISHRA, D.: Protease activity during rice leaf senescence. — Biol. Plant. **19** : 365—369, 1977.

KUMAR, K. B., KHAN, P. A.: Levels of deoxyribonucleic acid and deoxyribonucleolytic activity in ragi leaves during senescence. — Biol. Plant. **26** : 174—180, 1984.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., RANDAL, R. J.: Protein measurement with Folin phenol reagent. — J. biol. Chem. **193** : 265—275, 1951.

MARTIN, C., THIMANN, K. V.: The role of protein synthesis in the senescence of leaves. I The formation of protease. — Plant Physiol. **49** : 64—71, 1972.

MOORE, S., STEIN, W. W.: Photometric ninhydrin method for use in the chromatography of amino acids. — J. biol. Chem. **176** : 367—388, 1948.

PEREZ, C. M., CAGAMPANG, D. B., ESMANA, B. V., MONSERRATE, R. V., JULIANO, B. O.: Protein metabolism in leaves and developing grains of rices differing in grain protein content. — Plant Physiol. **51** : 537—542, 1973.

PETERSON, L. W., HUFFAKER, R. C.: Loss of ribulose 1,5-diphosphate carboxylase and increase in proteolytic activity during senescence of detached primary barley leaves. — Plant Physiol. **55** : 1009—1015, 1975.

RACHIE, K.: The Millets: Importance, Utilization and Outlook. — ICRISAT, Hyderabad 1975.

VILLAREAL, R. M., JULIANO, B. O.: Some properties of rice leaf protease. — Kimika **1** : 20—25, 1976.

VANLOON, L. C., HAVERKORT, A. J., LOCKORST, G. J.: Changes in protease activity during leaf growth and senescence. — FESPP Abstr. **280** : 544—545, 1978.