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Proteolysis of Endogenous Substrates in Senescing Oat Leaves[1]

I. SPECIFIC DEGRADATION OF RIBULOSE BISPHOSPHATE CARBOXYLASE

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ABSTRACT

Proteolysis of ribulose bisphosphate carboxylase (RuBPCase) during senescence was monitored using oat leaf segments (*Avena sativa* cv Victory), kept in the dark. We here report the development of a novel approach for measuring protein degradation of endogenous substrates both *in situ* and *in vitro* in crude extracts using specific antibodies against highly purified polypeptides. The proteolytic products were separated on sodium dodecyl sulfate-gels. They were then electrotransferred onto nitrocellulose paper and identified with specific antibodies to both the large and small subunits of RuBPCase. We could show differences in pH optima between two proteases degrading the subunits of RuBPCase. While both subunits were best hydrolyzed in acid and basic pH, they degraded differently at neutral pH. Furthermore, the large subunit displayed a different pattern of degradative products at the different pH levels. Older leaf segments, which were incubated in darkness, underwent enhanced proteolysis, as compared with young ones. These results show the advantages of the assay in demonstrating: (a) *in situ* proteolysis of specific substrates in crude extracts without further purification; (b) *in vitro* differential proteolysis of endogenous substrates during senescence.

Loss of total protein is one of the most dominant features in leaf senescence (10, 14, 19). Correlated with this loss is increased proteolytic activity (5, 10, 13). RuBPCase[2] is one of the predominant proteins lost during the initial stages of senescence (6, 13). This soluble constituent of chloroplasts is responsible for CO_2 fixation in photosynthetic organisms (8, 11). It is synthesized during leaf expansion and the level remains constant for several days without any apparent turnover (14). RuBPCase constitutes about 50% of the total soluble protein in wheat leaves (21), thus serving through its hydrolysis as a leaf storage protein which provides reduced nitrogen that can be transported to developing leaves or fruits (15). Recently, Ben-David *et al.* (2) showed that differential changes in the chloroplast membranal proteins occurred during senescence, and that the Cyt b_6-f complex disappeared early.

Endopeptidases and exopeptidases have been purified from various leaf tissues, and their properties have been extensively studied (3, 10, 13, 20, 21). The major endopeptidases have an acid pH optima and their substrate specificity is low. In most studies they have been assayed with animal protein substrate such as gelatin, haemoglobin, casein, or azocasein (16). Since these proteinases also have a high affinity for RuBPCase, which is presumably their natural substrate (20), they have been assayed using purified ^{14}C-labeled RuBPCase. The products of endopeptidic cleavage of RuBPCase were visualized by SDS gel electrophoresis and fluorography (18). Only the large subunits (mol wt 55,000) were cleaved to a series of fragments, while no evidence of any breakdown pattern for the small subunit was obtained (18). In addition to the acid endopeptidases, there are also reports of leaf proteolytic enzymes with higher pH optima (4, 5). The multiplicity of proteases found in leaf tissue may reflect a corresponding variety of function and substrate specificity.

In this report we demonstrate endogenous proteolytic activity in crude extracts without any further need of purification or addition of exogenous substrate. By using electrotransfer of proteins and immunodetection with specific antibodies against both subunits of RuBPCase, we were able to show differential proteolysis of these two subunits under different conditions. Preliminary results have been published (15).

MATERIALS AND METHODS

Plant Material. Oat seedlings (*Avena sativa* cv Victory) were grown in vermiculite under continuous fluorescent lighting at 25 ± 1°C. The first leaves were collected 12 d after sowing. Twelve upper leaf segments, 6 cm in length and excluding the tips, were floated on water at 25 ± 1°C in darkness for 1, 2, and 3 d before being assayed for proteolysis.

Protease Assay. Leaf segments (500 mg) were homogenized at 4°C in 5 ml of 100 mM Tris-HCl (pH 8.0). The homogenates were centrifuged at 12,000*g* for 10 min. The supernatant fraction was used for the determination of the endogenous protease activity. This fraction was mixed with different buffers (v/v, 2:1): (a) 1 M acetate buffer, final pH 4.5; (b) 1 M K-phosphate, final pH 7.0; (c) original Tris buffer, pH 8.0. The reaction was carried out at 37°C, and was stopped by boiling for 5 μg in 1/5 volume of dissociation buffer (6% w/v Tris-HCl [pH 6.8], 100 μg/ml Bromophenol blue, 10% SDS, 0.3% EDTA, 50% glycerol), containing 1/20 volume of mercaptoethanol. Samples were kept frozen at −20°C until used for electrophoresis.

Electrotransfer of Polypeptides from Slab Gels to Nitrocellulose Paper and Immunodetection. Samples of 50 μl were run overnight on 12.5% SDS polyacrylamide slab gels at 8 mamp per gel (9). Methyl Green (0.1%) was used to mark the lanes. Gels were performed in duplicate. One gel was stained with Coomassie Brilliant Blue R, while the protein bands from the second gel were electrotransferred onto 0.45 μm nitrocellulose paper (Schlercher and Schull, West Germany). The electrotransfer was performed in buffer containing 16 mM glycine, 25 mM Tris-HCl (pH 8.7), 0.02% SDS, and 20% v/v methanol at 40 v, 0.4 amp for 2 h (12). Seventy % of the high mol wt proteins, including the large subunit and 100% of the small subunit were transferred. The nitrocellulose was then saturated with 50 ml of albumin buffer solution (25 mM Tris-Cl [pH 7.6], 140 mM NaCl, and 1% BSA [Sigma fraction V]) for 1 h with gentle shaking. Twenty μl of the antibodies against either the large or the small

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[2] Abbreviation: RuBPCase, ribulose bisphosphate carboxylase.

subunit of RuBPCase was added and the nitrocellulose was incubated at room temperature overnight with gentle shaking. The nitrocellulose paper was washed five times 50 ml of the washing buffer (25 mM Tris-Cl [pH 7.6] and 140 mM NaCl) and either 5 μCi ^{125}I-protein A (Amersham) or peroxidase-bound protein A (Sigma) was added. After incubation for 1 h with gentle shaking, the paper was washed with 50 ml of the washing buffer, then with 50 ml of washing buffer containing 1% Triton X-100 and three additional times with washing buffer. The nitrocellulose paper was then dried and exposed to X-ray film. When the protein-A peroxidase was used, the paper was stained, after being washed, with 30 ml of 10 mM Tris-HCl (pH 7.4), 3 ml 0.5% benzidine, and 10 μl H_2O_2. The reaction was stopped with excess of 10 mM Tris-HCl (pH 7.4), and the paper dried. Specificity and purity test of antibodies has been suggested by Nelson which includes partial cleavage by V-8 proteases (12). The antibodies against RuBPCase has been examined using this method (Zemel and Gepstein, unpublished results) and were found to be specific.

RESULTS AND DISCUSSION

Hydrolysis of the Large Subunit of RuBPCase. Using the electrotransfer technique, we observed different degrees of degradation for both subunits of RuBPCase at different pH levels. Figures 1–3 demonstrate the peptide cleavage pattern of the large subunit of RuBPCase, using specific antibody against this protein and detecting it with protein A peroxidase. Time 0 lane each day (Figs. 1–3) revealed the *in situ* degradative products of the large subunit from which it can be seen that these smaller polypeptides underwent degradation during autodigestion (Fig. 1). At all pH levels, proteolytic activity increased with the length of incubation of leaf segments in dark. The greatest hydrolysis was obtained when the endogenous reaction was incubated at pH 4.5 (Figs. 1–3). This proteolysis rate was observed either using acetate or citrate, indicating that the high proteolytic activity in the acidic pH is not due to specific types of buffers. In leaf segments which were kept for 3 d in the dark and then homogenized and incubated for 120 min of autodigestion, the majority of the main protein band had already disappeared after 30 min (Fig. 3). Also no degradation products were visible.

When autodigestion was carried out at a neutral and basic pH, different patterns of proteolysis were obtained. The degradation products seemed different from those obtained at the hydrolysis at acid pH. The difference in polypeptide pattern at different pH values are real and do not reflect the difference in the rate of degradation. By comparing the degradation patterns at pH 4.5 after 5 min to the pattern at pH 8 after 120 min (Fig. 1), when about the same amount of the large subunit was lost, it is evident that the degradation products are totally different. The proteolysis at pH 4.5 was faster than that at pH 7.0 and 8.0 and rises more sharply with age, reaching a maximum after 3 d of incubation in darkness. Martin and Thimann (10) showed a similar phenomenon using exogenous substrate. They suggested that there is a neutral enzyme which is more stable than the acidic one(s), and its activity rises more slowly with time after detachment.

Hydrolysis of the Small Subunit of RuBPCase. Thomas and Huffaker (18) showed no evidence of a comparable breakdown for the small subunit of RuBPCase, probably due to the difficulty of visualizing proteolysis of this subunit on SDS-PAGE, followed by protein staining. Using the antibody against the small subunit in the electrotransfer technique, we could demonstrate loss of this subunit of the enzyme at different pH levels, following the

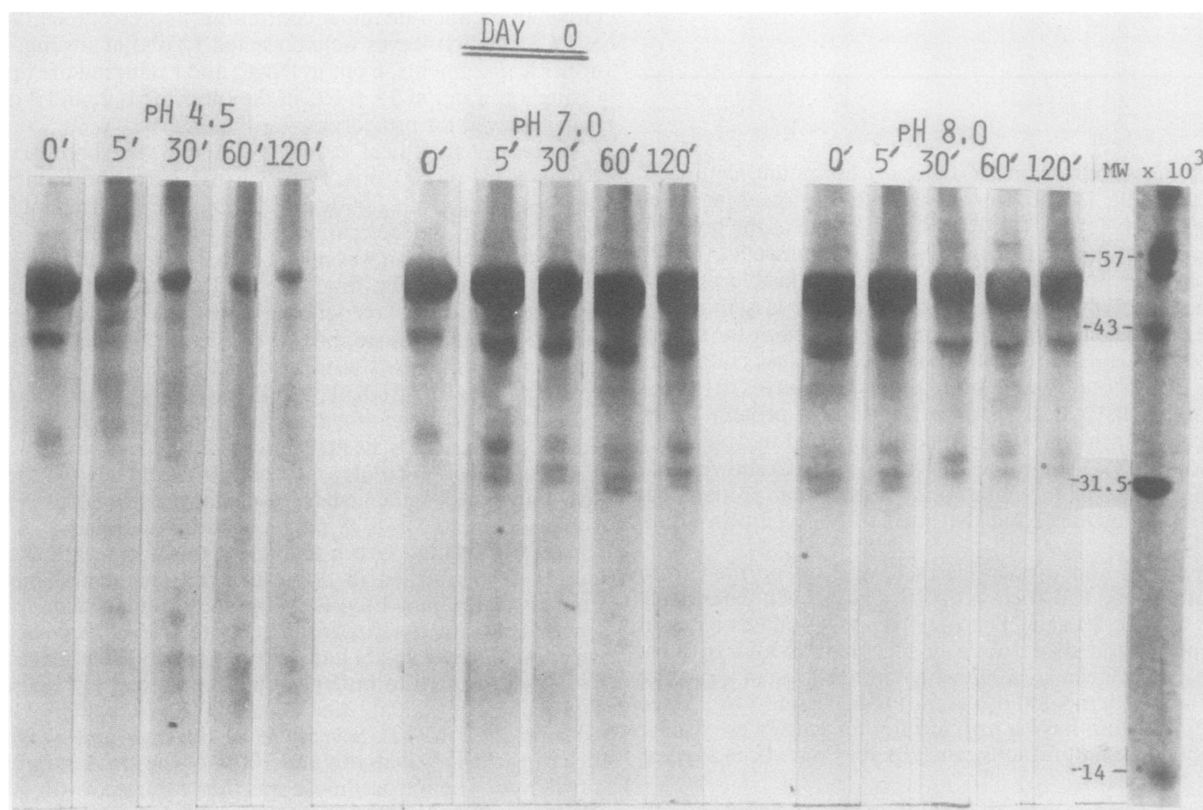

FIG. 1. Proteolysis of the large subunit of RuBPCase—day 0. Oat leaf segments were homogenized and incubated for 120 min of autodigestion at 37°C at pH 4.5, 7.0, and 8.0. Samples of 1.6 mg fresh weight were applied to 12.5% SDS-polyacrylamide gels, transferred to nitrocellulose paper, and treated with the antibody against the large subunit of RuBPCase, as described in "Materials and Methods". The large band represents the large subunit of RuBPCase.

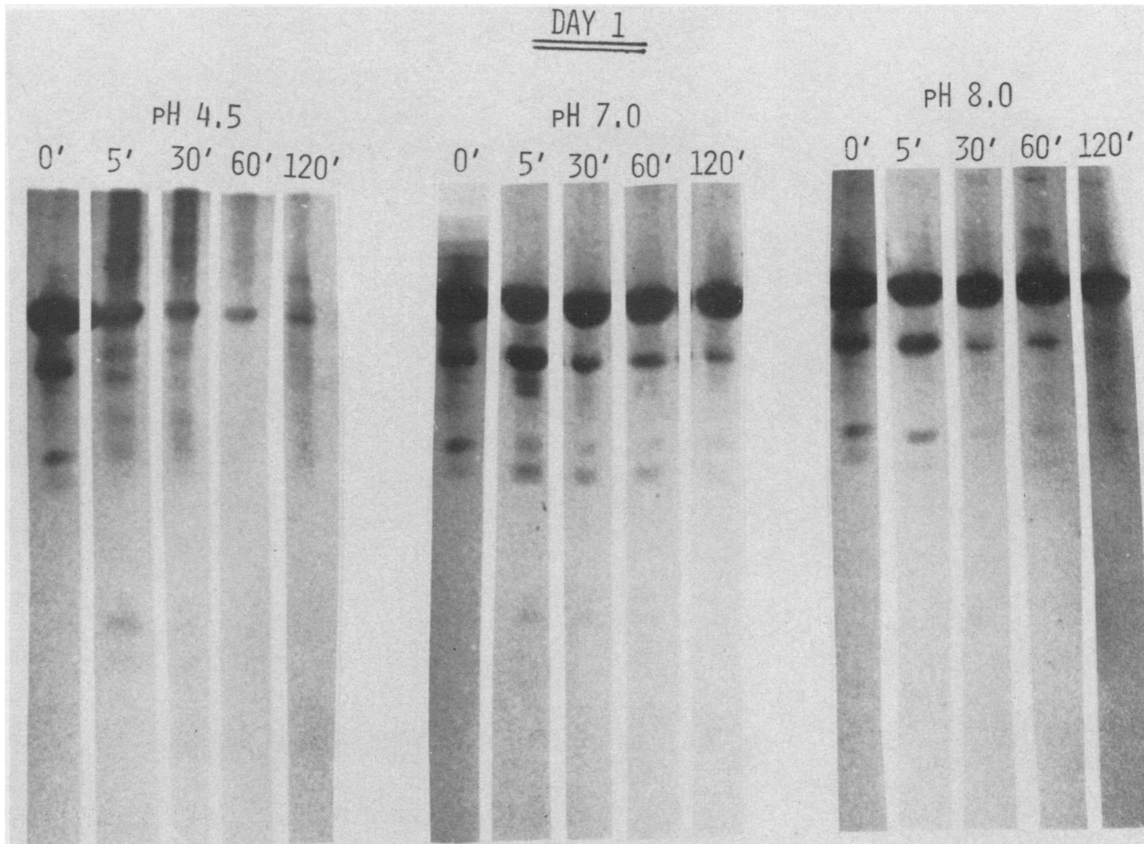

FIG. 2. Proteolysis of the large subunit of RuBPCase—day 1. Oat leaf segments were kept for 1 d in the dark and then homogenized and incubated as described in the legend of Figure 1.

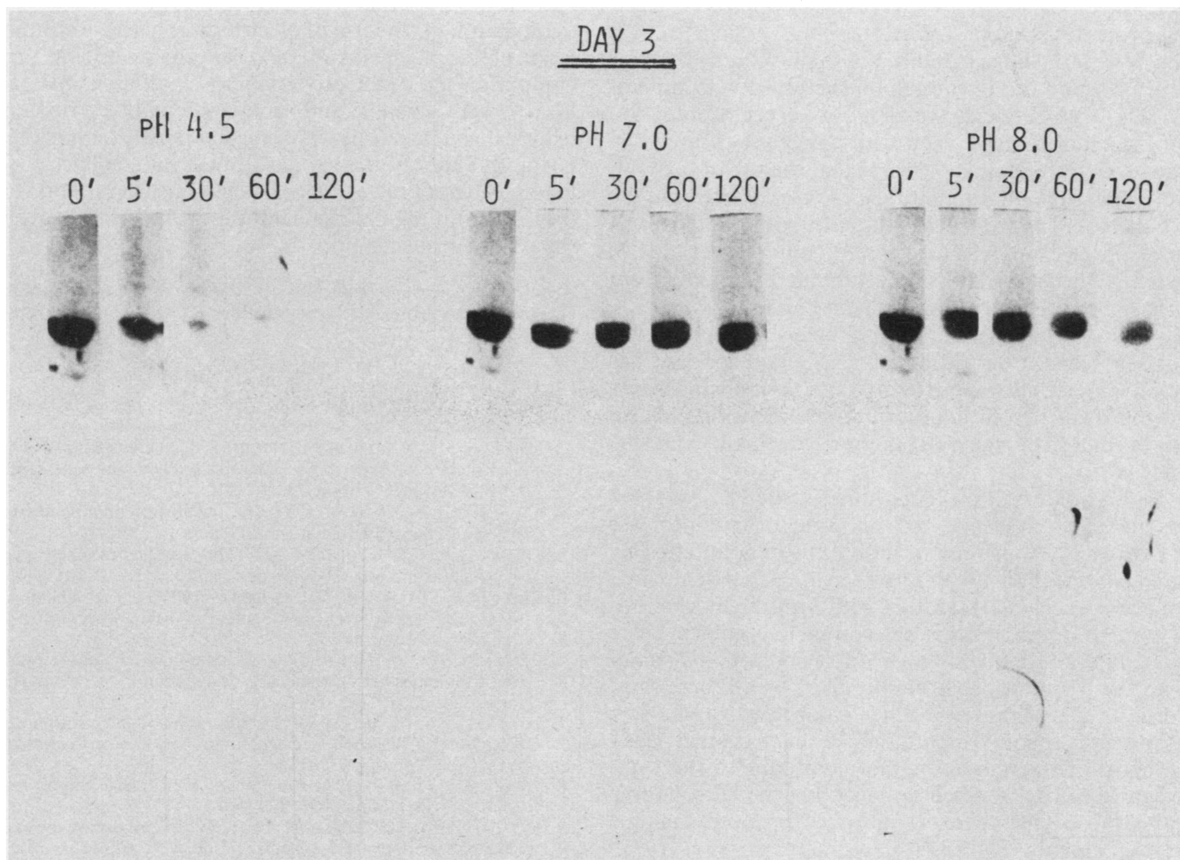

FIG. 3. Proteolysis of the large subunit of RuBPCase—day 3. Oat leaf segments were kept for 3 d in the dark, homogenized, incubated and transferred as described in Figure 1.

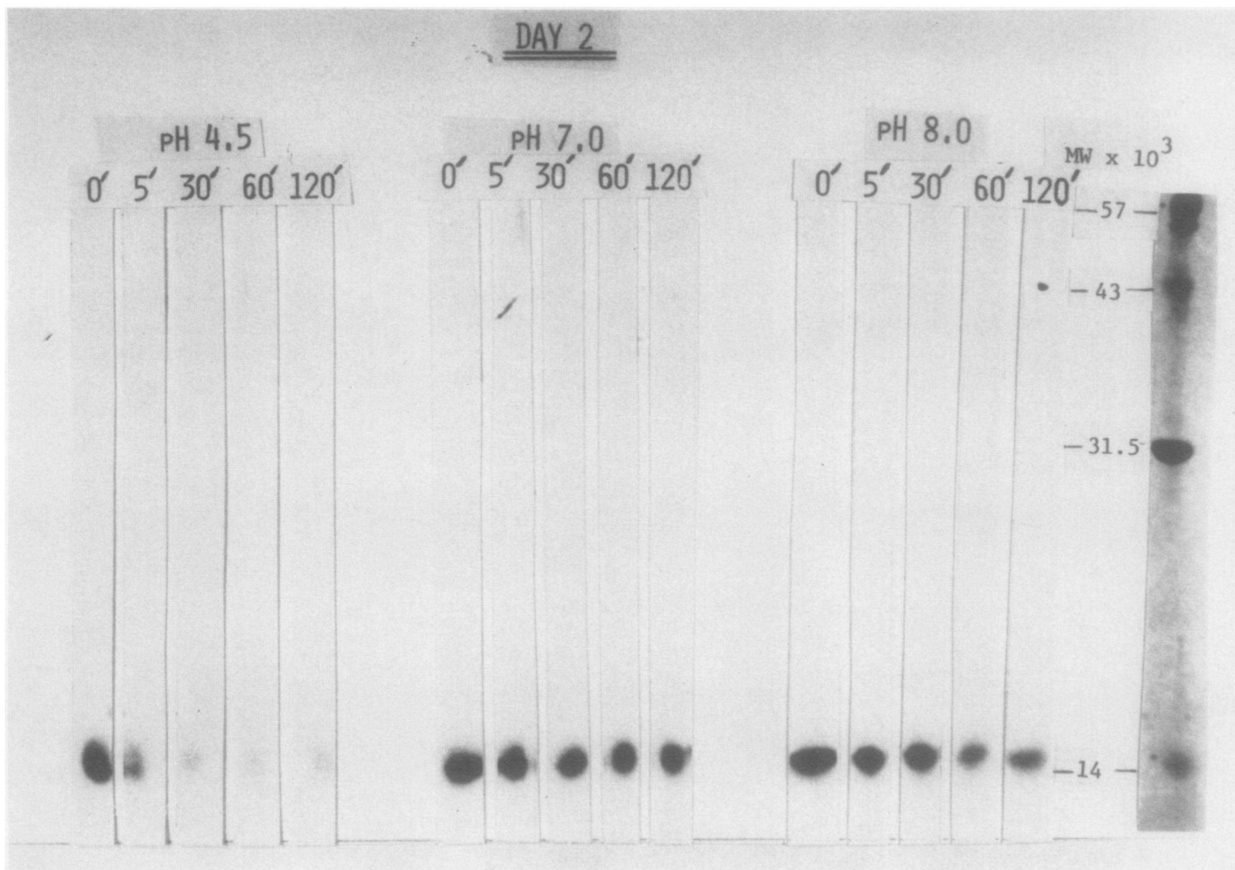

FIG. 4. Proteolysis of the small subunit of RuBPCase—day 2. Oat leaf segments were kept for 2 d in the dark, homogenized and incubated as described in Figure 1.

endogenous proteolysis reaction (Fig. 4).

Similarly, as with the large subunit, the small subunit was best cleaved at pH 4.5 and the phenomenon increased with time of senescence (Fig. 4). Although we did not detect degradation products of the small subunit, even with a higher percentage of gel (17%, data not shown), it is probable that the use of 0.20 μm nitrocellulose paper or other membrane filters will improve the detection of the very low mol wt peptides. In both the large and small subunits, proteolysis was detected in young leaves (day 0) and increased with time. The small subunit also underwent proteolysis at higher pH levels, especially at pH 8.0.

The proteolysis demonstrated above is most likely characteristic of leaf senescence. Activation of acid endopeptidase has been observed in detached leaves (10, 13) and in senescing leaves attached to the plant (5). In addition, there are reports of leaf proteolytic enzymes with higher pH optima, which are expressed in senescence (4, 5).

The fact that inhibitors of protein synthesis, such as cycloheximide, prevent leaf senescence, loss of protein, and protease activation, is evidence that protein mobilization is initiated by an increase in endopeptidase activity and synthesized de novo at the onset of senescence (7, 10, 13, 17). Although the mechanism and site of protein degradation is not completely known, there are two suggested alternatives. (a) Cytoplasmic and vacuolar proteases probably cannot penetrate the plastid envelope; thus, if they hydrolyze RuBPCase and other chloroplast proteins, it would only be after the envelope has aged and becomes leaky (7). (b) Stroma proteins may cross the membrane to the cytoplasm or vacuole during senescence, enabling the surrounding acid proteases to degrade them. It may be, however, that the chloroplast itself contains protease(s).

In summary, the use of electrotransfer and immunodetection is an efficient general method for characterizing proteases by comparing the degradative pattern at different pH ranges. The assay is very sensitive and rapid and can be carried out without using exogenous substrates or further purification of the enzymes. Using this technique, we have obtained evidence for proteases having different pH optima in senescing leaves. This is a valuable initial step in the characterization of these proteases which will require their purification.

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