Differential Induction of Endoproteinases during Senescence of Attached and Detached Barley Leaves

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ABSTRACT

Endoproteinase activities and species were compared during dark-induced senescence of attached and detached primary barley leaves by isoelectric focusing and polyacrylamide gel electrophoresis of cell-free extracts. Neither of the two major endoproteinases (EP1 and EP2) changed in amounts during senescence of attached leaves, nor did new endoproteinases appear. In contrast, during senescence of detached leaves, both EP1 and EP2 activities increased and four new species of endoproteinases appeared. Proteolytic activity was evenly distributed throughout attached leaves, but activity in the detached leaf increased sharply from the tip to the base with the four new higher molecular weight species of proteinases present only in the bottom half of the leaf nearest the cut end. Thus a wound response may be superimposed on the processes of senescence in detached leaves. Cycloheximide and kinetin both inhibited the increase of EP1, EP2, and the induction of the four new endoproteinases; chloramphenicol had no effect. Indications are that both the increases in activity and the induction of new species of proteinases were the result of activity of cytoplasmic ribosomes.

Hydrolysis of total protein and ribulose-1,5-bisphosphate carboxylase protein in vivo was somewhat faster in detached than attached leaves. The difference, however, was much less than would be expected from the great increase in proteolytic activity in detached leaves.

Large-scale hydrolysis of proteins is an event occurring early in leaf senescence. Large increases in proteolytic activity occur in detached oat (17), barley (25), and corn (7) leaves; attached wheat (35) leaves senescing in darkness; and attached barley (8) and wheat (4) leaves naturally senescing with age under normal growing conditions.

Chloroplasts are the major sites of protein degradation in cereal leaves during senescence. RuBPCase is localized in chloroplasts, constitutes from 50 to 60% of the total soluble protein in many C3 plants, and accounts for about 90% of the protein degraded during the early stages of senescence (8, 24, 25, 35). The concentration of other soluble proteins remains quite stable during the time that RuBPCase is disappearing (25). There is now good evidence that chloroplasts contain proteinases that can degrade their protein constituents (5, 28, 31, 34) and degrade their Chl as well (18). Recent reports show a sequential loss of chloroplast constituents and chloroplast numbers; both RuBPCase protein and activity disappeared faster than chloroplast number in senescing leaves of wheat (16, 36) and barley (19). There may be an interaction between vacuoles and chloroplasts during the latter part of the sequence. Peoples et al. (24) and Wittenbach et al. (36) produced electron micrographs showing a shifting of chloroplasts toward the vacuoles as wheat leaves age. Wittenbach et al. (36) further showed a possible invagination or engulfing of chloroplasts by vacuoles.

Earlier work showed that almost all of the endoproteolytic activity in senescing leaves resides in the vacuole (2, 9, 14, 36). Two major endoproteinases (EP1 and EP2) constitute over 99% of that activity in barley leaves (21, 22) and both are located exclusively in the vacuole (30). At present their function is unknown. If barley leaf chloroplasts are degraded within vacuoles, then EP1 and EP2 may be used in the final degradation of chloroplasts. It has also been proposed that the vacuolar proteinases may also function in turnover of cytoplasmic proteins (20). Little is known about the regulation of specific endoproteinases concerning whether they increase in amounts, activities, or if new endoproteinases appear during senescence.

A comparison of endoproteinases in senescing attached and detached barley leaves offered a unique means of following the ontogeny of EP1 and EP2 prior to and during senescence, the induction of new endoproteinases, and the correlation of these findings to the loss of RuBPCase and total soluble protein. Many investigators question the significance of using detached leaves for studies of senescence because they differ in several responses from attached leaves (11, 26). This study also gives information on the feasibility of using detached leaves.

MATERIALS AND METHODS

Plant Treatments. Barley (Hordeum vulgare L. variety Numar) was grown in vermiculite in 15-cm plastic pots and sub-irrigated with full-strength nutrient solution (12). Plants were grown for 7 d under continuous light (550 μE/m2·s) at 27°C and 55% RH. Seven-d-old intact barley plants were placed into continuous darkness to induce senescence. In addition, the primary leaves of similar seedlings were detached at the base, and the top 10 cm were recut under water. Ten detached leaves were placed base down in beakers containing 10 ml of 0.2 mM CaSO4. In addition to CaSO4, some treatments included 25 μg CAP/ml, 5 μg CHI/ml, or 10 μg kinetin/ml. The beakers containing the leaves were then placed in continuous darkness. Both the intact plants and detached leaves were held at 27°C and 55% RH.

Preparation of Cell-Free Extracts. Ten leaves were ground in a chilled mortar and pestle with 0.1 M K-phosphate (pH 6.0) containing 1 mM DTT (1 g of tissue/3 ml of buffer). The homogenate was centrifuged at 50,000 g for 15 min, and the supernatant was used for the assays described below.

Separation of Endoproteinases. Separation of the endoproteinases present in a cell-free extract was done using thin-layer isoelectric focusing, and polyacrylamide disc gel electrophoresis. For isoelectric focusing, 7.5 g of Sephadex G-75 superfine, 0.1 g lysine (free base), and 0.1 ml arginine (free base) were added to

1 Abbreviations: RuBPCase, ribulose 1,5-bisphosphate carboxylase; CAP, chloramphenicol; CHI, cycloheximide.
100 ml of deionized H2O. This slurry was brought to 2% in amphotolytes (1 part pH 4–6, 1 part pH 6–8, and 1 part pH 2–11). One ml of cell-free extract for every 12 ml of slurry was added. Finally, enough DTT was added to make a final concentration of 2 mM. Eighteen to 20 ml of this final mixture was spread on a 20 × 10-cm glass plate, and the slurry surface was made smooth and uniform by gently tapping the underside of the plate. The slurry was dried under N2 at room temperature until 1- to 2-mm cracks along the edges indicated that it had lost ~25% of its moisture (Operating Manual 171D for thin-layer isoelectric focusing, Desaga/Brinkman). Paper wicks soaked in 0.2 M H2SO4 or 0.4 N ethylenediamine were used in conjunction with platinum ribbon electrodes. Focusing was performed using an applied voltage of 250 V for 4 h followed by 600 V for 3 h in a moist N2 atmosphere in a Desaga/Brinkman double chamber maintained at 4°C.

After isoelectric focusing, a strip of Brinkman print paper that had been soaked in 0.2 M K-phosphate (pH 5.4) and then air-dried was rolled over the slurry surface. Endoproteinases were transferred to the paper strip during absorption of the liquid for 20 s from the slurry. The paper strip was then transferred to an agarase-gelatin tray. After incubation for 1 to 2 h at 40°C, the paper strip was removed and the agarase-gelatin layer was ‘developed’ as previously described to identify the location and relative amounts of endoproteinases (21).

Polyacrylamide disc-gel electrophoresis was done according to Davis (6) and Ornstein (23) using 7.5% acrylamide gels that had a monomer-to-bis ratio of 39:1. To each disc gel was added 0.1 ml of cell-free extract. After electrophoresis, the gels were extruded, sliced longitudinally, and placed flat surface down onto an agarase-gelatin layer to identify the location and relative amounts of endoproteinases in the gel. The assay was then conducted as described previously (21). The mol wt of the proteinases were determined by the Hedrick and Smith method (10) with urease, catalase, aldolase, BSA, lactic acid dehydrogenase, hexokinase, pepsin, albumin, γ-amylose, chymotripsin A, Cyt c, insulin, and RNase t1 serving as mol wt standards. The percentages of polyacrylamide used were 5 to 10%.

Distribution of Proteolytic Activity in Senescing Detached and Attached Leaves. Intact 7-d-old seedlings were placed into continuous darkness. After 5 d, the leaves were cut into 2-cm sections and azocasein activity in the cell-free extracts was measured. Alternatively, the top 10 cm of 7-d-old primary leaves were detached, placed base down in 0.2 mM CaSO4, and allowed to senesce in continuous darkness for 3 d. The leaves were cut into 2-cm sections, and the activity against azocasein of the cell-free extracts was measured.

Protein Assays. Total soluble protein was determined by the method of Lowry et al. (15). To determine the total amount of RubPCase protein, the total soluble proteins were separated by SDS electrophoresis in 10% polyacrylamide slab gels using the buffer of Laemmli (13). The gels were fixed in 10% TCA for 10 min, stained overnight with Coomassie brilliant blue, destained, and scanned at 540 nm with a Gilson gel scanner. The amount of RubPCase protein was determined by summing the areas under the large and small subunit peaks of RubPCase.

Azocasein, Chl Assays. Activity of the cell-free extracts against azocasein was done as previously reported (25). Total Chl was assayed by the method of Arnon (1) by extracting the pellet after centrifugation of the cell-free extract with 80% acetone.

Each experiment was repeated at least three times; results are presented which showed consistent trends.

RESULTS

Endoproteinases of Attached and Detached Barley Leaves. Figure 1A shows the two major endoproteinases, EP1 and EP2 (21, 22) present in primary leaves of intact seedlings that were placed in darkness at 7 d of age for 5 d of dark-induced senescence; the endoproteinases were separated by isoelectric focusing of cell-free extracts. The second, fainter band (EP2) is slightly more basic and partly obscured by the major enzyme. Neither the species nor the activities of endoproteinases changed during senescence in the attached leaves. The greater size of the bands of EP1 and EP2 for the detached leaves shows that the activities of these endoproteinases increased substantially during senescence (Fig. 1B). The additional proteinases seen in Figure 1B appeared during senescence of the detached leaves. To test whether the induction of new endoproteolytic activity in detached leaves may have resulted from a response to injury when the leaves were cut, isoelectric focusing patterns of the upper and lower parts of the detached leaves were compared after 3 d of senescence. The new species were detected only in the lower
basal half of the leaves in a pattern exactly the same as shown in Figure 1B. The Hedrick and Smith method (10) used to determine mol wt showed that only two additional bands were resolved by Ornstein-Davis polyacrylamide disc-gel electrophoresis (Fig. 2C). The two bands had mol wt of 72 and 109 kD, respectively.

Effect of Kinetin, CHI, and CAP on Proteinases. Treatment of the detached leaves with CHI or kinetin prevented the appearance of the new species of endoproteinases and the increased activity of EP1 and EP2 (Fig. 1A). If detached leaves were treated with 25 \mu{g}/ml CAP, the new species of endoproteinases and the increased activities of EP1 and EP2 appeared during the 3 d of dark-induced senescence (Fig. 1B).

Distribution of Proteolytic Activity in the Senescing Leaf. After 3 d in darkness, the attached leaf had proteolytic activity distributed fairly evenly throughout (Fig. 3). The detached leaf, however, showed a sharply increasing gradient of activity. The lowest activity was in the leaf tip (the oldest tissue of the leaf) and the highest activity was in the leaf tissue closest to the cut surface. The rate increased roughly 6-fold from the tip of the leaf to the cut base. When cell-free extracts of the sections from leaves of intact seedlings were subjected to PAGE electrophoresis, only the two endoproteinases present in Figure 2, A or B (EP1 and EP2) were found in the tip sections. The two additional higher mol wt proteinases shown in Figure 2C were detected only in the lower half of the leaf.

Changes in Chl, Soluble Protein, RuBPCase Protein, and Proteolytic Activity. After the changes in both species and amounts of endoproteinases were determined, actual losses of Chl, soluble protein, and RuBPCase in attached and detached leaves were compared (Fig. 4). The loss of Chl from barley leaves followed the same general pattern during dark-induced senescence of detached or attached leaves, although the rate of loss was significantly lower in attached leaves (Fig. 4A). Detached and attached leaves lost total protein at the same rate until they had been in darkness 3 d. Fifty per cent of the protein had been lost by this time (Fig. 4B). Loss of total protein was somewhat more rapid thereafter in detached leaves. Detached leaves lost RuBPCase somewhat faster than attached leaves after 2 d in darkness (Fig. 4C). The differences in in vivo loss of proteins between attached and detached leaves (Fig. 4, B and C) were small compared to differences in the development of proteolytic activity (Fig. 4D). After a short lag, the proteolytic activity increased rapidly in detached leaves, peaked at day 4 (which was 4.5-fold greater than the initial level found in the green unsecessed leaf), and then declined. By contrast, proteolytic activity increased little during senescence of the attached leaf.
cytoplasmic polyribosomes.

An induced wound response may have been superimposed on the processes of senescence in the detached leaf. Evidence includes the four newly synthesized higher mol wt endoproteinases, detected only in the lower half of the leaf; appearance of the new species of endoproteinases correlated well with the progressive increase in proteolytic activity (against azocasein) toward the base (cut end) of the leaves, which had senesced for 3 d (Fig. 3), and the proteolytic activity progressively increased (against azo-casein) in the leaves for up to 4 d during senescence (Fig. 4).

Differences in loss of soluble and RuBPCase protein were small between the attached and detached leaves during senescence when compared to the greater concentration of endoproteinase activity in the detached leaves. It has been established that EP1 and EP2 are localized in vacuoles of barley leaves (30). The new species of endoproteinases, whose intracellular locations are unknown, may also be sequestered away from chloroplasts in which RuBPCase is localized. Hence, the vacuolar EP1 and 2 and the new species of endoproteinases may have little to do with initial proteolysis within the chloroplast. The above results seem to fit the current hypothesis that chloroplasts are the likely site of degradation of their protein components (5, 16, 19, 28, 31, 34). Endo- and exoproteolytic activity associated with chloroplasts have now been reported for barley (5, 34). Evidence exists that chloroplasts may be invaginated or engulfed by vacuoles during dark-induced senescence of wheat leaves (36); hence, the final disintegration of chloroplasts may possibly occur within vacuoles. In that case, the engulfing of chloroplasts by vacuoles may represent the rate-limiting step in loss of chloroplasts rather than the concentration of vacuolar proteinases. This might account for the lack of a major effect of the increased amount of EP1 and EP2 and the new species of proteinases on the loss of RuBPCase protein and total protein in the detached leaves (Fig. 4).

Much work has been reported showing a correlation between increasing proteolytic activity and loss of leaf protein (4, 7, 17, 25, 32). Other workers have detected no clear-cut relationship between increasing protease activity and protein degradation in senescing leaves from various plants (24, 27, 29, 32). Peoples et al. (24) found that the flag leaf of wheat had two peaks of acid proteinase activity, but only the second peak coincided with the rapid loss of RuBPCase protein. Subsequent work has shown that the vacuoles of wheat (14, 34), corn (33), barley (9, 30), and pineapple (2) leaves contained the major leaf proteolytic activity. The separation of protein substrates from the major hydrolytic enzymes by cellular compartmentation and the differences between attached and detached leaves help explain the difficulties in establishing meaningful correlations between assays for total proteolytic activity and protein degradation.

The detached leaf is very convenient for use in studies of senescence, because it allows easy introduction of chemical treatments through the cut surface. However, information obtained using detached leaves must be interpreted with care. Differences exist between senescing attached and detached leaves: in development of species and amounts of proteinases (Fig. 1), in development of isozymes of glutamate dehydrogenase enzymes (26), and in ultrastructural changes in chloroplasts (11). Even though large differences in proteolytic activities occur, the degradation of protein during dark-induced senescence was remarkably similar in attached and detached leaves (Fig. 4, B and C). Hence, it seems feasible to use detached leaves in studies where the responses are similar to those of attached leaves. Senescing detached leaves also appear to be promising material to study the induction and synthesis of proteinases.

**LITERATURE CITED**


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**FIG. 4.** Comparison of Chl and protein loss, and the development of proteolysis during senescence of attached and detached leaves. Plants were grown for 7 d as described in "Materials and Methods" and then placed either attached or detached in continuous darkness. Measurements on detached leaves were not made beyond 5 d of treatment because of tissue deterioration. A, Total Chl; B, total protein; C, RuBPCase; D, proteolysis measured with azocasein. (●), Detached; (○), attached.

**DISCUSSION**

Endoproteolytic enzymes differed markedly between dark-senesced attached and detached barley leaves. Neither new species of endoproteinases nor increases in activities of EP1 and EP2 were detected during dark-induced senescence of attached leaves (Fig. 1). Both EP1 and EP2 increased greatly in activity as the detached leaves senesced (Fig. 1B). In addition, four new species of endoproteinases (identified by isoelectric focusing) appeared in the detached leaves as they senesced. Treatments of CHI or kinetin, which effectively inhibit dark-induced senescence of detached leaves (17, 25, 32), prevented both the increased activity of EP1 and EP2 and the appearance of the new species of endoproteinases (Fig. 1, A and B). Senescence of detached leaves was not inhibited by 25 μg/ml of CAP (17, 25, 32), and during the 3 d of dark-induced senescence, both increased activity of EP1 and EP2 and the appearance of four new endoproteinases were observed. We have previously established that detached leaves rapidly absorb CAP from a treatment solution (3). This indicates that the increased activity of EP1 and EP2 and the appearance of the new endoproteinases may be facilitated by