Leaf Proteolytic Activities and Senescence during Grain Development of Field-grown Corn (Zea mays L.)*

Received for publication June 17, 1976 and in revised form September 27, 1976

URS K. FELLER,* TAI-SEN T. SOONG, AND RICHARD H. HAGEMAN
Department of Agronomy, University of Illinois, Urbana, Illinois 61801

ABSTRACT
Some proteolytic enzymes occurring in the leaves of field-grown corn (Zea mays) (B73) were identified and partially characterized. Changes in activities of several proteolytic enzymes in in concentrations of protein and chlorophyll as a function of intraleaf segments (tip to base), leaf position, and leaf senescence during grain development and maturation were followed in crude leaf extracts.

The aminopeptidase (not affected by sulfhydryl or fluoride reagents) was most active at pH 7, while the carboxypeptidase(s) (sensitive to fluoride, but insensitive to sulfhydryl reagents) was most active in the acid range, pH 3 to 6. The presence of two or more endopeptidases is indicated. Endopeptidase (caseolytic) activity at pH 5.4 appeared to be stimulated by sulfhydryl groups or EDTA, while caseolytic activity at pH 7.5 was not.

Visually, individual leaf senescence starts at the leaf tip and the necrotic (brown) V-shaped area enlarges progressively toward the leaf base. Canopy senescence occurs in two phases. Foliar symptoms are first observed on the bottom leaf and then in sequential order up the plant. Subsequently, senescence occurs on the top leaf and moves downward. These foliar senescence symptoms are paralleled by decreases in exopeptidase activities, protein, and chlorophyll concentrations and by increases in endopeptidase activities.

During development and maturation of the grain, both aminopeptidase and carboxypeptidase activity of the middle half of the ear leaf increased (2- to 3-fold) during the onset of the visual reproductive phase (tassel and ear emergence). However, during grain development and plant senescence, both activities decreased rapidly and concurrently with the loss of protein and chlorophyll from this leaf section. In contrast, caseolytic activity at both pH 5.4 and 7.5 increased gradually during the early reproductive phase and rapidly with leaf senescence. The fastest rate of increase in caseolytic activities was concurrent with the most rapid loss of protein from the leaves. The coincidence of these events suggests a major role for the caseolytic enzymes in initiating the rapid hydrolysis of leaf protein.

Although the number of proteolytic enzymes detected in various plant tissue is large, their physiological role during plant maturation (leaf senescence) is largely unknown (10, 18). Little information is available concerning the relationship between proteolytic leaf enzymes and leaf protein mobilization during grain development and maturation of crop plants.

A CP* purified from barley leaves had a pH optimum at 5.2

1 This work was supported by a Frasch Foundation grant and by Hatch funds.
2 Present address: Pflanzenphysiologisches Institut, Universität Bern, Altenbergstrasse 21, CH-3013 Bern, Switzerland.
3 Abbreviations: ME: mercaptoethanol; PHMB: p-hydroxymercuricbenzoate; NEM: N-ethylmaleimide; PMSF: phenylmethylsulfonylfluoride; AP: aminopeptidase; CP: carboxypeptidase; CA: caseolytic proteases.

(21) while a CP from Aspergillus had a pH optimum between 3 and 4 (11).

An acid sulfhydryl endopeptidase which functions in the mobilization of storage proteins in germinating seeds (4-6, 8, 9, 23) also occurs in leaf tissue (1, 3). The level of acid sulfhydryl endopeptidase activity remained relatively constant during senescence (2, 19) suggesting that this enzyme has a minor role in the final mobilization of leaf proteins. With detached leaves, extractable protease activity was correlated with leaf senescence and protein synthesis was required for senescence (14, 16). Martin and Thimmann (14) found two proteolytic enzymes with different pH optima (pH 3 and 7.5) in tips of excised oat leaves. With hemoglobin as substrate, acid protease activity decreased after an initial increase, while neutral activity continued to increase throughout the senescence period. Endopeptidases insensitive to sulfhydryl reagents and with a neutral pH optimum have been found in germinating seeds (15, 20). A bean leaf endopeptidase (pH optimum 9-10) that hydrolyzes denatured leaf protein has been reported (17).

The objectives of this work were to identify some of the major proteolytic enzymes occurring in field-grown corn leaves and to study the changes in their activity patterns and the mobilization and loss of leaf protein during grain development and maturation.

MATERIALS AND METHODS

Plant Material. Leaves from field-grown corn (inbred B73, planted May 6, 1975, in fertile soil on the Agronomy South Farm) were the source material. The leaves, excised at the sheath and deribed, were placed in plastic bags and stored on ice for transport to the laboratory. Each sample was comprised of equivalent leaves from at least three plants. Ear leaves were used for all experiments except where indicated. All of the work was done with fresh tissue except for the temperature and inhibitor studies, where frozen (−20°C) tissue was used. No differences in enzyme activities were detected in extracts from comparable fresh or frozen tissue.

Extraction. Leaves were cut into 1-cm² sections and thoroughly mixed prior to subsampling. Six g of leaf segments and 24 ml of 0.05 M acetate buffer (pH 5.4 with KOH) containing 1% (w/v) insoluble PVP and 0.1% (v/v) ME were homogenized (VirTis 45 homogenizer) for 1 min at medium and 1 min at high speed. The homogenates were filtered through four layers of cheesecloth and then centrifuged for 10 min at 3,000g. The supernatant was stored overnight at 0°C before desalting (amino acid removal) on a Sephadex G-25 column. The column (0.9 × 11 cm) was prepared as described (13) except the equilibration medium was 0.05 M acetate buffer (pH 5.4). Before loading of the extract, the column, held in a conical tube, was centrifuged for 5 min at 30g followed by 5 min at 300g and the excess buffer removed from the retaining centrifuge tube. After loading with ml enzyme extract, the column was held at 0°C for 10 min and then centrifuged as previously indicated. The collected eluate was used directly for assays. For enzyme characterization stud-
ies. 5 ml supernatant from the initial centrifugation were de-
salted by dialysis against 1,000 ml of 0.05 M acetate buffer (pH 5.4) containing 0.1% (v/v) ME. Dialysis was done at 3 C for 15
hr with two changes of medium.

**Aminopeptidase Assay.** The substrate medium (L-leucine-p- 
nitroanilide) was prepared as described by Chrispeels and Boul-
ter (6) except that the final pH was 7. The enzyme extract (0.02
ml) and substrate medium (0.5 ml) were incubated at 37 C for 1
hr. The reaction was stopped by placing the reaction tubes in
boiling water for 5 min. The samples were then diluted with 2 ml
H2O and measured against a zero time blank. One unit of activity
was defined to an absorbance change of 1/hr at 410

**Carboxypeptidase Assay.** The substrate, N-carbobenzy-
lyphenylalanne-l-alanine, was prepared as described by Visuri et
al. (21) except that the final pH was 5. At pH 5, AP activity was
negligible. Enzyme extract (50 ml) and substrate solution (0.3
ml) were incubated at 37 C for 1 hr. The reaction was stopped
by addition of 0.2 ml of 15% (w/v) trichloroacetic acid. For a
blank (autodigestion), the enzyme was incubated with 0.05 M
acetate buffer (pH 5) containing 0.5 mM EDTA. The free amino
groups were detected with ninhydrin (22).

**Assay for Casolytic Activity.** A solution of 5% (w/v) casein
in water was prepared by repeated adjustment of the pH with
KOH to pH 7 until the casein was completely dissolved. From
this stock solution, the substrate media were prepared with a
final casein concentration of 0.5% (w/v). The media were
buffered with 0.05 M acetate (KOH) at pH 5.4 or with 0.05 M
tris-HCl at pH 7.5. Immediately before use, 0.1% (v/v) ME was
added to both media. Enzyme extract (0.005-0.1 ml depending
on activity) were brought to 0.1 ml with H2O and incubated for 3
hr with substrate solution (0.3 ml) at 37 C for the pH 5.4 assay
and 45 C for the pH 7.5 assay. The reaction was stopped by
addition of 0.2 ml 15% (w/v) trichloroacetic acid. After standing
at 3 C for 30 min, the precipitate was removed by centrifugation
(10 min. 300g). The free amino groups in the clarified superna-
tant were determined with ninhydrin (22). Boiled enzyme blanks
were used. The increase in free amino groups was linear over a
4-hr period.

The CA may reflect the hydrolytic action of both endo- and
exopeptidase enzymes under the assay conditions employed (un-
purified extracts). While this is undesirable, the loss of activity
incurred through separation techniques is even more objection-
able for survey purposes. The CA (pH 7.5) is undoubtedly low
because ME was added to all assays. The inhibitory effect of ME
(Table 1) was not found until the seasonal survey work was well
underway.

**Inhibitors and Activators.** Aliquots of the enzyme extract
were pretreated with the inhibitors or activators for 15 hr at 3 C,
except ME, which was added to the assay mixture. Controls
were aliquots of enzyme extract pretreated with an equivalent
volume of the solvent used for each inhibitor or activator. All
solutions were freshly prepared and water was the solvent except
for PMSF which was dissolved in ethanol (final ethanol concen-
tration for preincubation 0.2%).

**Chlorophyll Concentration.** An aliquot (0.1 ml) of the crude
homogenate was diluted to 1 ml with water. Four ml of acetone
were added to each aliquot, and after standing 15 min at 3 C in
the dark, the absorbance at 665 nm was measured against 80%
(v/v) acetone.

**Protein Concentration.** To an aliquot (1 ml) of the crude
extract, held in a 12 ml conical centrifugation tube, 2 ml of 15%
(w/v) trichloroacetic acid were added with stirring. The samples
were allowed to stand at 3 C for 30 min and the precipitate
collected by centrifugation at 120g for 20 min. The nitrogen
content of the precipitate was determined with Nessler's reagent
after digestion. Protein was estimated by multiplying µg N by
6.25.

**Expression of Data.** Difficulty was encountered in selecting a
basis for expression of data because the usual parameters, fresh
and dry weight or protein and Chl concentrations, change during
senescence. It was difficult to measure leaf area because senes-
cing leaves of field-grown corn were ragged, shrunk, or wrinkled.
Data obtained with tissues from different leaf segments or
different leaves were expressed on a fresh weight basis. Because
fresh weight was also changing, the data permit only qualitatively
rather than quantitative comparison. In the survey work during
grain development when the middle half of the ear leaf was used
for assays, leaf area was measurable and activities were ex-
pressed on an area basis.

### RESULTS AND DISCUSSION

Three types of proteolytic enzyme activities were detected in
mature, photosynthetically active tissue and senescing corn leaf
tissue.

**Aminopeptidase Activity.** The pH optimum for the enzyme-
hydrolizing L-leucine-paranitroanilide was between 6.5 and 7
(Fig. 1). The symmetrical shape of the pH response curve sug-
gests the presence of only one enzyme. The optimum tempera-
ture for a 1-hr standard assay at pH 7 was 45 C. Activity was not
significantly affected by treatments with EDTA, PHBMB, NEM,
PMSF, or ME (Table 1).

**Carboxypeptidase Activity.** The pH response curve (Fig. 1)
with pH optima at 3.5 and 5 suggest the presence of at least two
enzymes in corn leaves. When assayed at pH 5, the CP en-
zyme(s) was more tolerant to temperature treatments and had a
broad range (35-65 C) with an optimum at 55 C (1-hr assay).
Activity was not affected by EDTA or thiol reagents, but PMSF,
a hydroxyl group probe, caused a 50% loss of activity (Table 1).

**Casolytic Activity.** With mature but photosynthetically active
leaves (ear leaves harvested August 1), the CA exhibited a
broad and multipeaked pH profile with highest activity at pH 5
(Fig. 1). With senescing leaves (bottom leaves harvested August
28), the pH profile was again broad and multipeaked with highest
activity at pH 6.5. Also, there was a marked increase in CA
in the neutral and alkaline pH range but a decrease in activity
in the acid pH range (below pH 5).

Table 1. Inhibition and Activation of Aminopeptidase.
Carboxypeptidase, and Casolytic Activities Extracted from Corn Leaves

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mature but photosynthetically active</th>
<th>Senescing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CA pH 5.4</td>
<td>CA pH 7.5</td>
</tr>
<tr>
<td></td>
<td>AP</td>
<td>CP</td>
</tr>
<tr>
<td>PHMB 0.1</td>
<td>94</td>
<td>97</td>
</tr>
<tr>
<td>NEM 2.5</td>
<td>107</td>
<td>101</td>
</tr>
<tr>
<td>PMSF 0.5</td>
<td>99</td>
<td>48</td>
</tr>
<tr>
<td>EDTA 0.1</td>
<td>90</td>
<td>99</td>
</tr>
<tr>
<td>ME 2.0</td>
<td>85</td>
<td>112</td>
</tr>
<tr>
<td>ME 10.0</td>
<td>96</td>
<td>104</td>
</tr>
</tbody>
</table>

* % of control

With senescing leaves, the CA at pH 7.5 had a broad tempera-
ture profile with maximum activity at 45 C (standard 3-hr as-
say). Assays made at 35 and 55 C showed 75% of the activity at
45 C.

The CA was affected differently when extracts of mature and

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Fig. 1. Proteolytic activities from corn leaves as a function of pH of assay medium. The activities of the carboxypeptidase (CP) and aminopeptidase (AP) are expressed as per cent of the maximum. The caseolytic activities (CA) are expressed as \( \mu \text{mol} \text{ free amino groups produced hr}^{-1} \text{ g fresh wt}^{-1} \). CP, AP, and CA enzymes were extracted from the middle half of the ear leaf (active, mature) harvested August 1. The CA activities were also determined with extracts prepared from bottom leaves (senescing) harvested August 28. The buffers used were 0.1 M citrate-phosphate (below pH 8) and 0.1 M tris-HCl (above 8). Vertical lines indicate the pH at which assays are run.

senescing leaves were treated with inhibitors and activators (Table 1). EDTA and ME depressed pH 7.5 activity in both mature and senescing tissue, but stimulated pH 5.4 activity in mature tissue and depressed it in senescing tissue. PMSF depressed the CA at pH 5.4, but had little effect at pH 7.5.

Data obtained with crude extracts of corn leaf tissue indicate the presence of two caseolytic enzymes (or group of enzymes), one with an acidic and one with a neutral pH optimum. Previous work has shown that an acidic endopeptidase, activated by sulphydryl reagents or EDTA, is the principle enzyme involved in the degradation of storage protein in germinating corn seeds (8).

The CA could be due to the action of both endo- and exopeptidases (CP at pH 5.4 and AP at pH 7.5). However, the concentration of substrate for both exopeptidases would be relatively low until free amino or carboxyl groups are made available by the action of the endopeptidases. The bulk of the CA assayed either at pH 5.4 or 7.5 was probably due to endopeptidase activity. This was confirmed for the endopeptidase activity at pH 7.5 by showing that the amount of trichloroacetic acid-soluble ninhydrin-reactive material present after incubation constituted only a minor part of the total. The changing properties of the CA at pH 5.4 (Table 1) suggest that these measurements are not representative of one enzyme. The depression of the CA by PMSF at pH 5.4 could be due to the inhibition of CP, which is PMSF-sensitive (Table 1) and active at this pH (Fig. 1). The inhibition by ME or EDTA of CA at pH 5.4 from senescing corn leaves also indicates that a portion of the CA may be due to CP activity. The different effects of PHMB or NEM or CA (pH 7.5) from mature and senescing leaves (Table 1) and the multiphasic pH profile imply that more than one neutral endopeptidase is present in the extracts.

CHANGES IN PROTEOLYTIC ENZYMES, CHOROPHYLL, AND PROTEIN IN CORN LEAVES

Ear Leaf (Longitudinal Segments). The changes in AP, CP, and CA activities, protein and Chl concentrations in four equal length segments of the ear leaf (harvested August 5 and 25 and September 8) are shown in Figure 2. The AP activities of all four leaf segments decreased with each successive harvest. There was no difference in AP activity along the length of the leaf at the first harvest. By the third harvest, a decreasing gradient in AP activities from leaf base to tip was observed. Although the changes in CP activities were relatively minor, activity decreased in the two leaf tip segments with each successive harvest. In contrast to the decreases or constancy of the activities of the exopeptidases, the CA increased with leaf senescence and activity at pH 7.5 increased more than activity at pH 5.4. At all harvests there was an increasing gradient of CA activities from base to leaf tip.

Visually, senescence of corn leaves was first observed at the tip and leaf margin appearing as a V-shaped area of brown or
apparently necrotic tissue. This V-shaped area enlarges and moves toward the leaf base with time until the entire leaf becomes necrotic. The data (Fig. 2) obtained with leaf segments show that the decreases in AP activities, Chl and protein concentrations, and the increases in CA are consistent with the visual pattern of leaf senescence.

**Leaf Position.** The changes in measured protease activities and protein and Chl concentrations of the various leaves, harvested at intervals during grain development, are shown in Figure 3. Data (not included) obtained with three other corn genotypes are similar to those obtained with genotype B73.

Visually, senescence was first observed on the bottom leaf and then sequentially by leaf up the plant. Subsequently, a similar pattern of senescence starts with the top leaf and develops downward. The last leaf to show symptoms of senescence was at leaf position +2 (second leaf above the ear). These data (Fig. 3) show that the changes in measured protease activities and protein and Chl concentrations of the various leaves at the different harvest dates reflect the development of the visual patterns of canopy senescence. With each successive sampling, increases in CA activities were concurrent with decreases in protein and Chl. In contrast, AP or CP activities decreased or remained unchanged.

**Ear Leaf (During Grain Development).** The changes in AP, CP, and CA, activities as determined by sampling the middle half of the ear leaf during development and maturation of the grain are shown in Figure 4.

The AP and CP activities increased with the onset of the visible reproductive phase (tasseling observed July 15). Maximum AP activity was obtained by July 22, while maximum CP activity was measured on August 5. After August 5, CP activities declined rapidly, while AP activities remained constant until July 22 before declining. The differences in activity patterns may be due to the intracellular localization of the two exopeptidases (7, 12) and/or to hormonal regulations (2, 16).

AP and CP activities increase while leaf protein concentration is high and relatively constant. They decrease concurrently with the loss of leaf protein. In contrast to the changes in exopeptidase activities, the increases in CAs (assayed at pH 5.4 and 7.5) coincide with the initial decrease and the eventual loss of the bulk of the leaf protein. The most rapid rate of protein loss was concurrent with the most rapid increase in CAs. There was a decrease in CAs only after two-thirds of the protein had been lost.

The coincidence of the loss of leaf protein with the increase in CA activities (Figs. 2–4) suggests a major role for the caseolytic enzymes in initiating the rapid hydrolysis of leaf protein. However, these data do not rule out a role for the exopeptidase activities in the mobilization of leaf protein during grain development.

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**Figure 4.** Changes in proteolytic activities, protein and Chl concentrations, and specific leaf weight of corn leaves during the grain development and maturation period. All activities are expressed on a basis of leaf area (dm²). Amino- (AP) and carboxy- (CP) peptidase and caseolytic proteases (CA) were extracted from the middle half of ear leaves harvested on the dates indicated. Pollination occurred approximately July 15. The extraction and assays were as detailed under “Materials and Methods.”
LITERATURE CITED