The Hydrolysis of Endosperm Protein in Zea mays

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ABSTRACT

Degradation of the major storage proteins in maize endosperm, zein and glutelin, begins during the 2nd day of germination. The protein most abundant in the mature endosperm is degraded most rapidly. The patterns of protein loss are essentially similar in germinating seeds and excised endosperms. Cycloheximide, added at the beginning of the incubation period, prevents the development of α-amylase and protease activities and the disappearance of starch and protein reserves. Late additions (70 hours) of cycloheximide still inhibit the increase in hydrolase activity but have no effect on the hydrolysis of storage reserves. The results indicate that the hydrolytic enzymes are synthesized de novo in the maize endosperm.

Cereal endosperm storage proteins are of two major classes, the prolamines and globulins (19). In maize these proteins, zein and glutelin respectively, are different in both amino acid composition and intracellular location. Zein is deficient in lysine and tryptophan, and to a lesser extent in arginine, histidine, and methionine (12). It is located mainly in protein bodies (5). Glutelin, on the other hand, has a balanced amino acid composition and forms a cytoplasmic matrix in the cell (3, 20). When the seeds germinate, both proteins are degraded to supply the embryo with amino acids required for its growth. In this paper we examine the sequence of events of endosperm protein breakdown during germination. The results show that loss of zein and glutelin begins early during germination, and that the rate of digestion of either protein is related to its abundance in the dry endosperm. The proteases required for this digestion appear to be synthesized de novo in the endosperm.

MATERIALS AND METHODS

Zea mays hybrid W19 × 38-11 was used in all experiments. The seeds were purchased from the Agricultural Alumni Association, Lafayette, Indiana or were grown in Hamilton. Germinated seeds, excised endosperms incubated in buffer, and endosperms of mature ungerminated seeds were used. Intact seeds were germinated as described by Harvey and Oaks (8).

Incubation of Excised Endosperms. Seeds were sterilized in Javex (a commercial bleach containing 6% sodium hypochlorite), rinsed, and allowed to imbibe in sterile water for 2 hr at 28 C. The embryos + scutella were then dissected out. The endosperms (10 per flask) were transferred to sterile incubation flasks, each containing 10 ml of 1 M acetate buffer, pH 5.0, with 1 mM CaCl2, Penicillin (10 μg/ml), chloramphenicol (10 μg/ml), and streptomycin (250 μg/ml) were included in the incubation medium to minimize bacterial contamination. These antibiotic concentrations were found to have no effect on endosperm starch and protein hydrolysis during incubation. A total of 50 endosperms were used per treatment. The flasks were incubated with shaking (20 oscillations/min) at 28 C. After incubation aliquots of medium were plated on nutrient agar to test for contamination. Occasionally fungal infected samples were found and discarded.

Enzyme Assays. Endosperm protease was extracted and assayed as described in the accompanying paper (8). Extracts (0.1–0.25 ml) were incubated with 1 ml of 5% gliadin and 1 ml of 0.05 M acetate buffer, pH 3.8, containing 2.5 mM EDTA, in a total volume of 2.25 ml. Aliquots of incubation medium (0.25–0.5 ml) were incubated with 1 ml of gliadin and 1.5 ml of the assay buffer in a total volume of 3.0 ml. After 10 min the reaction was stopped by the addition of an equal volume of 5% trichloroacetic acid, and the increase in absorbance at 280 nm of the trichloroacetic acid soluble fraction was measured. Activity was expressed as mg tryptophan released from protein per hr per endosperm.

α-Amylase was extracted from frozen endosperms with 5 mM acetate buffer, pH 5.0, which contained 1 mM CaCl2. Two ml of buffer were used per g fresh weight of endosperm tissues. When necessary, extracts were diluted for assay. Endosperm extracts or aliquots of incubation medium were incubated for 10 min at 28 C with 0.5 mg of soluble starch, in a total volume of 6.5 ml. The reaction was stopped by addition of 2 ml of 15% trichloroacetic acid, and residual starch was measured by color reaction with iodine (13). Activity was expressed as mg starch degraded per min per endosperm. With intact seeds, values are recorded for endosperm extracts. With excised endosperms, values represent the sum of activities from endosperm extract and medium.

Starch and Protein Hydrolysis. Total protein hydrolysis was measured by release of total nitrogen from the endosperm into the incubation medium. Total nitrogen was assayed by Kjeldahl digestion (9) followed by Nesslerization (15). Dry weight loss from endosperms of germinated seeds (50 per sample) was measured as a rough estimate of starch loss. An indication of starch hydrolysis in excised endosperms was obtained by measuring reducing sugars released into the incubation medium. Aliquots of medium were diluted as required and assayed for reducing sugars by Nelson’s method (17).

Protein Fractionation. The endosperm proteins, albumin, globulin, zein, and glutelin, were extracted according to their...
solubilities, as described by Osborne (19) and Jimenez (12). Lyophilized and powdered endosperms were defatted by grinding for 5 min in n-butanol. The butanol extract was retained for nitrogen measurement, and the endosperm powder again lyophilized. One gram samples of the defatted endosperm powder were subjected to consecutive extractions with distilled water (albumins), 5% sodium chloride (globulins), 70% ethanol (zeins), and 0.2% sodium hydroxide (glutelins). The powder was stirred for 24 hr in 50 ml of solvent at 3°C, then centrifuged at 28,000g for 20 min. The residue was washed with cold-distilled water, and the washings were combined with the extraction solvent. Nitrogen in each fraction was determined by Kjeldahl digestion followed by Nesslerization. After the final extraction the insoluble residue was lyophilized, and its nitrogen content was measured. The protein composition per endosperm was calculated and compared with total nitrogen per endosperm measured directly on endosperm powder. This extraction method gives a 90 to 110% recovery of endosperm nitrogen. Variability between replicate experiments is in the order of ±10%. This analysis is sufficiently accurate to demonstrate major differences or changes in endosperm protein fractions.

RESULTS

Pattern of Protein Breakdown. Figure 1 shows the course of endosperm protein digestion during germination of intact seeds. Zein and glutelin loss began after about 20 hr of germination, but total protein loss was not apparent until about 60 hr. In this interval, nitrogen increased in the water and sodium chloride extracts. Most of this increase was dialyzable and thus presumably represents nitrogen released by degradation of the storage proteins. Glutelin breakdown was more rapid than zein degradation from 40 to 80 hr. Subsequently, hydrolysis continued at approximately the same rate for each protein until the endosperm protein reserves were depleted.

In a second experiment, various batches of the Wf9 × 38-11 seed of different protein composition were tested. In these, total protein varied from 30.0 to 42.5 mg protein-nitrogen per 10 endosperms. The zein content ranged from 6.0 to 21.5 mg nitrogen per 10 endosperms, while the glutelin content remained fairly constant. The seeds were germinated and their protein composition analyzed when 20 to 30% of endosperm protein had been degraded. This represents the most sensitive stage for detection of different rates of degradation of zein and glutelin (Fig. 1). In high glutelin seeds, glutelin was degraded more rapidly than zein, whereas in high zein seeds, zein was digested more rapidly (Table 1). Zein and glutelin were lost at equal rates from seeds containing equal amounts of the two proteins.

Influence of the Embryo on the Loss of Endosperm Protein. The course of protein breakdown and changes in protease activity were followed in endosperms of germinated seeds and in excised endosperms incubated in buffer. Protease activity appeared earlier in excised endosperms than germinated seeds (Fig. 2). Consistent with this, protein breakdown also commenced earlier in excised endosperms, and proceeded at a somewhat faster rate. In each case, the maximum enzyme activity occurred when about 75% of the endosperm protein had been degraded. Protein analysis showed that degradation of zein and glutelin began later and progressed slightly more slowly in intact seeds. There was, however, no qualitative difference in the pattern of protein breakdown in the presence and absence of the embryo. The embryos of germinating seeds thus appear to retard protease production and endosperm hydrolysis.

Hydrolase Activity and Degradation of Endosperm Reserves. The development of protease and α-amylase activity in germinating seeds are compared in Figure 3. The appearance, increase, and then decline in activity show the same trend for both hydrolytic enzymes. α-Amylase was detected at about 2 days after imbibition of the seeds, slightly earlier than the protease, and its maximum activity was measured at 9 days, about 24 hr later than the peak in protease activity.

A small dry weight loss, principally starch, occurred during the first 48 hr of germination (Fig. 3). No nitrogen loss was evident in this interval, although the distribution of endosperm protein altered (Fig. 1). Some protein hydrolysis therefore...
does occur during the initial 48 hr of germination i.e. before the development of protease activity. This can probably be attributed to proteases known to be present in ungerminated cereal seeds (5, 13, 17) but which were not detected by our assay. After 48 hr, starch and protein loss was rapid. Protein breakdown continued at a steady rate until day 8, then leveled off. This reduction in the rate of hydrolysis occurred when little endosperm nitrogen remained. The linear rate of starch breakdown continued until day 9. The decline in both protease and $\alpha$-amylase activity began when endosperm reserves were almost depleted. The rapid phase of strach and protein degradation coincided with the period of increasing protease and $\alpha$-amylase activity.

**Requirement for Protein Synthesis.** Figure 4A shows that cycloheximide (5 $\mu$g/ml) added at or near the beginning of the incubation period prevented the degradation of protein and starch. When added at 30 hr, cycloheximide partially inhibited

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<th>Table I. Endosperm Protein Breakdown during Germination of Seeds of Different Zein to Glutelin Ratios</th>
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<td>Seeds were germinated for 3.5 days, then their endosperm proteins were analyzed. The zein to glutelin ratios were: A, 0.3; B, 0.7; C, 1.0; and D, 1.5. Batches B and D were supplied by the Agricultural Alumni Association, Lafayette, Indiana. Batches A and C were grown in Hamilton.</td>
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<th>Seed Batch</th>
<th>Initial Values</th>
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<tr>
<td></td>
<td>Total Zein Glutelin</td>
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<td>mg N/10 endosperms</td>
<td>mg/10 endosperms</td>
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<tr>
<td>A</td>
<td>30.0 6.0 18.0</td>
<td>9.5 2.2 7.0</td>
</tr>
<tr>
<td>B</td>
<td>39.5 11.5 17.0</td>
<td>12.5 4.8 7.0</td>
</tr>
<tr>
<td>C</td>
<td>41.5 16.5 16.2</td>
<td>8.5 5.5 5.5</td>
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<tr>
<td>D</td>
<td>42.5 21.5 14.5</td>
<td>13.8 12.25 6.0</td>
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**DISCUSSION**

The results of the current investigation show that both zein and glutelin are degraded from the earliest times of germination and that the grand period of hydrolysis from 3 to 8 days coincides with the appearance of an acid protease. The rate of disappearance of each storage protein is related to its initial concentration (Table I) and appears to be independent of protein type and location within the seed. The protein hydrolysis which occurs during the initial 48 hr before the development of the acid protease activity can probably be attributed to proteases known to be present in ungerminated cereal seeds (6, 14, 18) but which were not detected by our assay.

In an early survey of changes in biochemical events during germination in Zea mays, Toole (22) indicated that the endosperm was able to digest its own starch and protein reserves. Later Dure (4) found that excised endosperms produce little $\alpha$-amylase and more recently Ingle and Hageman (10) have suggested that gibberellic acid additions are necessary for the digestion of endosperm storage reserves. Our results suggest that an embryo factor or exogenous gibberellic acid are not necessary for the autolysis of endosperm in a wide variety of hybrid or inbred lines of Zea mays (Fig. 2, and unpublished results). They also show that all the protease and amylase found in normally germinating seeds could originate within the endosperm itself. Two observations suggest that although gib-
berellic acid additions to the medium do not enhance the production of hydrolases, it is, nevertheless, involved in their development. (a). Abscisic acid inhibits the development of \( \alpha \)-amylase and protease in maize endosperms and this inhibition is overcome specifically by additions of gibberellic acid (Harvey and Oaks, unpublished results). (b). Endosperms from dwarf corn which might be expected to be deficient in endogenous gibberellic acid give a good response to added gibberellic acid (Harvey and Oaks, unpublished results). Thus, the differences in the results of various investigators probably reflect differences in the endogenous levels of gibberellic acid in the endosperm tissue. In barley a number of enzymes increase in activity during germination or in excised aleurone layers. Of these \( \alpha \)-amylase and protease require gibberellic acid for induction, whereas ribonuclease and \( \beta \)-glucanase do not. In each case, however, the enzyme has been shown with the density labeling technique to be synthesized de novo and their synthesis is sensitive to cycloheximide (1, 7, 11). In our experiments, cycloheximide inhibits the development of \( \alpha \)-amylase, protease, and the degradation of maize endosperm reserves. This indicates that the \( \alpha \)-amylase and protease in the maize endosperm are also synthesized de novo.

During germination protease and \( \alpha \)-amylase increase in activity at a time when the storage reserves are being lost at a rapid rate. Increasing hydrolytic enzyme activity throughout periods of protein, starch, phytin, and RNA breakdown has also been observed in oats (20). The maximum in hydrolytic enzyme activity coincides approximately, with the depletion of endosperm reserves. Sutcliffe and Baset (21) suggested that new hydrolase could be produced with each new wave of degradation of storage reserves. Our results with cycloheximide show that after 70 hr, new enzyme is not required for a maximum rate of degradation of storage reserves. At this time about half of the total possible hydrolase is present. New
end enzyme is therefore not required to digest new interfaces of storage reserves.

LITERATURE CITED