AN ABSTRACT OF THE THESIS OF

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Dr. Te May Ching

Preharvest rain damage to starch quality of wheat (Triticum aestivum L.) seeds is known to be caused by amylase activity in the endosperm. Cultivars with seed dormancy or with glumes containing inhibitors or resisting water penetration are resistant to such damage. There are a number of isozymes and other amylolytic enzymes which might be involved in starch degradation upon wetting by rain. If resistant cultivars contain different components of amylolytic enzymes than the susceptible ones, then these differences could be used in selecting cultivars to avoid rain damage and to reduce financial losses, or for breeding materials in synthesizing future cultivars. Therefore, this study was instigated to compare the quantitative, qualitative, and developmental differences of amylolytic enzymes in a rain resistant cultivar (Maris-Hobbit) and a rain susceptible cultivar (Stephens).
A quantitative distribution of amylolytic enzyme in different parts of wheat grain during maturation was recorded for the first time. A high percentage of total enzyme activity was found in the pericarp at a very early stage of maturation, followed by a tremendous increase of amylolytic enzyme was observed in endosperm. In addition to previously reported three α-amylases in the pericarp, two new and one known β-amylases were also observed. Amylolytic enzyme activity in the endosperm was high at later stages of maturation, which amounted to 50% of that found during germination. A total of sixteen amylolytic isozymes: three α-amylases, three phosphorylases or debranching enzymes, one β-amylase and nine $60^\circ$-stable β-amylases or debranching enzymes, were observed in endosperm at later stages of maturation. β-Amylase activity is the major enzyme found in the testa and aleurone layer in which four β-amylases and three minor α-amylases were observed. No amylolytic activity was detected in the embryo extract however, three or four minor bands were observed in the zymogram upon longer incubation.

Isozyme pattern of 1-day germinated seeds was similar to that of mature seed. On second day of germination, five or six α-amylases and one β-amylase were synthesized. They increased in intensity or quantity with time of germination.
At the peak of starch degradation, seventh day of germination, a total of ten bands were found in Maris-Hobbit and 11 bands in Stephens.

Little quantitative and qualitative differences in amylolytic enzymes between the susceptible and resistant cultivars during seed maturation and germination were observed. The presence of preexisting α-amylase and β-amylase at early germination indicates the possibility that α-amylase can be activated by rain to initiate the degradation of starch, following by the hydrolysis on the partially degraded starch by β-amylase, thereby degrading flour quality. The newly harvested seeds of Maris-Hobbit exhibited partial dormancy in a germination test at 25°C. These studies suggest that the differences in their susceptibility to rain damage is due to seed dormancy in Maris-Hobbit and/or the presence of inhibitors in glumes.
Amylolytic enzymes in wheat seeds

by

Wha-gi Lee

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AMYLOLYTIC ENZYMES IN WHEAT SEEDS

INTRODUCTION

Wheat is a major staple food around the world, and producing sufficient wheat for the world's consumption has been one of the goals of agronomists for many decades. Currently, improving wheat quality for nutritional needs is the major thrust. One of the major problems is the reduction of starch quality by pre-harvest rain or sprouting. This damage results in serious loss of marketable value (McEwan 1976), because the damaged flour produces bread with low volume and short noodles, neither of which are acceptable to consumers. It has been reported that the preharvest rain damage on starch quality is due to the action of amylolytic enzymes (Moss et al., 1972; Derera et al., 1977; Gordon et al., 1977; Gordon, 1978). Resistance to rain damage can be obtained in cultivars with seed dormancy (Belderok, 1976), with low rate of water penetration through glumes (Chang, 1943), with germination inhibitors in bracts (Smith, 1948; Derera et al., 1976, 1977), with slow response on \( \alpha \)-amylase production in endosperm (Gale and Marshall, 1973; Gale, 1976; Derera et al., 1976; Derera et al., 1977), or with high fiber content in seed coat (Moss and Kirby, 1976). However, these characteristics of resistance are usually not present in cultivars with high productivity, and breeding high producers with seed dormancy
is a difficult and time consuming task. Therefore, selecting cultivars with low amylolytic enzymes at maturity may be a more fruitful and rapid procedure for planting purposes or for breeding selection of less susceptible varieties to alleviate the problem of preharvest damage and financial loss to the farmers. In selecting suitable cultivars, the qualitative and quantitative amylolytic enzyme activity should be discerned. This is the purpose of this study.

Two cultivars of wheat are selected for this study. Stephens is a new cultivar with very high productivity which are used in production in the Northwest; however, it is susceptible to pre-harvest rain damage. An English variety, Maris-Hobbit, is resistant to pre-harvest rain damage in the Northwest of U.S. and it is known to have seed dormancy (personal contact with Dr. W. E. Kronstad). If cultivar differences in amylolytic enzymes, quantitatively or qualitatively are present at later stages of maturation, in fully matured seeds, or at early stages of germination, then the procedure may be used as a selection tool by plant breeder. If no differences are found, then other characteristics of resistance should be the selection criterion. It does not, however, rule out the possibility that other cultivars may exhibit these differences in enzyme activity and isozyme patterns.

This study used polyacrylamide gel disc electrophoresis
(PAGDE) differential temperature denaturation, and specific substrate to discern the isozymes of α-, β-amylases, debranching enzyme and phosphorylase during maturation and germination. Quantitative assay was also conducted in the original and temperature denatured extracts of various stages during maturation and germination. The distribution of amylolytic enzyme in different parts of the seed and their changes during maturation were also studied, as little quantitative information of this nature was reported in the literature.

The experimental results indicated that some α-amylase was present in mature seeds that are easily activated by hydration in both cultivars. The differences may be due to seed dormancy in Maris-Hobbit that inhibits the activation of the pre-existing amylases and thus results in resistance to pre-harvest rain damage. Glume resistance to water penetration and the presence of inhibitors in glumes, however, are not ruled out for the rain damage resistance in Maris-Hobbit. More research is needed into the mechanism of seed dormancy in Maris-Hobbit.
MATERIALS AND METHODS

Materials

Two cultivars of wheat (*Triticum aestivum* L.) seeds, one soft white winter type—Stephens and the other hard red winter type—Maris-Hobbit, were produced in 1978 and 1979 on the Hyslop Experimental Farm in Corvallis, Oregon. These two cultivars differ significantly in the response to preharvest rain damage. Maris-Hobbit seeds, being dormant on the heads, are resistant to damage, while Stephens seeds are not dormant and susceptible to damage. Therefore these two cultivars were selected to discern if any relationship exists between the pattern of amylolytic enzymes during maturation and germination and pre-harvest rain damage to the starch quality. For the changes of amylolytic enzymes during germination, seeds were harvested in 1978 and stored under room conditions. For the maturation study, seeds were grown in 1979 and sampled at 14, 21, 28, 35, 42, 49, 56, 63 and 70 days after anthesis (DAA). Stephens flowered one week earlier than Maris-Hobbit, thus its 14-DAA sample was missed. The collected heads were stored at -80°C until analysis.
Enzyme preparation

Germination: Two replications of 100 seeds were germinated in paper towel rolls at 20°C in a germination chamber. A sample of 10-20 seeds were removed from each roll daily, embryos or seedling axes were dissected from endosperms and their fresh weights were obtained. The embryo and seedling dry weight was determined by drying in an oven at 100°C for one hour and then at 85°C for 23 hours. The endosperms were homogenized in 10 ml of cold grinding buffer (0.05M Tris, 0.01M CaCl₂, pH 6.5) with a polytron (Brinkman Instruments Model PT20ST), set at 5.5 for 30 sec in a cold room. The slurry was centrifuged at 10K rpm for 10 minutes, and the supernatant was used as the cell free enzyme preparation. The precipitate of this enzyme preparation was used for the determination of starch content in endosperm.

Maturation: Fresh weight and dry weight of the seeds at different maturing stage were determined in two replications of 10 each. Two replications of 30 seeds from each sampling were dissected into four parts: pericarp, "seed coat and aleurone layer", endosperm, and embryo. Each part was collected on ice, washed with ice cold grinding buffer to remove contaminants and then ground with a polytron in 2 ml of grinding buffer as stated previously. The cell-free supernatant was used as the enzyme preparation. The
starch content was determined in the precipitate of pericarp and endosperm only, as no starch grain was observed microscopically in the "seed coat and aleurone layer" or in the embryos.

Assay of amylases

In seeking the best method for separating β-amylase activity from others, one known β-amylase inhibitor, iodoacetamide (Spradlin and Thoma 1970) failed to completely inhibit the activity of crude barley β-amylase purchased from Sigma Chemical Co., while 60°C or 70°C heat treatment did fully inactivate the crude barley β-amylase (Figure 1). Furthermore, according to Kruger (1973), wheat β-amylases can be inactivated by a heat treatment of 60°C and the debranching enzyme was inactivated by heat treatment of 70°C. It, then, can be assumed that β-amylase, debranching enzyme (r enzyme) and α-amylase of cereal grains can be separated by differential heat treatment. Therefore, each enzyme preparation was divided into three equal portions: one was heated in a water bath for 20 minutes at 70°C (70°C-extract) to retain only α-amylase (Briggs 1972). Another portion was heated at 60°C for 20 minutes (60°C-extract) to inactivate heat labile enzymes, β-amylase etc., and retain and r amylases. The last unheated portion was used for the determination of total amylolytic enzyme activity.

The assay method of amylases follows that of Bernfeld
Fig. 1 Electrophoretic results of crude β-amylase from barley after heat and inhibitor (idoacetamide) treatments.

UH: 100 μg of enzyme in 100 μl of grinding buffer was charged on the polyacrylamide gel.

60°C (70°C): 10 ml of enzyme in 1 μg/μl of grinding buffer was heated at 60°C (70°C) for 20 minutes, 100 μl of the supernatant was charged.

IAA: the same kind of enzyme preparation was incubated in one additional volume of idoacetamide (20mM in 0.1M phosphate buffer pH 8.8) at room temperature for 30 minutes. Then 100 μl of the incubated mixture (50 μg enzyme) was charged.

Pi: Control of the IAA treatment containing 50 μg enzyme.
with some modification by incubating 2 ml of the diluted heat treated or untreated enzyme preparations at 30°C with 2 ml of 1% soluble potato starch in 10 mM Ca-acetate pH 4.8. An aliquot of 0.5 ml was taken at 0, 5, and 10 min and mixed with 0.5 ml of 1% 3,5-dinitrosalicylic acid. The mixture was boiled for five minutes and its absorbance was read at 575 nm against a reagent blank. Glucose and maltose were used as the standards of the enzyme product.

Electrophoretic analysis

Polyacrylamide gel disc electrophoresis (PAGDE) of enzyme preparations: Isozymes was separated by the method of Davis (1964) using 7% polyacrylamide gel. An aliquot of 50-100 μl of the enzyme extract was charged onto each tube. Electrophoresis was conducted at 4°C, and a direct current of 2 mA was applied to each gel tube for 15 minutes to concentrate the protein bands, then the current was increased to 5 mA per gel for approximately two hours or 30 minutes longer than the time required for the dye marker to reach the lower tube end.

Detection of total starch hydrolytic enzymes: To detect electrophoretically separated amylolytic enzyme bands, several buffer systems for incubation were compared, all containing 1% soluble starch in (a) 0.1 M Ca-acetate pH 5.6 (Briggs 1972), (b) 0.02 M Na-phosphate pH 6.5 (Juliano and Varner 1969), or (c) 0.05 M tris-maleate pH 5.6 (Juliano and
Varner 1969). It was found that the tris-maleate buffer resulted in the same isozyme pattern as the phosphate system, however, the bands were sharper than that of the phosphate incubation. The Ca-acetate system did not reveal all the bands that the other two systems did. Therefore, the tris-maleate buffer was used throughout the study for the gel incubation in detecting amylolytic enzymes.

After electrophoresis the gels were incubated in 1% soluble starch in 0.05M tris-maleate pH 6.5 for one hour at room temperature (26°C) with occasional stirring. After incubation the gels were rinsed with distilled water, and stained with an iodine solution (0.5% I₂ in 1% KI) for 30 seconds. The stained gel was rinsed with distilled water and immersed in 2% acetic acid for immediate photographing. The clear enzyme bands on a blue background disappeared rapidly in acetic acid. The enzyme banding pattern on the gel, however, can be retained for 3-4 days in a filter paper lined chamber moistened with I₂-KI solution.

Detection of α-amylases on PEGDE separated gel: The specific substrate of α-amylase, β-limit dextrin (βLD), was prepared from waxy-maize amylopectin (AMAIZO American Maize-products Co. Hammond, Indiana) using pure sweet potato - amylose (Sigma Chemical Co.) by the method of Kruger (1972). The prepared βLD (0.4% in 4mM acetate buffer pH 4.6) was then incorporated into a 1% agar slab for the detection of α-amylases by Kruger's method (1972) with some modification. A 4% agar solution was made into 5 ml of βLD solution, and
this mixture was added with rapid stirring into 15 ml of boiling LD solution. The agar slab was molded on a glass plate that was previously coated with a dried thin film of 1% egg albumin as an adhesive. The prepared slab gel was stored in a moist chamber and used within three hours of preparation. Old slabs resulted in faint background and diffused bands.

To detect the isozymes of α-amylase, the electrophoresed gel was cut in half with a very thin steel wire, moistured with 0.2M Na-acetate pH 5.3, and placed on βLD-agar slab for 30 minutes to one hour at room temperature (26°C) in a moist chamber. After the incubation, the PEGDE half gel was removed and the slab was stained with an iodine solution (0.5% I₂ in 1% KI), rinsed with water and 2% acetic acid for immediate photographing.

**Determination of soluble protein content in the enzyme preparation**

The protein content of the enzyme preparation was estimated by the rapid Bradford method (1976). The reagent is composed of 0.01% Coomassie brilliant blue G-250 in 4.75% ethanol and 8.5% phosphoric acid solution. To estimate the content of protein in enzyme preparation, two replications of 0.1 ml enzyme extract were mixed with 3 ml reagent and then the absorbance of the mixture was read at 595 nm against a water blank. After corrected for reagent blank, the A₅₉₅ of samples was converted to µg of proteins based on a
standard curve of Bovine Serum Albumin.

**Starch determination**

The residue of enzyme extraction as stated in enzyme preparation was suspended in 10 ml of 85% ethanol and the mixture was filtered with the aid of vacuum through a Watman No. 1 filter paper. The residue was dried at 80°C for 24 hours. The sugar free residue was refluxed in 20 ml 0.2N H$_2$SO$_4$ for one hour (Smith *et al.*, 1964). The hot mixture was filtered through Watman No. 42 filter paper. The sugar content in the filtrate was determined by the anthrone method (Yemme and Willis, 1954) and the starch was calculated using a correction factor of 0.9.
RESULTS AND DISCUSSION

The experimental results will be summarized as maturation and germination studies in which the changes in weight, starch content, enzymatic activity and electrophoretic patterns will be discussed separately.

MATURATION

A. Changes in seed weight and water content

The data of kernel fresh weight, dry weight (Figure 2), and water content (Figure 3) indicated that both the Maris-Hobbit and Stephens cultivars became completely ripened at 56-DAA. At 42-DAA, seeds of both cultivars reached maximum kernel fresh weight of 100mg/seed and 95 mg/seed, respectively. Water content was 40% and 24%, respectively. At 56-DAA, seeds of both cultivars had lowest water content of 9% and 12%, and highest kernel dry weight of 54 mg/seed and 58 mg/seed, respectively. This pattern complies with the general developmental changes in other seeds (MacGregor et al., 1971).

B. Starch content

The data in Figure 4 shows that during the early stages of seeds development, the starch content in the pericarp of these two cultivars decreased sharply. Between 14- and 28-DAA, the rate of starch decrease in pericarp was more than 50% per week. This indicates the pericarp's functional role
Fig. 2 Changes in seed fresh weight (---) and dry weight (-----) of Maris-Hobbit (*) and Stephens (x) during maturation.
Fig. 3 Changes in water content of Maris-Hobbit (•) and Stephens (×) during maturation.
Fig. 4 Changes in starch content in the endosperm (—) and pericarp (-----) of Maris-Hobbit (•) and Stephens (x) at various stages of maturation.
as the temporary depot of photosynthate which is mainly synthesized in the green parts of the plant, e.g. leaf, stem, awn etc. As the starch synthesizing system was developed in the endosperm 28-DAA, the starch content in the pericarp was maintained at a constant level as the transporting site of photosynthate. Endosperm starch content increased gradually for two weeks, and then rapidly for the following two weeks to reach a maximum at 42-DAA. At the full maturity, Stephens had 9% more starch than Maris-Hobbit.

C. Amylolytic enzyme activity and electrophoretic pattern of maturing kernel

At earlier stages of kernel development, 14, 21, 28, 35, 42 and 49-DAA, each seed was dissected to pericarp, "seed coat and aleurone layer", endosperm, and embryo in order to determine the distribution of amylolytic enzymes in anatomical parts of the seed. Table 1 summarizes the quantitative changes in total amylolytic activity in different parts of the maturing grain of the two cultivars. The data indicated that most of the amylolytic enzymes were located in the pericarp at the very early stage. For example, 14-DAA, 86% of the amylolytic activity of the Maris-Hobbit was located in the pericarp. At later stages, a tremendous increase in enzyme activity and distribution was observed in the endosperm. At 56-DAA a maximum total activity of 7 and 8 mg maltose produced per seed per minute was obtained in Maris-
Table 1. The quantitative distribution of total amylase activity in various parts of maturing seed of Maris-Hobbit and Stephens cultivars.

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<th>DAA*</th>
<th>Parts</th>
<th>Maris-Hobbit</th>
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<th>Stephens</th>
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<tr>
<td></td>
<td></td>
<td>Dry wt.</td>
<td>Total act.** % of total</td>
<td>Dry wt.</td>
<td>Total act.** % of total</td>
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* DAA: Days after anthesis.
** Total activity: ug maltose produced/seed.min.
Table 1. Continue.

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<td><strong>% of total</strong></td>
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* DAA: Days after anthesis.
** Total activity: ug maltose produced/seed.min.
Hobbit and Stephens, respectively. This rate amounts to 50% of the maximum rate of the germinating seed. What is the function of these starch degrading enzymes in a starch assimilating tissue remains to be explored in future research.

The data in Figures 5-20 summarizes the quantitative and qualitative changes in amylolytic enzymes in different parts of the grain during maturation.

A rapid increase in total activity (unheated extract) of amylolytic enzymes in endosperm during maturation of both cultivars was observed (Figure 5), reached a maximum activity of 7-8 mg maltose/seed.min at 56-DAA when the seeds became completely matured, and then decreased somewhat. Even in one year old dry-stored seeds, a total activity of 3.4 and 5.3 mg maltose/seed.min was found for Maris-Hobbit and Stephens, respectively (Figure 28). The enzyme activity of 60\textdegree-extract increased sharply at 42-DAA, reached a maximum activity of 0.475 mg maltose/seed.min at 49-DAA that amounted to 7% of the total enzyme activity of the endosperm and then decreased during the following weeks. There was little enzyme activity in 70\textdegree-extract, indicating the lack of active \(\alpha\)-amylase during maturation. However, using \(\beta\)LD as substrate and longer incubation time the presence of low \(\alpha\)-amylase was shown in Figure 10 and 11. It is apparent that the major amylolytic enzymes in maturing endosperm were heat labile (the activity of unheated minus 60\textdegree-extract), among which \(\beta\)-amylase, phosphorylase, glucosidase, transglucosidase,
Fig. 5 Changes in total activity of amylolytic enzyme in endosperm of Maris-Hobbit (•) and Stephens (x) at various stages of maturation.

- - - - : unheated enzyme extract.
- - - - -: 60°C heated enzyme extract.
- - - - - - -: 70°C heated enzyme extract.
Fig. 6 Changes in specific activity of amylolytic enzyme in endosperm of Maris-Hobbit (*) and Stephens (x) at various stages of maturation.

---: unheated enzyme extract.
----: 60°C heated enzyme extract.
······: 70°C heated enzyme extract.
etc. (Briggs, 1972) are the likely candidates. Based on the electrophoretic pattern in Figures 7-9, the major one probably was β-amylase, as it exhibited the highest activity (band E1, 2, 3, 4).

The data in Figure 6 indicate that the changes in the specific activity of amylolytic enzymes in endosperm of both cultivars had the same trend as the changes in total activity (Figure 5), except Maris-Hobbit had a small peak at 21-DAA in 60°C-extract and another one in the 70°C-extract at 42-DAA. Both of which, however, were transitory.

The changes in amylolytic enzyme patterns in maturing endosperm of both cultivars are shown in Figure 7-11. The zymogram of unheated-extract composed of a total of sixteen bands in Maris-Hobbit and 15 bands in Stephens are shown in Figure 7. The fastest moving band, band E1, was visible on the gel even after 70°C treatment (Figure 8, 9), though the intensity was much reduced. This band was not observable on the LD slab (Figure 10, 11). Therefore it is more likely to be an extremely active β-amylase which was not completely inhibited by the high temperature treatment, or a heat stable one as found in maturing soybean (Adams et al., 1979). The second & third fast moving bands, band E2 & E3, might be debranching enzyme or heat-stable α-amylase, since they were present in 60°C-extract (Figure 8, 9) but not in 70°C-extract (Figure 9).

Band E4 appeared to be β-amylase since it did not hydrolyze SLD, and it did not resist to the heat and not
Fig. 7 Diagram of isozyme patterns of amylolytic enzymes in endosperm of cultivar Maris-Hobbit and Stephens at various stages of maturity (unheated enzyme extract).

- XXXX: highest intensity.
- XXX: medium intensity.
- ===: minor intensity.
Fig. 8 Diagram of isozyme patterns of amylolytic enzymes in endosperm of cultivar Maris-Hobbit at various stages of maturity (60°C and 70°C heated enzyme extract).

60°C: 60°C heated enzyme extract.
70°C: 70°C heated enzyme extract.

xxxx: highest intensity.
xxxx: medium intensity.
xxxx: minor intensity.

(1.5 seed in 100 ul of grinding buffer was charged on the polyacrylamide gel)
Fig. 9 Diagram of isozyme patterns of amylolytic enzymes in endosperm of cultivar Stephens at various stages of maturity (60°C and 70°C heated enzyme extract).

60°C: 60°C heated enzyme extract.
70°C: 70°C heated enzyme extract.

××××: highest intensity.
×××××: medium intensity.
×××: minor intensity.

(1.5 seed in 100 ul of grinding buffer was charged on the polyacrylamide gel)
Fig. 10  Diagram of isozyme patterns on 2-D slab gel of \( \alpha \)-amylase in pericarp (P), testa (T), aleurone (A), and endosperm (E) of cultivar Maris-Hobbit at various stages of maturity (unheated enzyme extract). (1.5 seed in 100 ul of grinding buffer was charged on the polyacrylamide gel)
Fig. 11 Diagram of isozyme patterns on βLD-slab gel of α-amylase in pericarp (P), "testa and aleurone layers" (T), endosperm (E) of cultivar Stephens at various stages of maturity (unheated enzyme extract).

(1.5 seed in 100 ul of grinding buffer was charged on the polyacrylamide gel)
survive the 60°C treatment. Band E5, E6 and E7 were present on βLD slab and in 70°C-extract (Figure 10); thus they are α-amylase. Bands E8, E12-E16 were present in 60°C-extract (Figure 8, 9) and they did not hydrolyse βLD (Figure 10 & 11); therefore they might be heat stable β-amylase.

Bands E9, E10 and E11 were pink in color when soluble starch was used as substrate, presented in 60°C-extract and not shown in βLD slab; thus they might be phosphorylase or debranching enzyme.

The developmental changes of these isozymes (Figure 7) indicated that bands E1, E2, E3 and E4 were formed at the very early stage of kernel development. The intensity of bands E1 and E2 first increased at 14-DAA, and bands E3 and E4 increased rapidly in the following week. At 21-DAA, three pink bands, bands E9, E10 and E11 formed, increased their intensity at the following stages of maturation, and decreased slightly at full maturity. Bands E8, E12-E16 were formed at 28-DAA, and their intensity increased during maturation. Bands E5, E6 and E7 appeared in the middle stage of maturation. As full maturity approached, the level of these α-amylases decreased.

As shown in Figure 12, the total amylolytic activity of unheated extract in the pericarp was high at 14-DAA, reached a maximum at 21-DAA, and then decreased rapidly. The major enzyme activity is the heat labile one (the difference between unheated & 60°C-extract), and the enzyme
Fig. 12 Changes in amylolytic activity in pericarp of Maris-Hobbit (*) and Stephens (x) during maturation.

- - - - - : unheated enzyme extract.
---: 60°C heated enzyme extract.
........: 70°C heated enzyme extract.
Fig. 13 Changes in specific activity of amylolytic enzyme in pericarp of Maris-Hobbit (*) and Stephens (x) at various stages of maturation.

- - - - : unheated enzyme extract.
---- : 60°C heated enzyme extract.
-------- : 70°C heated enzyme extract.
might be β-amylase. The activity difference between 60°C & 70°C heat treatment was not significant, indicating that there was no debranching enzyme or 60°C stable --70°C labile enzyme in the pericarp. Probably the heat-resistant ones were α-amylase type. The enzyme activity peak also coincided with the rapid rate of starch reduction in pericarp (Figure 3). The total activity of heated extracts increased slightly at 21-DAA to 10-15 µg maltose/seed.min, and then decreased to 5 µg maltose/seed.min.

The specific activity in pericarp was shown in Figure 13, the pattern of which is similar to total activity except that the peak was delayed somewhat.

The zymogram of pericarp was very clear and shown in Figure 14, 15 and 16. The three faster moving bands, P1, P2 and P3 appeared to be β-amylase as they were not present in heat extracts and βLD-slabs (Figure 10 and 11). Based on their high intensity at all stages, they are the consistent major enzymes. Bands P4, P5 and P6 of both cultivars appeared very early in the kernel development, increased at 28-35-DAA, and then decreased as the kernel ripened. Heat treatment and the positive results on the βLD slab indicated that these three bands are α-amylase, and they have the same mobility as the α-amylase, E5, E6 and E7, in the endosperm.

The activity of P1 and P2 was very high at early stages of maturation, and then decreased during the remaining period of maturation. The activity of P3 increased continuously to 42-DAA, then decreased somewhat during maturation. The
Fig. 14 Diagram of isozyme patterns of amylolytic enzymes in pericarp (P) and "testa and aleurone layer" (T) of cultivar Maris-Hobbit and Stephens at various stages of maturity (unheated enzyme extract).

- : highest intensity.
- : medium intensity.
- : lowest intensity.

(1.5 seed in 100 ul of grinding buffer was charged on the polyacrylamide gel)
Fig. 15 Diagram of isozyme patterns of amylolytic enzymes in pericarp (P) and "testa and aleurone layer" (T) of cultivar Stephens at various stages of maturity (60°C and 70°C heated enzyme extract).
60°C: 60°C heated enzyme extract.
70°C: 70°C heated enzyme extract.
(1.5 seed in 100 ul of grinding buffer was charged on the polyacrylamide gel)
Days after anthesis

Fig. 16  Diagram of isozyme patterns of amylolytic enzymes in pericarp (P) and "testa and aleurone layers" (T) of cultivar Maris-Hobbit at various stages of maturity (60°C and 70°C heated enzyme extract). 60°C: 60°C heated enzyme extract. 70°C: 70°C heated enzyme extract. (1.5 seed in 100 ul of grinding buffer was charged on the polyacrylamide gel)
intensity of P1, 2 & 3 bands (Figure 14) paralleled the total quantitative assay results (Figure 12). The intensity of α-amylase P4, 5 & 6 varied a little during maturation, further indicating that the β-amylase (P1, 2 & 3) is the major amylolytic enzymes in the pericarp. These results support the conclusion of Meredith and Jenkins (1973) that the pericarp has true α-amylase and heatlabile β-amylase. According to Kruger (1972), the major β-amylase in maturing wheat, β-2 and β-3, were found largely in the endosperm, with only a small amount present in the pericarp. The results of this study complied with his finding. The fact that most of the β-amylase in immature wheat seeds resided in the endosperm, whereas the bulk of the α-amylase though low in activity (Figure 12) is in the pericarp (Figure 14), also confirms the previous results of Sandstedt and Beckord (1946).

Even though the pericarp β-amylase, P1, P2 and P3, has similar mobility to the endosperm β-amylase, E1, E2, E3 and E4, the pericarp β-amylases were very sensitive to 60°C heat treatment (Figure 16), while endosperm β-amylase was more heat resistant (Figure 9). Therefore, the β-amylases in pericarp possibly are different from that of the endosperm.

Changes in total activity and specific activity of amylolytic enzyme of the seed coat and aleurone layer are shown in Figure 17 and 18. The enzyme activity of unheated
extract of both cultivars increased during the early stage of kernel development, while the 60°-extract and 70°-extract showed little or no enzyme activity. These data indicated that β-amylase is the major amylolytic enzyme in testa and aleurone layer, and there was no detectable debranching enzyme and α-amylase.

In the electrophoretic pattern shown in Figure 14, 15 and 16, three very faint α-amylase bands (T5, 6 & 7) were detected in the testa and aleurone layer. The intensity of the α-amylase bands did not change during maturation. Just as in the endosperm of the very early stage of maturation, there were four β-amylases in the testa and aleurone layer (T1, 2, 3 & 4). The activity of T1 and T2 increased rapidly during maturation, while T3 and T4 remained about constant. When the testa and aleurone layer was separated and analyzed, the rapid increase of T1 & T2 occurred only in the aleurone layer and not in the testa (Figure 19). The isozyme pattern of 60° and 70°-extract further indicated that three weak α-amylase isozymes (T5, 6 & 7) were in the testa and aleurone layer as in unheated extract. There was one extra faint band still left after 70°C heat treatment, which was assumed to be heat stable β-amylase as found in maturing soybean (Adam et al., 1979).

Very little or no amylolytic activity was found in the embryo at different stage; therefore no quantitative data could be shown. Only three or four minor bands were detected
Fig. 17 Changes in amylase activity in "testa and aleurone layer" of Maris-Hobbit (•) and Stephens (×) at various stages of maturation.

---: unheated enzyme extract.

-----: 60°C heated enzyme extract.

······: 70°C heated enzyme extract.
Fig. 18 Changes in specific activity of amylolytic enzyme in seed coat and aleurone layers of Maris-Hobbit (•) and Stephens (x) at various stages of maturation.

--- : unheated enzyme extract.
---- : 60°C heated enzyme extract.
***** : 70°C heated enzyme extract.
Fig. 19  Diagram of isozyme patterns of amylolytic enzymes in testa (T) and aleurone (A) of cultivar Maris-Hobbit at various stages of maturity (unheated enzyme extract).

(1.5 seed in 100 μl of grinding buffer was charged on the polyacrylamide gel)
by electrophoresis in unheated-extract (Figure 20). These bands probably were \( \beta \)-amylase by their Rms and their absence on \( \beta LD \)-slabs.

The function of these amylolytic enzymes is of interest since the maturing grain is actively accumulating starch. Perhaps they are engaged in increasing the amount of primer for starch synthetase to build larger molecules at early stages (Fekete and Viewey, 1974). Later than 49 days, starch accumulation was slowed down (Figure 4) while the activity of amylolytic enzymes still remained high. This is difficult to explain. It may be that they were in an inactive complex form with protein body (Briggs 1972) or with starch grain (Okamoto & Akazawa 1979), and upon extraction they were relieved and expressed in \textit{in vitro} assay as shown in this study.

D. Soluble protein content in maturing grains

The changes of soluble protein content were shown in Figures 21 and 22. The soluble protein content in unheated extract of endosperm increased rapidly, and reached a maximum at 35 to 42-DAA. This result is similar to the results of MacGregor \textit{et al.} on maturing barley (1971). These soluble proteins not only composed of enzyme and various factors, but also include newly synthesized storage proteins which eventually will became insoluble and be deposited in protein bodies (Bewley, 1978). In the 60\( ^\circ \) and 70\( ^\circ \)-extracts,
Fig. 20  Diagram of isozyme patterns of amylolytic enzymes in embryo of cultivar Maris-Hobbit and Stephens at various stages of maturity (unheated enzyme extract).
(1.5 seed in 100 ul of grinding buffer was charged on the polyacrylamide gel)
Fig. 21 Changes in soluble protein content in endosperm of Maris-Hobbit (•) and Stephens (×) during matur maturity.

- - - - : unheated enzyme extract.
--- --- : 60°C heated enzyme extract.
---------- : 70°C heated enzyme extract.
the protein content increased gradually during maturation. These heat stable proteins again may be composed of many kinds, and the peak observed between 42 and 56-DAA may reflect the activity of 60°-stable β-amylases and debranching enzymes (Figure 5).

The soluble protein of unheat-extract in pericarp (Figure 22) decreased continuously during maturation, while the heated ones varied somewhat at different stages.

The soluble protein content in the testa and aleurone a layer is shown in Figure 23. It increased from 14-DAA, reached a maximum at 21-DAA, and then decreased probably to the end of maturation. It was impossible to separate the testa and aleurone layers from the endosperm after 35-DAA. Even though aleurone grains usually increase with maturation, the soluble protein will decrease with drying as the seed ripens.

GERMINATION

A. Changes in seedling weight and water content

Changes in fresh weight, dry weight, and water content of one embryo or seedling axis from both cultivars was shown in Figures 24 to 26. For the first three days, the weight increased slowly and then rapidly all the way up to endosperm exhaustion, 12 days of germination. It also showed that the weight of cultivar Stephens was slightly higher
Fig. 22 Changes in soluble protein content in pericarp of Maris-Hobbit (•) and Stephens (×) during maturation.

- : unheated enzyme extract.
- - - : 60°C heated enzyme extract.
- - - - : 70°C heated enzyme extract.
Fig. 23 Changes in soluble protein content in testa and aleurone layer of Maris-Hobbit (●) and Stephens (×) during maturation.

- - - - - : unheated enzyme extract.
- - - - - : 60°C heated enzyme extract.
- - - - - : 70°C heated enzyme extract.
Fig. 24 Changes in fresh weight of one embryo or seedling axis from Maris-Hobbit (*) and Stephens (×) wheat seeds during germination at 20°C.
Fig. 25 Changes in dry weight of one embryo or seedling axis from Maris-Hobbit (•) and Stephens (x) wheat seeds during germination at 20°C.
Fig. 26 Changes in water content of embryo or seedling axis from Maris-Hobbit (•) and Stephens (×) wheat seeds during germination at 20°C.
than that of the Maris-Hobbit. The difference between these
two cultivars may be related to the total reserve (Figure 4),
the seed weight of Maris-Hobbit and Stepnens was 41, 52 mg/
seed, respectively.

The pattern of seedling weight changes during germina-
tion of these two cultivars was similar to the results of
Ching and Rynd on Yamhill wheat seed (1978).

The water content increased rapidly in the embryo of
germinating seeds (Figure 27) and the peak of 92% was
reached on 5th day of germination. This pattern paralleled
with the germination of other cereals.

B. Changes in starch content in endosperm

The data in Figure 27 show the changes in starch
content in endosperm during germination. For the first
three days the starch content decreased slowly, then
decreased rapidly to nine days of germination, and then
slowed down. The peak reduction coincides with high
amylolytic activity (Figure 28). Apparently the starch
reserve in endosperm provides the carbon backbones and
substrate for energy required by the rapid growth of seed-
ling during germination. The starch reserve in the
endosperm of Stephens was about 10% higher than that of
Maris-Hobbit, which is reflected in their weight difference.
The changes in starch content of germinating seed was
similar to the changes in rice seeds (Murata et al. 1968).
Fig. 27 Changes in starch content in the endosperm of Maris-Hobbit (*) and Stephens (x) during germination.
C. Amylolytic enzyme activity and electrophoretic pattern of germinating seed

Starch hydrolytic enzymes were analyzed during the germination of these two cultivars and the results are shown in Figures 28-31. A rapid increase in total activity in unheated extract during germination was observed (Figure 28). Maximum activity was reached at 6-7 days of germination, after which activity decreased rapidly. As a whole, the amylolytic enzyme in unheated extract of Stephens seems to have higher activity than Maris-Hobbit at early stages of germination, and decreased earlier than Maris-Hobbit at later stages. The enzyme activity of 60°-extract also increased rapidly at early stages, reached a maximum of 50% of unheated-extract at six days of germination, and then decreased. The amylolytic activity of 70°-extract (α-amylase) had a similar trend to the unheated one, except the peak amounted to only 10% of the unheated one.

The changes in amylolytic isozyme patterns in germinating seeds were shown in Figure 29, 30 and 31. In the germinating seed, there were a total of 18 bands in endosperm extract of Maris-Hobbit and 19 bands in that of Stephens. Band G2, G3, G17 and G18 were only visible on PAGDE gel of unheated extract and not in heated extracts or on βLD slab. Therefore these four seem to be β-amylase isozymes. Since bands G15 and G16 were 60°-stable and 70°-labile enzyme, and they did not show in βLD slab, probably
Fig. 28 Changes in total activity of amylolytic enzyme in endosperm of Maris-Hobbit (•) and Stephens (x) wheat seeds germinated at 20°C.

- - - - - : unheated enzyme extract.
- - - - - - : 60°C heated enzyme extract.
- - - - - - - - : 70°C heated enzyme extract.
they are debranching enzyme or heat stable α-amylase, as is found in maturing soybean (Adam, 1979). Bands G4-8, G10, G12-14 and G19 were visible in 70°-extract and on LD slab, and thus they must be α-amylase. Bands G7, G9 and G11 were 60°-stable and 70° labile enzyme with similar mobility of E9, E10 and E11 of pink bands of maturing seed described in the section dealing with maturation, therefore they might be phosphorylase or debranching enzyme. The fastest moving band, G1, although visible on PAGDE gel after 70°C heat-treatment, was much reduced in intensity compared to the unheated and 60°-extracts, and it was not observed on LD slab. Thus, it may be heat stable β-amylase as mentioned before.

In the 1-day-germinated seed, the zymogram was similar to that in mature seed (Figure 7). Two major bands, G2 and G3, were prominent. Four minor bands of β-amylase, G15-18, and three minor pink bands G7, G9 and G11 were observed. All these minor bands disappeared after three days of germination. Three α-amylase bands, G4-G6, were detectable in the 1-day material with G4 having higher activity than the other two. All these three α-amylases increased in activity by their intensity with germination time (Figure 30 & 31). On the second day of germination, a new slower moving β-amylase, G3, was observed, with increased activity with time during germination, and then decreased. The two pre-existing β-amylases, G1 and G2, disappeared with time and
Fig. 29  Diagram of isozyme patterns of amylolytic enzymes in endosperm of cultivar Maris-Hobbit and Stephens during germination (unheated extract).

- - - - - - : highest intensity.
- - - - - - : medium intensity.
- - - - - - : minor intensity.
Fig. 30  Diagram of isozyme patterns of amylolytic enzymes in endosperm of cultivar Maris-Hobbit and Stephens during germination (60°C heated enzyme extract).

- - - - - - : highest intensity.
- - - - - - - : medium intensity.
- - - - - : minor intensity.
(0.1 seed in 100 ul grinding buffer was charged on the polyacrylamide gel)
Fig. 31 Diagram of isozyme patterns of amylolytic enzymes in endosperm of cultivar Maris-Hobbit and Stephens during germination (70°C heated enzyme extract).

\[\text{Zzzi-E} : \text{highest intensity.}\]
\[\text{nNnNn} : \text{medium intensity.}\]
\[\text{---} : \text{minor intensity.}\]

(0.1 seed in 100 ul grinding buffer was charged on the polyacrylamide gel)
were hardly visible after seven days of germination. The G3 may be released from its complex with protein body as there is usually no new synthesis of $\beta$-amylase during germination (Hardie, 1975). From PAGDE gel of 70°-extract or $\beta$LD slab, it was found that after two days germination $\alpha$-amylase, G7, G9, G11-14 in both cultivars and G-0 in Stephens were synthesized, which increased in activity with time to seven days of germination, and then decreased.

Based on the data in Figure 28, the major enzymes in endosperm on the first day of germination were heat labile, such as $\beta$-amylase, debranching enzyme, phosphorylase etc. These enzymes appeared to be pre-formed in endosperm during maturation (Briggs, 1972). Additional isozymes of $\alpha$-amylase were synthesized during germination as shown th the zymograms (Figure 30 & 31). At later stages, though the intensity of $\alpha$-amylase bands were intensified, and appeared to be the major amylolytic enzyme on the PAGDE gel with germination (Figure 29 & 31), it seems that $\beta$-amylases, the activity difference between unheated and 60°-extract as shown in Figure 28 was the major enzyme in endosperm during germination of these two cultivars. The possible discrepancy between these two detection systems may be attributed to the assay products analyzed and the length of incubation. For the Bernfeld method used for Figure 28, the reducing sugar is quantified, while the intensity of electrophoretic bands was based on the amount of starch-I$_2$ blue complex left over
Fig. 32 Diagram of isozyme patterns on αLD-slab gel of α-amylase in endosperm of cultivar Maris-Hobbit during germination.

- UH₁: unheated enzyme extract.
- 60°C: 60°C heated enzyme extract.
- 70°C: 70°C heated enzyme extract.

(0.1 seed in 100 μl grinding buffer was charged on the polyacrylamide gel)
Fig. 33 Diagram of isozyme patterns on βLD-slab gel of α-amylase in endosperm of cultivar Stephens during germination.

UH: unheated enzyme extract.
60°C: 60°C heated enzyme extract.
70°C: 70°C heated enzyme extract.

(0.1 seed in 100 ul grinding buffer was charged on the polyacrylamide gel)
after enzyme action. Thus gel detection method is not absolutely quantitative, and often favors the products of \( \alpha \)-amylase. As Ching mentioned (1972), "Better clarity and precision on properties and control mechanism of individual enzymes and their catalytic activities could be obtained by studying purified enzyme rather than the total soluble enzyme extracts of the tissue".

The specific activity of amylolytic enzymes in endosperm during germination was shown in Figure 34. The general pattern follows that of the total activity indicating true increase and fall of these enzymes during germination.

D. Soluble protein content in germinating seeds

The soluble protein content in the unheated extract of endosperm increased rapidly, reached a maximum at seven days of germination, and then decreased sharply (Figure 35). These changes were typical of germinating cereals such as Zea mays (Ingle et al., 1964). The extracted soluble protein consists mainly of enzymes, solubilized storage protein, and protein factors for various metabolic processes of the endosperm. Therefore the rise and fall of the soluble proteins is related to the catabolic activity of the endosperm. In the 60\(^{\circ}\)- and 70\(^{\circ}\)-extracts, a similar pattern to that of unheated extract was observed, but at a much lower level due to heat denaturation. Besides \( \beta \)-amylase and
Fig. 34 Changes in specific activity of amylolytic enzyme in endosperm of Maris-Hobbit (•) and Stephens (×) wheat seeds germinated at 20°C.

--- : unheated enzyme extract.
--- : 60°C heated enzyme extract.
--- : 70°C heated enzyme extract.
debranching enzymes which are known to be heat resistant (Kruger, 1972), other heat stable soluble proteins are difficult to discern.

The presence of maturing $\alpha$-amylase (E5, 6 and 7) at early germination (G4, 5 and 6) indicates the possibility that these enzymes can be activated by rain to initiate the degradation of starch molecules in the endosperm. Then the latent or preexisting $\beta$-amylase (E1, 2 and or G1 and G2) continues the hydrolysis on the products of the $\alpha$-amylase and thus furthers the degradation of starch quality. Since little quantitative or qualitative differences were observed between these two cultivars in their amylolytic enzymes, the difference in their susceptibility of rain damage would have to be attributed to the seed dormancy or special glume characteristics in Maris-Hobbit.

The data of germination test in Table 2 indicated that Maris-Hobbit has seed dormancy while Stephens does not. However, the relationship between the seed dormancy and the amylolytic enzymes involved is not absolutely established. Further experiment is required to explore the mechanism of resistance in pre-harvest sprouting damage in wheat cultivars.
Fig. 35 Changes in soluble protein in endosperm of Maris-Hobbit (*) and Stephens (x) wheat seeds germinated at 20°C.

- - - : unheated enzyme extract.
- - - - : 60°C heated enzyme extract.
- - - - - : 70°C heated enzyme extract.
Table 2  Accumulated germination percentage ± standard deviation of Maris-Hobbit and Stephens wheat seed. Seeds were collected on Aug. 3, 1979 and stored in air tight container in cold room to preserve seed dormancy. On Aug. 16, 1979, four replications of 100 seeds were germinated on filter paper at 25°C in the dark.

<table>
<thead>
<tr>
<th>Days of Germination</th>
<th>Germination percentage (%. Mean±SD)</th>
<th>Maris-Hobbit</th>
<th>Stephens</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>8 ± 4</td>
<td>29 ± 2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>16 ± 7</td>
<td>62 ± 5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>26 ± 5</td>
<td>81 ± 8</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>36 ± 2</td>
<td>93 ± 3</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>43 ± 1</td>
<td>96 ± 2</td>
<td></td>
</tr>
</tbody>
</table>
SUMMARY AND CONCLUSIONS

Samples of two wheat cultivars, Maris-Hobbit and Stephens, were collected at regular intervals during kernel maturation. Fresh weight, dry weight, water content, starch content, the activity of different amylolytic enzymes and isozyme patterns were determined during maturation and germination.

From the experimental data it was found that the amylolytic activity was high during maturation, which amounted to 50% of that during germination (Figure 5 & 28). The zymogram of unheated extract at later stages of maturation, composed of fifteen bands in Stephens or sixteen bands in Maris-Hobbit, is shown in Figure 7. Three α-amylases and three phosphorylases or debranching enzymes with pink dextrin-I₂ as product were observed. Nine α-amylases in Stephens or ten in Maris-Hobbit were found, most of which were 60°C heat stable.

It is known that amylolytic enzyme is present in the pericarp to hydrolyze the temporary stored starch in that tissue; however, this is the first time a quantitative distribution of these enzymes in different parts of wheat grain during maturation has been recorded. Six amylolytic isozymes, three α-amylases and three β-amylases, were observed in the pericarp at early stages of maturation (Figure 14). The finding of these three β-amylases is
different from the results of Krugers (1972) who observed one minor $\beta$-amylase band in the pericarp of cultivar Marquis and Prairie Pride. Four $\beta$-amylases and three minor $\alpha$-amylases were observed in testa and aleurone layer with $\beta$-amylase being the major ones. No amylolytic activity was detected in embryo extract. However, three or four minor bands were observed in its zymogram upon longer incubation in substrate.

On the first day of germination, the enzyme pattern was similar to that of mature seed (Figure 7-29). Most of the bands which existed in the later stages of maturation were visible. On the second day of germination, five or six $\alpha$-amylase bands and one $\beta$-amylase were synthesized and they increased in intensity or quantity with time of germination. At the peak of starch degradation, on seventh day of germination, a total of ten bands were found in Maris-Hobbit and 11 bands in Stephens.

The presence of maturing $\alpha$-amylase at early germination indicates the possibility that these enzymes can be activated by rain to initiate the degradation of starch molecules. Then the latent $\beta$-amylase continues the hydrolysis on the products of the $\alpha$-amylase, and thus furthers the degradation of starch quality. Since little quantitative and qualitative differences were observed between these two cultivars on their amylolytic enzymes, the difference in their
susceptibility to rain damage would have to attribute to the seed dormancy or other quality in Maris-Hobbit.
BIBLIOGRAPHY


