

# Changes in Peroxidase Activity and Peroxidase Isozyme Patterns of Wheat during Kernel Growth and Maturation<sup>1</sup>

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## ABSTRACT

Peroxidase activities in whole and dissected parts of hard red spring and amber durum wheat kernels were determined at various stages of kernel growth and maturation. *o*-Dianisidine was used as hydrogen donor for the assay. Peroxidase activity was formed early in kernel development and resided largely in the pericarp and green layers. With further kernel development, these enzymes decreased and peroxidases in the other tissues slowly appeared. Polyacrylamide-slab electrophoresis with 3-amino-9-ethyl carbazole as hydrogen donor indicated that up to 12 peroxidase enzymes were present in immature wheat kernels and were located in different anatomical parts of the kernel. Changes in intensities of the various isozymes throughout development reflected quantitative changes in peroxidase in the various tissues.

Changes in biochemical components, particularly of enzymes, during the growth and maturation of different wheat varieties are currently being investigated in this laboratory. Such changes may be related to the final quality of various wheat varieties. Although the peroxidase enzyme systems of sound, mature wheats have been studied recently, there is little information available relating to changes in these systems during the growth and maturation of such wheats. Honold and Stahmann (1) and Evans and Mechem (2) have investigated the peroxidases and peroxidase isozymes in wheat mill fractions, as these enzymes may have a role in protein polymerization, and, as a consequence, may modify dough properties. Kobrehel et al. (3) recently obtained a high correlation between macaroni brownness and peroxidase activity for various durum semolinas. The only study reported on quantitative changes in peroxidase during wheat grain development was in 1926 by Bach et al. (4). The peroxidase enzymes increased irregularly, reached a maximum, and then decreased with further ripening.

The present study was undertaken, therefore, to obtain more information concerning the changes that occur in the peroxidase enzyme system of wheat during growth and development, and, in particular, the changes that occur in individual tissues of the developing kernel. Samples of a hard red spring wheat, Manitou, and a durum wheat, Hercules, were analyzed at various stages of kernel development for quantitative changes in peroxidase activity, using *o*-dianisidine as the hydrogen donor. Changes in peroxidase isozymes, as well as the anatomical distribution of the isozymes, were also determined by polyacrylamide-slab electrophoresis, using 3-amino-9-ethyl carbazole as hydrogen donor.

## METHODS AND MATERIALS

Manitou and Hercules wheats were planted on May 10, 1972, at the University of Manitoba experimental plots, Winnipeg, Manitoba. Manitou flowered on July 4, and Hercules on July 10. The varieties were sampled thereafter at 2- to 3-day intervals. At each sampling date, excised heads were stored intact in a deep freeze prior to analysis for peroxidase.

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### Dissection Techniques

Dissections were performed similar to those described previously for barley (5). The tissues, in order of removal during dissection, were as follows: pericarp, or outer epidermis of the ovary wall; green layer, a layer of chlorenchyma cells and underlying translucent testa layer, which together comprise the integuments of the ovule; embryo and scutellum; aleurone and endosperm. At later stages of kernel development, the aleurone and endosperm tended to fuse and were separated by scraping the endosperm tissues from the inside layer of the aleurone with a scalpel. The aleurone tissue was then immersed in droplets of water and scraped carefully to remove final traces of adhering endosperm material.

### Extraction of Peroxidase Isozymes

Five kernels of wheat or different tissues dissected from five kernels of wheat were ground using a mortar and pestle with 2 ml. of 12.5% sucrose solution. The suspensions were then centrifuged at  $25,000 \times g$  for 10 min., and the clear extracts were used for quantitative assays. For isozyme studies of tissues dissected from kernels, 30 seeds were dissected, and the tissues were extracted with 1.5 ml. of 12.5% sucrose solution. To facilitate detection of peroxidase isozymes in the embryo and scutellum during early stages of kernel development, these tissues were dissected from an additional 20 kernels and added to the previous samples prior to extraction with sucrose.

### Moisture

Duplicate 1-g. samples of kernels from freshly harvested wheat were analyzed for moisture by the AACC vacuum-oven method (6).

### Enzyme Activity

Activity was determined by a method similar to that described in the *Worthington Enzyme Manual* (7) with *o*-dianisidine as hydrogen donor. Substrate solution was prepared by mixing 40 ml. of 0.09% hydrogen peroxide in 0.05M phosphate buffer, pH 6.5, with 20 ml. of 1% *o*-dianisidine in 20% methyl alcohol. The substrate solution was diluted to 100 ml. with 0.05M phosphate buffer, pH 6.5. To 2.5 ml. of substrate solution was added 0.5 ml. enzyme or diluted enzyme solution at zero time; the reaction was monitored at 500 nm. at 25°C. for 5 min. on a Unicam SP-1800 ultraviolet spectrophotometer equipped with a recorder. One unit of peroxidase activity was defined as that amount of enzyme that consumed 1  $\mu$ mole of peroxide per min. at 25°C.

### Polyacrylamide-Slab Electrophoresis

Electrophoresis was performed at pH 4.75 by the method of MacGregor and Meredith (8), as described previously (9). An Ortec Model 4200 slab-electrophoresis system was used, and 12 samples could be run simultaneously. In one experiment, slab, disc electrophoresis at pH 8.9 was used (10).

### Detection of Peroxidase Isozymes

In a previous paper (9), various hydrogen donors were assessed for their ability to detect peroxidase. Of the five hydrogen donors tested, namely, catechol, guaiacol, benzidine, *o*-dianisidine, 3-amino-9-ethyl carbazole, the latter was found to produce the sharpest isozyme bands for peroxidase isozymes from various barley cultivars. A

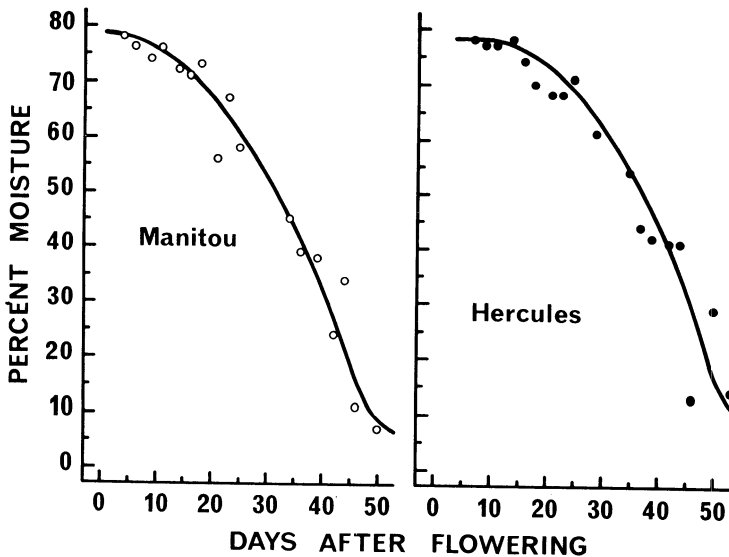


Fig. 1. Changes in moisture of Manitou (left) and Hercules (right) wheat kernels during growth and maturation.

similar result was found in the present study for wheat peroxidases. Consequently, 0.02M 3-amino-9-ethyl carbazole was used as substrate (11). The substrate was dissolved in 0.05M acetate buffer, pH 4.5, and contained 0.1% hydrogen peroxide so that immediate visualization of isozyme band formation could be observed over the period of enzyme incubation with substrate and donor.

## RESULTS AND DISCUSSION

### Peroxidase Activity in Developing Kernels of Manitou and Hercules Wheat

Kernels of Manitou and Hercules were sampled following flowering, and moisture content was used as an indication of kernel maturity (Fig. 1).

Total peroxidase and changes in anatomical distribution of peroxidase were determined on these samples, and the results were plotted in terms of units per kernel (Fig. 2). Total peroxidase in the Manitou kernels rose rapidly from day 10 after flowering, reached a maximum of 3.5 units at day 30, and then decreased thereafter to full maturity. Similar changes were noted in Hercules; peroxidase activity increased very rapidly from day 15 after flowering, quickly reached a maximum of 3 to 3.5 units at day 22, and then decreased, although not as rapidly nor to the same extent as in Manitou. The largest amount of peroxidase activity during very early growth occurred in the pericarp, and the rise and fall in peroxidase activity in this tissue mirrored that of the whole kernel for both varieties. Activity in the green layer also rose and fell in a similar manner during development, although the amount of activity in this tissue was lower. Peroxidase activity in the other tissues was very low in early stages of kernel maturity, but increased during development with the largest increase occurring in the endosperm.

It was interesting to compare peroxidase activities of the various tissues of the

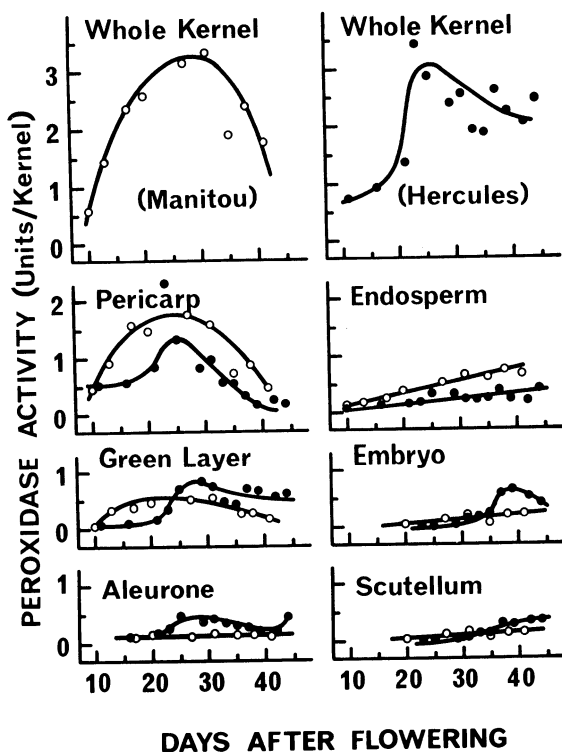


Fig. 2. Total and anatomical distribution of peroxidase activity during growth and maturation of Manitou (○) and Hercules (●) wheat kernels.

kernel on a percentage basis (Fig. 3) as changes in distribution of activities during development and differences between the two varieties were magnified. The decreasing contribution of the pericarp peroxidase throughout development was particularly marked. Whereas in early development this tissue contained over 75% of the activity, this decreased to about 20 and 10%, respectively, in Manitou and Hercules at full maturity. Small varietal differences in percentage of peroxidase activity contributed by the green layer, aleurone, scutellum, and embryo were observed, but the most noticeable change was found in the endosperm. Whereas the peroxidase activity in the endosperm from Hercules increased from 8% at day 10 after flowering to 15%, peroxidase activity in endosperm from Manitou increased from 17 to 40%.

#### Peroxidase Isozymes in Manitou and Hercules Wheat and Their Anatomical Distribution

Kernels of Manitou and Hercules at 35 and 28 days after flowering, respectively, were dissected into pericarp, green layer, endosperm, aleurone, scutellum, and embryo. These tissues, as well as the whole seeds, were extracted with sucrose. Peroxidase isozymes were separated by slab, disc electrophoresis at acid pH, and stained with 3-amino-9-ethyl carbazole. Immature kernels had 11 to

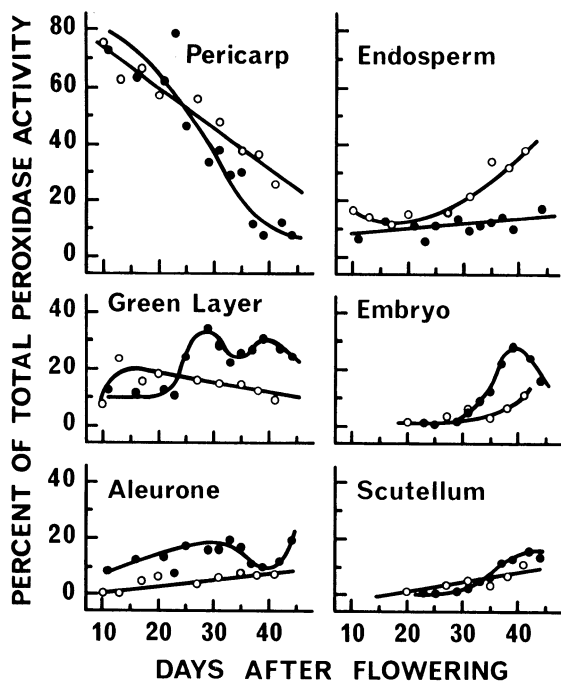


Fig. 3. Percentage distribution of peroxidase activity in anatomical parts of Manitou (○) and Hercules (●) wheat kernels during growth and maturation.

12 peroxidase isozymes (Fig. 4) and the various anatomical tissues contained different peroxidase isozymes. Comparison of the peroxidase isozymes in terms of  $R_f$  values, as shown in Table I, indicated that the individual isozymes found in similar anatomical tissues of both varieties had the same relative mobilities. The endosperm contained two bands of high mobility (bands 11 and 12). These bands appeared as one band because of similar mobilities and high intensity of staining. Although not clearly visible from Figure 4, endosperm tissue also contained two minor peroxidases (bands 7 and 9). Band 7 stained more intensely in the Hercules than in the Manitou. Peroxidase isozymes of high mobility were found in the scutellum tissue (band 10) and in embryo tissue (band 9). Both isozymes had extremely similar mobilities and could be satisfactorily discerned from one another only when individual tissue sections were compared. The embryo also contained an additional minor isozyme (band 6). Very intense peroxidase isozymes (bands 1 and 2) were observed in the aleurone tissue of Hercules but were not detected in the green layer of the Hercules wheat, along with four or five less intense bands of increased mobility. The green-layer tissue from Manitou contained only one of the two intense components (band 2) at this stage of kernel development. As will be shown later, band 1 is also present in Manitou but has disappeared by 27 days after flowering. Three minor peroxidase isozyme bands were also present in the Manitou. The pericarp contained four isozymes with only one of moderate intensity (band

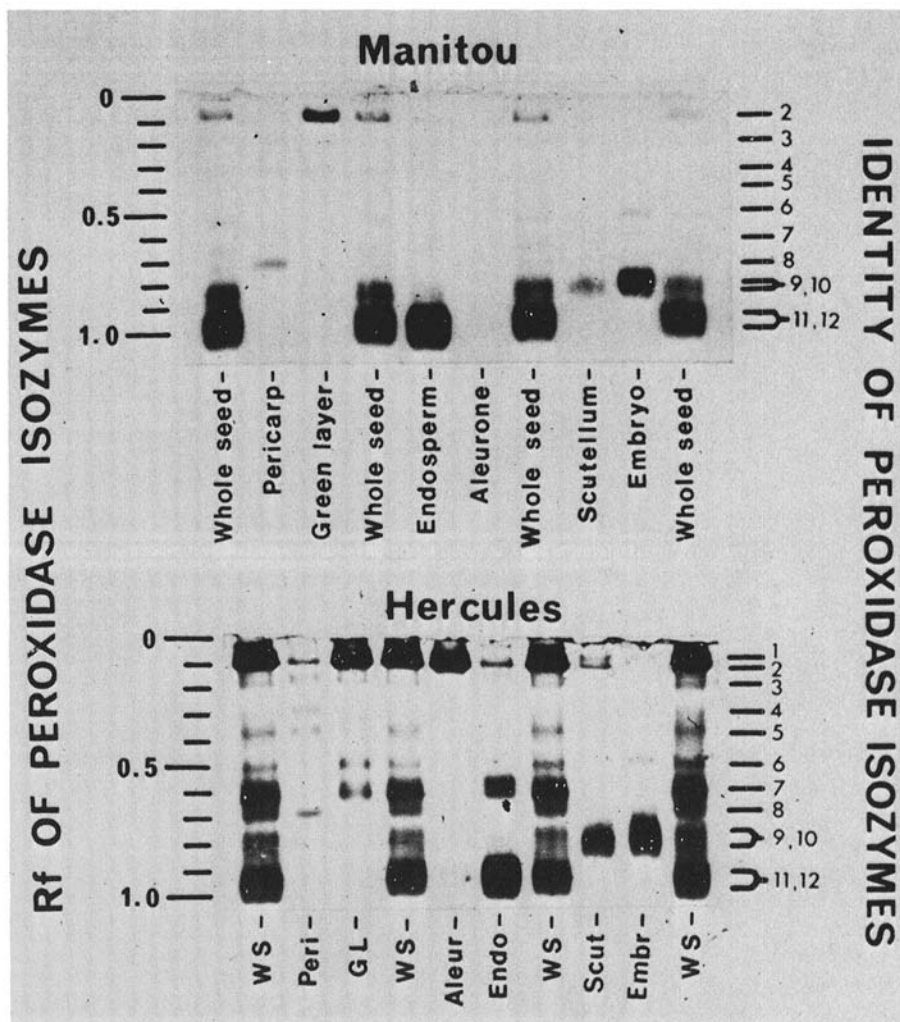


Fig. 4. Peroxidase isozymes in whole and anatomical parts of Manitou (top) and Hercules (bottom) kernels at 35 and 28 days, respectively, after flowering.

8). Hercules contained a fifth component (band 2) but this band may be due to slight contamination of pericarp tissue with tissue from the green layer. This would also explain the minor bands 1 and 2 that are present in the scutellum and embryo of the Hercules but not the Manitou.

#### Changes in Peroxidase Isozymes during Growth and Maturation

Kernels of Manitou at 12 stages of growth were extracted and the extracts were submitted to electrophoresis at basic and acid pH. The resolution and clarity of bands were much better on acid gels (Fig. 5B) than on alkaline gels (Fig. 5A) using 3-amino-9-ethyl carbazole as hydrogen donor. Changes in the intensity of number

TABLE I.  $R_f$  VALUES FOR PEROXIDASE ISOZYMES OF WHOLE SEEDS AND KERNEL DISSECTIONS<sup>a</sup>

| Isozyme | Whole Seed |      | Pericarp    |             | Green Layer |                          | Endosperm   |             | Aleurone |             | Scutellum   |      | Embryo      |             |
|---------|------------|------|-------------|-------------|-------------|--------------------------|-------------|-------------|----------|-------------|-------------|------|-------------|-------------|
|         | M          | H    | M           | H           | M           | H                        | M           | H           | M        | H           | M           | H    | M           | H           |
| 1       | ...        | 0.05 | ...         | ...         | ...         | <u>0.05</u> <sup>b</sup> | ...         | ...         | ...      | <u>0.05</u> | ...         | 0.05 | ...         | 0.05        |
| 2       | 0.07       | 0.09 | ...         | 0.09        | <u>0.07</u> | <u>0.09</u>              | ...         | 0.09        | ...      | <u>0.09</u> | ...         | 0.09 | ...         | 0.09        |
| 3       | 0.18       | 0.17 | 0.16        | ...         | 0.17        | 0.17                     | ...         | ...         | ...      | ...         | ...         | ...  | ...         | ...         |
| 4       | 0.31       | 0.29 | 0.29        | 0.28        | 0.29        | 0.28                     | ...         | ...         | ...      | ...         | ...         | ...  | ...         | ...         |
| 5       | 0.36       | 0.36 | 0.35        | 0.36        | ...         | 0.35                     | ...         | ...         | ...      | ...         | ...         | ...  | ...         | ...         |
| 6       | 0.52       | 0.51 | ...         | ...         | 0.52        | 0.50                     | ...         | ...         | ...      | ...         | ...         | ...  | 0.50        | 0.49        |
| 7       | 0.63       | 0.61 | ...         | ...         | ...         | 0.61                     | 0.63        | <u>0.59</u> | ...      | ...         | ...         | ...  | ...         | ...         |
| 8       | 0.70       | 0.69 | <u>0.68</u> | <u>0.69</u> | ...         | ...                      | ...         | ...         | ...      | ...         | ...         | ...  | ...         | ...         |
| 9       | 0.81       | 0.81 | ...         | ...         | ...         | ...                      | 0.80        | 0.81        | ...      | ...         | ...         | ...  | <u>0.82</u> | <u>0.80</u> |
| 10      | 0.84       | 0.83 | ...         | ...         | ...         | ...                      | ...         | ...         | ...      | <u>0.85</u> | <u>0.82</u> | ...  | ...         | ...         |
| 11      | 0.94       | 0.95 | ...         | ...         | ...         | ...                      | <u>0.93</u> | <u>0.93</u> | 0.97     | 0.98        | ...         | ...  | ...         | ...         |
| 12      | 1.00       | 1.00 | ...         | ...         | ...         | ...                      | <u>1.00</u> | <u>1.00</u> | 1.00     | ...         | ...         | ...  | ...         | ...         |

<sup>a</sup>M = Manitou; H = Hercules;  $R_f$  = mobility of peroxidase isozyme/mobility of fastest-moving peroxidase isozyme.

<sup>b</sup>Underlined isozymes refer to intensely stained bands in Fig. 4.

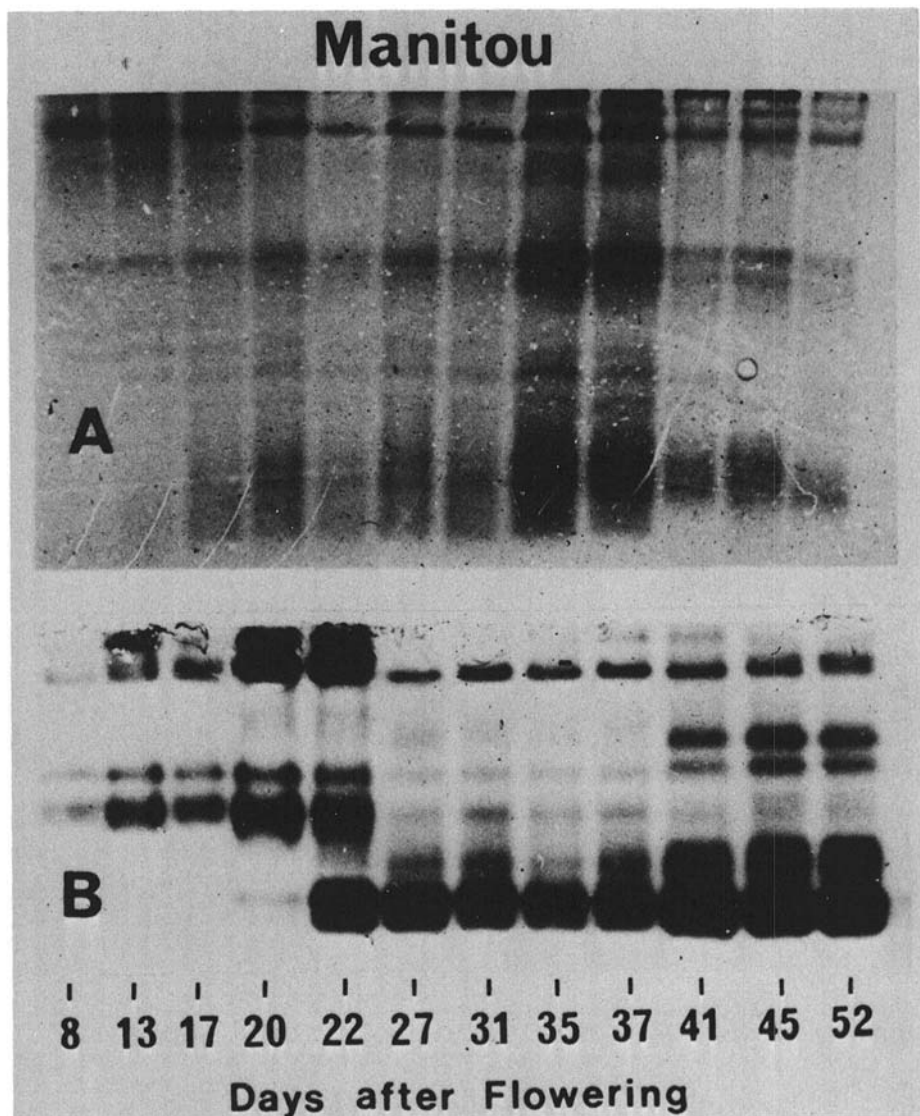


Fig. 5. Changes in peroxidase isozymes in Manitou wheat kernels during growth and maturation. A, electrophoresis at pH 8.9; B, electrophoresis at pH 4.75.

of bands occurred throughout growth. These changes, observed in Fig. 5B, were related to the changes in quantitative amounts of peroxidases in the various tissues throughout growth as previously shown in Fig. 2. Thus, during early kernel growth, the prominent bands were those attributable to the large amount of activity in the pericarp and green layers (bands 1, 2, 7, and 8 in Fig. 4). As the kernel developed and the peroxidase activity in these tissues decreased, there was also a decrease in the intensity of these isozymes. The electrophoretically slowest isozyme (band 1 in



Fig. 4) disappeared completely from kernels harvested 27 days after flowering. At the same time, in kernels harvested 22 days after flowering, there was an increase in intensity in the electrophoretically fastest-moving bands. These bands, by comparison with Fig. 4, were peroxidase isozymes present in the endosperm, scutellum, and embryo (bands 11, 12, 10, and 9). This was expected as the peroxidase activities in these tissues increased throughout growth and reached maximum values at full maturation.

### GENERAL DISCUSSION AND CONCLUSIONS

Peroxidase enzymes are present in the developing wheat kernel from a very early stage of maturation through to final harvest ripeness. In very early kernel development, peroxidase activity resides largely in the pericarp and green layer. The physiological reason for the presence of peroxidase in these tissues is not known with certainty. Suggested roles for these enzymes include oxidation of certain toxic phenols and amines which may be deleterious to the plant under certain circumstances (12), and elimination of hydrogen peroxide from plant tissues. It is known, however, that large metabolic changes are taking place in the pericarp and green layer tissues at this time, and other enzyme systems such as amylases (13) and proteases (14,15) are also present in substantial amounts, and then later decrease with increasing kernel maturity. As the kernel develops, peroxidase activity in the aleurone, endosperm, scutellum, and embryo slowly appears and continues to increase until full maturity. Since peroxidase enzymes are present in the endosperm at a time when the storage proteins are being laid down, it is interesting to speculate whether these enzymes can have an influence on such proteins and ultimately the quality of a variety.

Electrophoresis on slabs of polyacrylamide at acid pH indicates that the peroxidase in immature wheat kernels exists in multiple forms. These isozymes are located in different anatomical parts of the kernel, and variations in intensities of the bands during development reflect changes in quantitative amounts of the peroxidase activity in various tissues. During early growth, the peroxidase bands formed in the pericarp and green layer are dominant. As kernel development proceeds, these isozymes diminish in intensity at the same time that bands present in the endosperm, embryo, and scutellum increase. These latter isozymes consequently constitute the bands of greatest intensity at full maturity.

A comparison of the number of isozymes found at full maturity in the present study with those of other workers is difficult because of the widely varying electrophoretic conditions that have been used. In addition, the particular hydrogen donor used to detect peroxidase can have significant effects upon the resolution and number of peroxidase isozymes found (9). Thus, Honold and Stahmann (1) detected eight isozymes using polyacrylamide-gel electrophoresis and guaiacol as donor. Brad et al. (16) found a maximum of 10 bands by agar-gel electrophoresis, using benzidine as substrate. Using starch-gel electrophoresis, Evans and Mecham (2) found three isozymes in the break and reduction flours from common wheat, and an additional fourth component in the shorts and bran.

Determining the anatomical origin of peroxidase isozymes or multiple forms of other enzyme systems in mill fractions is very difficult for it is not possible to completely separate sound kernels of wheat into their component parts for use in

comparisons. By determining the anatomical distribution of wheat isozymes at an early stage in kernel development when tissues are easily separable and following the isozymic changes in these tissues as far into development as possible, however, one is able to estimate the probable isozymic composition in various portions of the mature kernel.

It was found that quantitative changes in peroxidase activity occurred in different parts of the wheat kernel throughout development. It is important, therefore, to realize the importance of kernel maturity in assessing quantitative enzyme activities. Thus, the presence of varying amounts of immature kernels in a wheat to be milled could have an influence on the enzyme activities present in different mill fractions.

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