CHANGES IN THE POLYPHENOL OXIDASES OF WHEAT DURING KERNEL GROWTH AND MATURATION[1]

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ABSTRACT   Cereal Chemistry 53(2): 201-213

Polyphenol oxidase (PPO) activities were determined at various stages in the growth and maturation of hard red spring, soft white spring, and durum wheat kernels, using a number of phenolic substrates. PPO activity was formed early in kernel development and decreased with kernel maturation. Dissection studies indicated that a large part of the PPO in the immature wheat kernel was present in the endosperm. Polyacrylamide-slab electrophoresis of immature kernel extracts followed by addition of gallic acid or catechin indicated that up to 12 PPO isozymes were present. These isozymes were located in different parts of the kernel. Upon germination, PPO activity increased.

The presence of polyphenol oxidase (PPO) in wheat and wheat products has been well established. Thus, as early as 1907, Bertrand and Muttermilch (1) detected the presence of a tyrosinase in bran. Amounts present in sound wheats and in wheat flours appear to be quite low (2), with the bran containing most of the PPO activity (2−5). The enzyme also appears to exist in multiple forms as shown by polyacrylamide gel electrophoresis (6). Recent studies have indicated that high levels of PPO are present in the grain coat of dwarf wheats and have a deleterious effect, causing discoloration in whole-wheat dough and chapatties (7,8).

By contrast, very little information is known concerning the PPO system present in immature kernels and its possible effect on quality. For example, the presence of immature and frozen kernels is a degrading factor in Canadian wheat varieties and such kernels can cause adverse effects such as brownness in semolina. Whether such brownness results from the action of endogenous levels of PPO during processing is not known. A recent study has indicated that developing kernels from Indian wheats contain a high level of PPO activity as detected with catechol substrate (9). In addition, nine to ten isozymes of PPO activity appeared to be present.

The present study was undertaken to examine changes in PPO of different Canadian wheat varieties during kernel growth and maturation. A hard red spring wheat (Park), a soft white spring wheat (Lemhi 62), and a durum wheat (Hercules) were selected for investigation. Immature kernels of wheat at various stages of growth were examined for PPO activity using a number of polyphenolic substrates and for the number of PPO isozymes by polyacrylamide-slab electrophoresis. Dissection studies were also carried out to indicate the anatomical location of the PPO activity and PPO isozymes. In addition, the PPO activity and PPO isozymes of germinated Hercules wheat kernels were examined.

MATERIALS AND METHODS

Park, Hercules, and Lemhi 62 wheats were planted on May 10, 1972, at the University of Manitoba experimental plots, Winnipeg. Flowering dates were July 7, 1972, for Park and Lemhi 62, and July 10, 1972, for Hercules. 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 PPO. For germination studies, Park and Hercules were germinated under conditions as described previously (10) except that samples were stored frozen prior to analysis. Dissection of immature wheat kernels was performed as described previously (11,12).

Gallic acid, catechol, and pyrogallol were purchased from Aldrich Chemical Co., Milwaukee, Wis., and d-catechin, caffeic acid, and L-β-3,4-dihydroxphenylanine (L-DOPA) from Sigma Chemical Co., St. Louis, Mo.
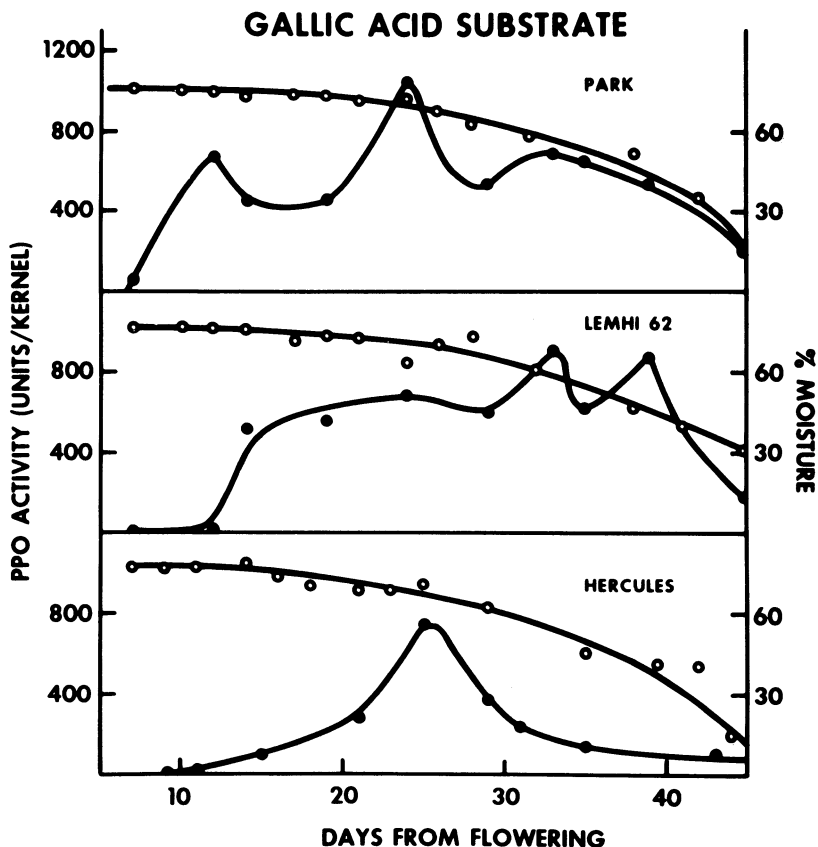

Fig. 1. Changes in PPO during kernel growth and maturation of Park, Lemhi 62, and Hercules wheat. The substrate used was gallic acid. O = per cent moisture; ● = PPO activity.

Extraction of PPO
    Twenty kernels of wheat, or tissues dissected from 20 kernels of wheat, were ground in a mortar and pestle with 8 ml of distilled water and then centrifuged at 25,000 × g for 10 min. The clear extracts were used for quantitative assays. For polyacrylamide-slab electrophoresis, the extractant was 12.5% sucrose, and additional dissected tissues were sometimes added before extraction, in the same volume of liquid, to facilitate detection of isozymes.

Moisture
    Duplicate 1-g samples of kernels from freshly harvested wheat were analyzed by the AACC vacuum-oven method (13) for moisture.

Enzyme Activity
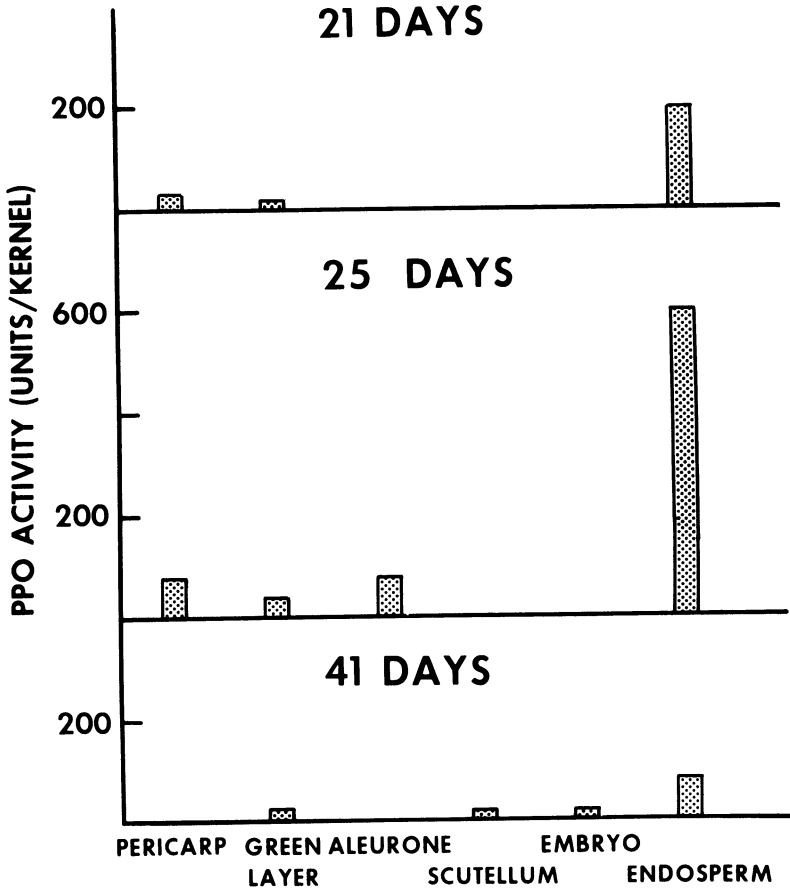   Substrates investigated in this study included gallic acid, L-DOPA, caffeic

Fig. 2. Anatomical distribution of PPO in immature Hercules wheat kernels at 21, 25, and 41 days after flowering. The substrate used was gallic acid.

acid, d-catechin, pyrogallol, and catechol. A $0.01M$ substrate solution was prepared in $0.1M$ McIlvaines citric acid-phosphate buffer, pH 6.8, which had been oxygenated for 5 min. Only 25 ml of substrate solution was prepared at one time and this was used within 10 min. The reaction was initiated by adding 0.2 ml of extract or diluted extract to 2.8 ml of substrate solution at 25° C and following the reaction on a Beckman Model 25 kinetic spectrophotometer equipped with a recorder. The increases in absorbance were measured at 350 nm with gallic acid as substrate and at 410 nm with the other substrates, using a quartz cell with a 1 cm path length. A linear relation between PPO concentration and absorbance change was found with all three substrates, except d-catechin. This substrate had a decreasing reaction rate with time, and initial velocities were estimated, therefore, from the changes in absorbance after the earliest possible reaction time.

A unit of polyphenol oxidase activity was defined as a change in absorbance of 0.001 in 1 min at 25° C.

Polyacrylamide-Slab Electrophoresis and Detection of PPO Isozymes

Electrophoresis was carried out on polyacrylamide slabs at pH 8.9, according to the method of Davis (14) and employing an Ortec Model 4200 slab electrophoresis system as described previously (11). PPO isozymes were detected by incubating slabs with $0.01M$ solutions of polyphenol substrate in $0.1M$ phosphate buffer, pH 6.8, at room temperature. Greenish or yellowish bands against a clear background indicated the location of PPO isozymes.

RESULTS

The reactivity of extracts of immature wheat toward a number of phenolic substrates was initially tested. There was negligible oxidation of tyrosine to o-dihydroxyphenylalanine. The dihydroxyphenols (catechol, L-DOPA, caffeic acid) and the trihydroxy phenols (pyrogallol, gallic acid) exhibited reactivity with conversion to o-quinone reaction products. Catechin, a flavonoid, was also quite reactive, although the rate of reaction decreased rapidly with time.

It was found in all cases that the reactivity of di- or trihydroxyphenols increased with increase in age of substrate. This increase was particularly noticeable with catechol, d-catechin, pyrogallol, and gallic acid. In some cases, the reaction rate increased by a factor of two or more with a substrate that had aged for 2 hr. Presumably the o-quinones produced from natural oxidation of the substrate are much more reactive toward attack by enzyme (15). As a consequence, substrate solutions, with the exception of catechol and pyrogallol, were prepared in small batches and used within 10 min throughout the study.

The PPO from immature wheat was particularly reactive toward gallic acid and the reaction rate was linear over a long period of time; therefore, this was the preferred substrate for most of the work in this paper.

Changes in PPO Activity during Kernel Growth and Maturation

Changes in the amounts of PPO activity with gallic acid substrate for Park, Lemhi 62, and Hercules wheats during kernel growth and maturation are shown in Fig. 1. Results are plotted in units per kernel. Kernel moistures are also shown and can be used as an indication of kernel maturity. PPO activity was present in

the immature kernels of all varieties. The enzyme was formed very early in kernel development and decreased as the kernel matured. PPO activity in the Park and Lemhi 62 wheats fluctuated during the growth cycle and maximum activities were greater than in Hercules wheat.

To ascertain the anatomical location of the PPO activity, kernels of Hercules wheat at 21, 25, and 41 days after flowering were dissected into pericarp, green layer, aleurone, scutellum, embryo, and endosperm. Analyses of these tissues for PPO activity were carried out with gallic acid substrate and plotted on a per kernel basis. The major amount of PPO activity in the immature kernel resided in the endosperm (Fig. 2). Furthermore, it was primarily the rise in PPO in this tissue from the 21st day to the 25th day after flowering and the fall from the 25th day to the 41st day after flowering that accounts for the observed fluctuation in the PPO activity of Hercules wheat shown in Fig. 1. With increasing maturation (day 41 after flowering), the level of PPO activity in the embryo and scutellum increased. Small amounts of PPO activity were also observed in the pericarp and green layer of the immature wheat kernel. PPO in the aleurone was found only at 25 days after flowering.

The PPO present in immature wheat was also examined with a number of other polyphenolic substrates. These included d-catechin, L-DOPA, catechol, pyrogallol, and caffeic acid. Because of slight variations in the age of substrate solutions, the results obtained with catechol and pyrogallol may be somewhat approximate, but do reflect changes within a particular variety over the different growth stages.

It was found that the PPO present in immature Park wheat exhibited reactivity toward L-DOPA, d-catechin, and pyrogallol but not toward caffeic acid (Fig. 3). Catechol was not tested.
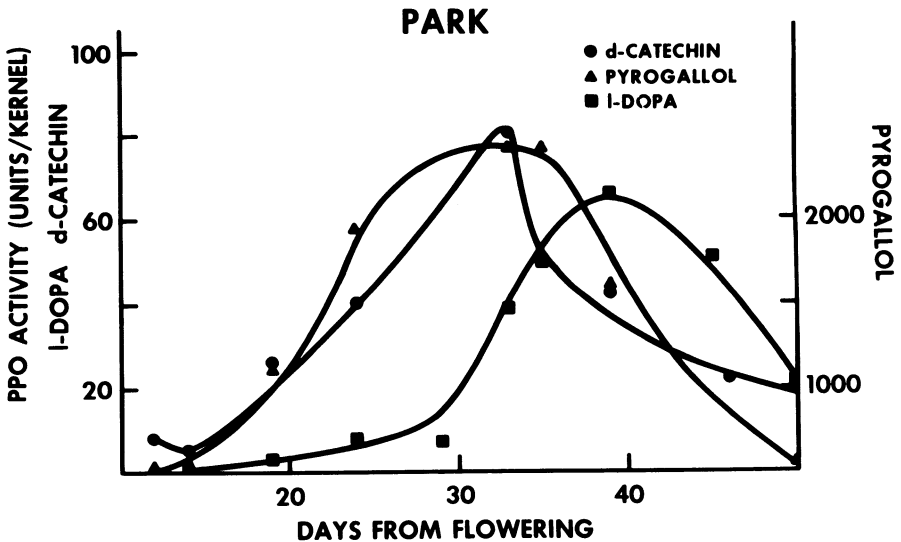

Fig. 3. Changes in PPO during kernel growth and maturation of Park wheat. The substrates used were d-catechin, pyrogallol, and L-DOPA.
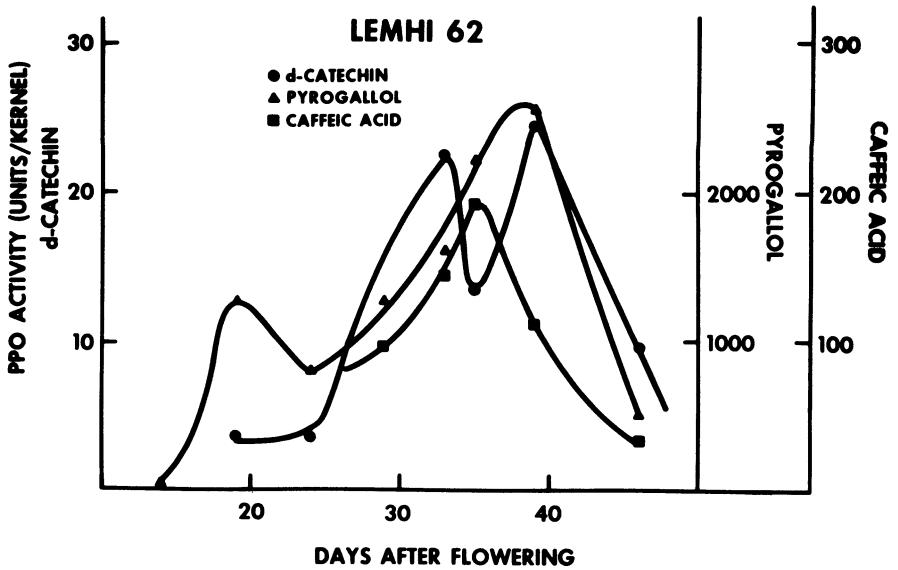
Fig. 4. Changes in PPO during kernel growth and maturation of Lemhi 62 wheat. The substrates used were d-catechin, pyrogallol, and caffeic acid.
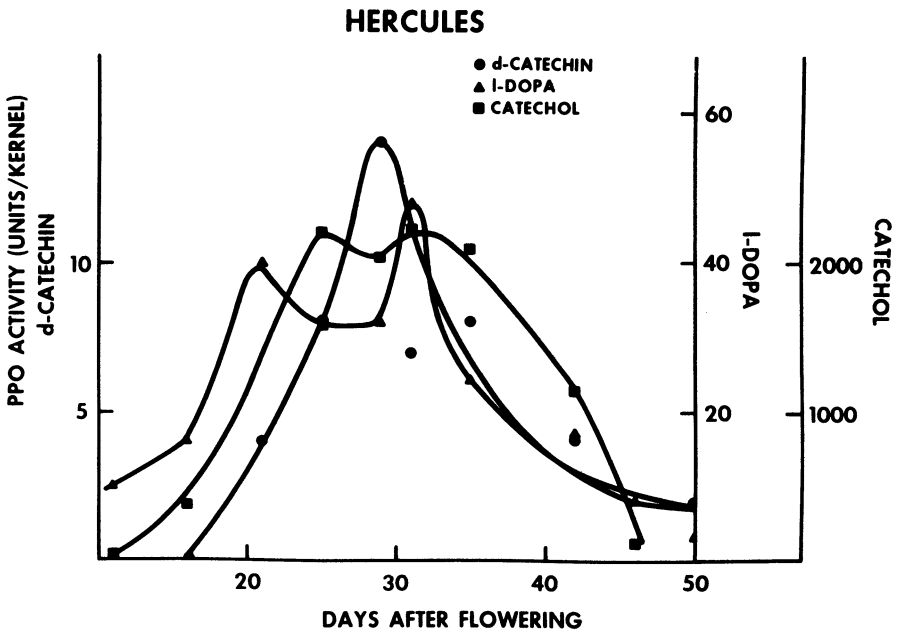

Fig. 5. Changes in PPO during kernel growth and maturation of Hercules wheat. The substrates used were d-catechin, L-DOPA, and catechol.

Immature Lemhi 62 kernels had PPO with reactivity toward d-catechin, pyrogallol, and caffeic acid but not L-DOPA (Fig. 4). Catechol was not tested.

With immature Hercules wheat, the PPO reacted with d-catechin, L-DOPA, and catechol substrates but not with caffeic acid (Fig. 5). Pyrogallol was not tested.

Changes in PPO Isozymes during Growth and Maturation of Wheat

Polyacrylamide-slab electrophoresis of immature wheat extracts, followed by incubation in either gallic acid or d-catechin, was used to detect PPO isozymes. As depicted in Fig. 6, a total of 12 isozymes were observed with widely varying mobilities. The number of bands detected depended on the stage of kernel maturity and the substrate used. Figure 7 shows the PPO isozymes detected at 12 stages in the kernel growth of Hercules wheat with gallic acid as substrate. The most intense bands were 2, 11, and 12. As the kernels matured, a large number of
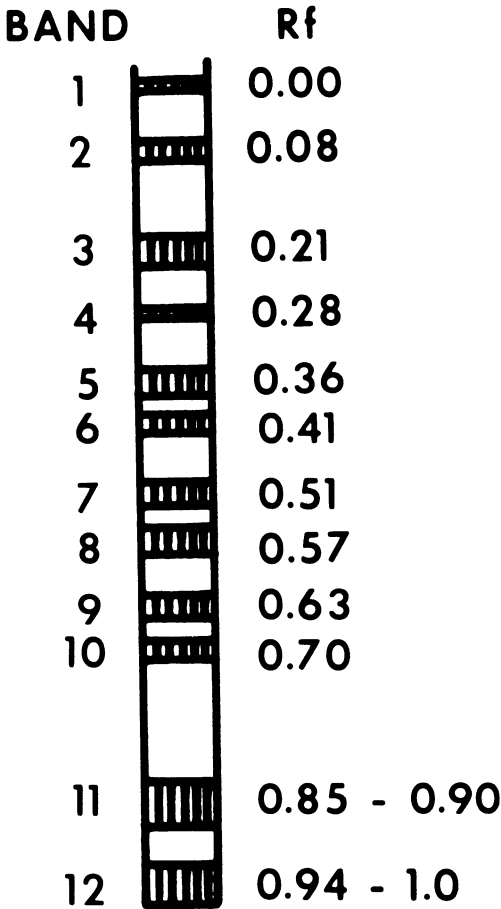

Fig. 6. Composite of all isozymes present in immature Hercules wheat kernels at various stages of maturity. R_f = mobility of PPO isozyme/ mobility of dye front.

the more mobile PPO isozymes disappeared and band 2 was the major band remaining. With d-catechin as substrate (not shown), the observed bands were more diffuse and some differences in isozyme reactivity were observed. Bands 2, 11, and 12 were still the most intense bands but bands 9 and 10 disappeared and bands 7 and 8 were found to be present. As with gallic acid substrate, band 2 was the major band in the near-mature kernels of wheat.

Immature kernels from 12 stages in the growth of Park wheat were examined in the same manner and no appreciable differences in isozyme patterns were observed.

Extracts of dissected tissues from immature Hercules and Park kernels at 25 and 24 days after flowering, respectively, were also examined by polyacrylamide-slab electrophoresis to determine the anatomical locations of individual PPO isozymes. The results obtained with gallic acid as substrate are shown in Fig. 8. Different PPO isozymes were present in different parts of the kernel. Band 1, which does not enter the gel, was found in most tissues, although the amount present varied from tissue to tissue. Band 2 was found predominantly in the outer tissues (pericarp, green layer, and aleurone); bands 5, 6, 9, 10, and 12 in the endosperm; band 11 only in the pericarp; and band 4 faintly detectable in the green layer. No isozymes were found in the embryo or scutellum, with the exception of the faint appearance of band 3 in the scutellum of Park wheat. This was expected as PPO activity develops in these tissues at a later stage of kernel growth.

With d-catechin as substrate, a similar banding pattern for the isozymes was
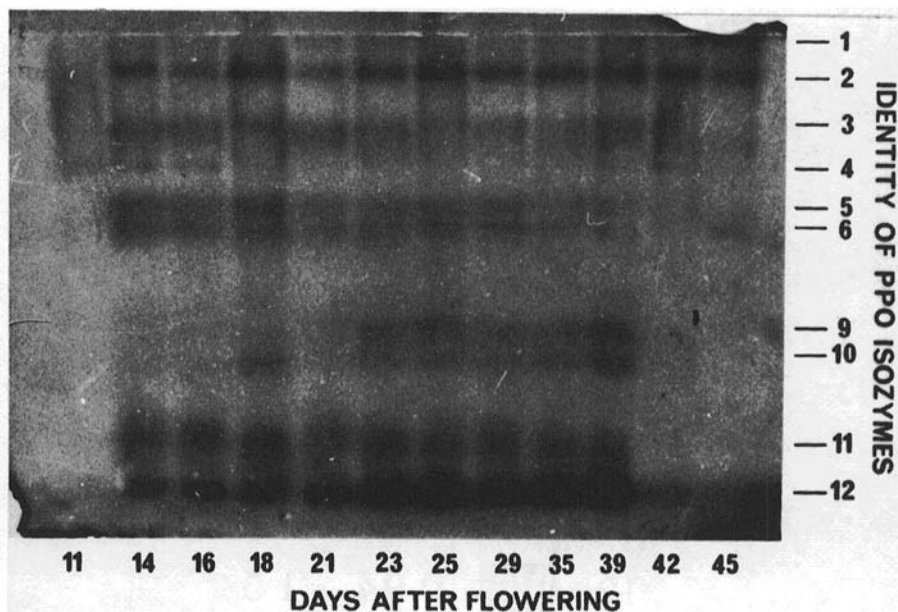

Fig. 7. Changes in PPO isozymes in Hercules wheat kernels during growth and maturation.

observed, with the exception that bands 9 and 10 in the endosperm were absent and bands 7 and 8 were faintly detectable in the embryo and scutellum tissues.

PPO Isozymes of Mature Wheat Varieties

To determine if appreciable varietal differences in PPO isozymes occurred between wheat varieties, 12 sound hard red spring and 11 durum wheats from 1972 Canadian plant breeders' varieties were extracted and submitted to polyacrylamide-slab electrophoresis, followed by gallic acid or d-catechin addition to detect isozymes. Quantitative PPO was determined on a number of the varieties using gallic acid substrate and plotted on a per kernel basis (Table I). In general, it was found that the PPO activities of the hard red spring wheats were higher than those of the durum wheats.

Comparing the isozymes of the varieties, all appeared to have bands 1, 2, and 3 with gallic acid as substrate. As expected, the strong and highly mobile bands 11 and 12 present in the immature kernels were not evident. The only difference found was the existence in some varieties of PPO isozymes, with mobilities corresponding to bands 7 and 8. These bands could be detected better if d-catechin were used as substrate, and they were apparent in the hard red spring wheat varieties, Neepawa and Thatcher, and in two unlicensed varieties, CT 609
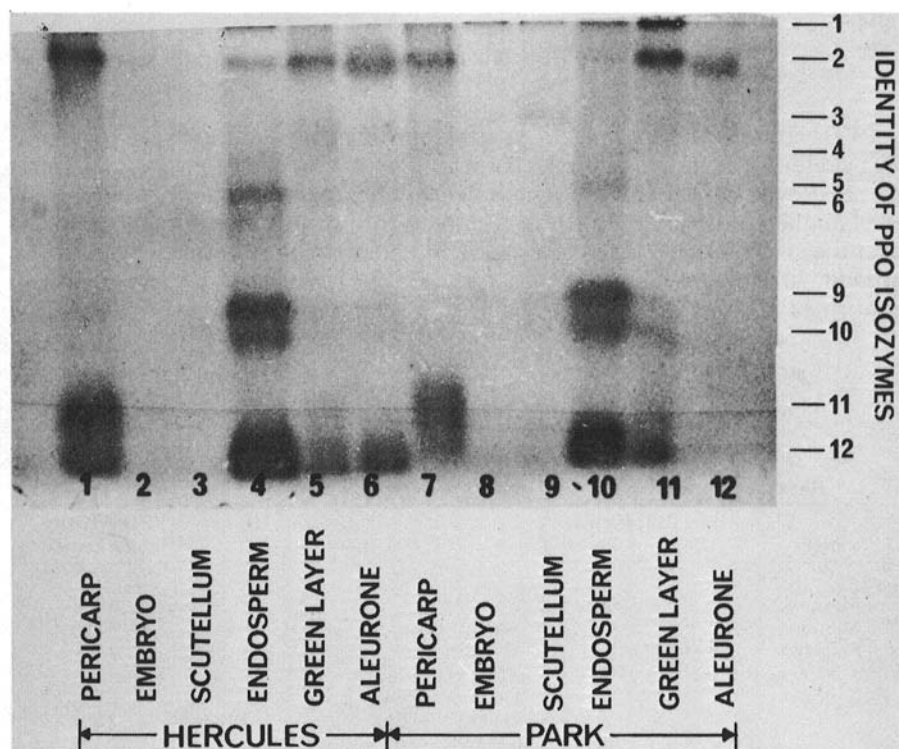

Fig. 8. PPO isozymes in the anatomical parts of Hercules and Park immature wheat kernels.

✕ CT 742 and CT 262 ✕ Manitou. Band 3 was also absent with d-catechin as substrate and illustrated the differences in substrate specificities observed with wheat PPO isozymes. Of the durum wheats analyzed, only Hercules and a nonlicensed variety, DT 182✕ DT 190, contained faint evidence of bands 7 and 8. A number of European and South American wheats were also examined. No differences in electrophoretic mobilities of the isozymes were found and bands 7 and 8 were apparent in only a few of the wheats. No correlation could be found between the presence of bands 7 and 8 and spaghetti brownness.

Changes in PPO during Germination

Upon germination, the enzyme systems in wheat generally undergo drastic changes. To determine what changes occurred in the PPO system of wheat, the variety Hercules was germinated for 7 days. Analysis of the PPO activity with gallic acid substrate indicated (Fig. 9) that the enzyme increased 33-fold up to day 5 and then decreased.

PPO isozymes were also examined for both Hercules and Park wheats, using gallic acid to detect the isozymes. With both varieties, the major isozyme that formed corresponded to band 2 already present in immature and mature wheat kernels. In addition, Hercules contained weaker bands with R_f values of 0.37 and 0.94–1.0 and Park contained weaker bands with R_f values of 0.24, 0.67, 0.74, and 0.94–1.0.

DISCUSSION AND GENERAL CONCLUSIONS

PPO with reactivity toward a number of dihydroxy- and trihydroxyphenols was present in immature wheat kernels. The enzyme developed during early kernel growth, remained throughout development, and then decreased to a low level as the kernel matured. PPO reactivity toward different substrates varied over the growth period and suggested that more than one PPO enzyme might be present in the growing wheat kernel. Polyacrylamide-slab electrophoresis confirmed this, as up to 12 isozymes were present that differed in substrate

TABLE I
Plant Breeders' Varieties of Wheat Analyzed by Polyacrylamide-Slab Electrophoresis

Hard Red Spring Wheat		Durum Wheat	
Variety	PPO Activity units/kernel	Variety	PPO Activity units/kernel
Marquis	78	Mindum	10
Manitou	36	Stewart 63	41
Neepawa	101	Hercules	13
Thatcher	83	Pelissier	16
Cypress	48	D6062 × D6142	29
		DT182 × DT192	16
		[(Yt54 × Norin 10-Brevor) × Ld357] × Tcn² × Lakota	44
		(DT188 × DT224) × DT182	5
		DT182 × DT224	7

reactivities, as well as in relative amounts during development. Different anatomical parts of the kernel also contained different PPO isozymes. Surprisingly, a large part of the PPO in early kernel development was present in the endosperm of the kernel and at a time when large metabolic changes such as protein synthesis and starch deposition were occurring. How the PPO interaction with phenolic compounds might participate in such metabolic events is a matter for conjecture. Phenolic compounds are abundant in plant tissue and one of their main functions may be in the metabolic regulation of red-ox potentials (16,17). PPO attack on these phenols leads to the formation of quinonoid compounds which may possibly interact with the sulfhydryl group of cysteine, with the ε-amino group of lysine and with the α-amino group of amino acids (18–22). Presumably, these addition products would form during the later stages of kernel maturation when dehydration of the kernel and disruption of the organized cell structure occurred. Evidence of undefined bound phenolic compounds in wheat flour and dough have been reported by Gallus and Jennings (23). Ferulic acid and its oxidation product have recently been found in the insoluble pentosan portion of wheat flour (24). It was suggested that a part of the insoluble pentosan could have been formed by oxidation by diferulic acid of the soluble pentosans in the ripening wheat kernel. Such diferulic acid would form by oxidase catalysis of ferulic acid.

As the kernel approached full maturity, the level of PPO in the endosperm dropped and that in the germ and scutellum increased. This would be consistent
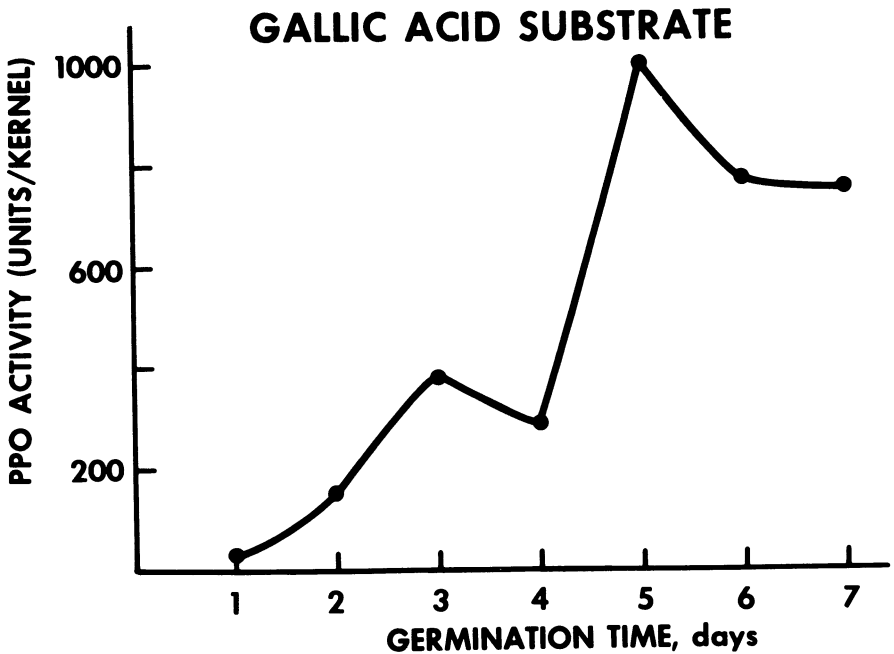

Fig. 9. Change in PPO activity of Hercules wheat during germination. The substrate used was gallic acid.

with findings that the bulk of PPO in mature wheats is located in the bran (2–5).

Comparison of hard red spring, durum, and white spring wheat immature kernels indicated that there were differences in PPO substrate specificity. Gallic acid and d-catechin were suitable substrates for all varieties. L-DOPA, however, was reactive with PPO from the hard red spring wheat, Park, and the durum wheat, Hercules, but not the white spring wheat, Lemhi 62. Park and Lemhi 62, on the other hand, were reactive toward caffeic acid, but Hercules was not. Whether these are general differences between hard red spring, durum, and white spring wheats remains to be established.

The findings reported here are similar to those reported recently by Taneja *et al.* (9) for two Indian varieties of wheat. They found that PPO reactive to catechol was present in developing wheat and decreased as the kernel matured. With L-DOPA as substrate, nine to ten isozymes could be detected on polyacrylamide disc gels at alkaline pH. Because of differences in experimental conditions, it is difficult to ascertain whether such isozymes correspond to the d-catechin and gallic acid isozymes reported in this paper. The electrophoretically mobile L-DOPA isozymes decreased with kernel maturation, however, as did the gallic acid and d-catechin isozymes.

PPO isozymes of a number of mature Canadian hard red spring and durum wheats were examined using gallic acid and d-catechin substrates to determine if inter-varietal differences existed. With both hard red spring and durum wheats, similar isozymes were found. One of the isozymes was reactive toward gallic acid substrate only. It was also found that some mature hard red spring and durum varieties contained two additional isozymes that were more reactive toward d-catechin than gallic acid. With durum wheats, however, no correlation could be found between these isozymes and semolina brownness. With catechol as substrate, electrophoretic differences have been reported recently between the isozymes present in durum and common wheat (6).

Upon germination, PPO activity increased and a major PPO isozyme present in immature and mature wheat kernels increased in intensity. Whether this increase was due to *de novo* synthesis of PPO or reactivation of existing enzymes remains to be determined.

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