

Activity and Inhibition of Polyphenol Oxidase in Extracts of Bran and Other Milling Fractions from a Variety of Wheat Cultivars

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ABSTRACT

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Studies were conducted to compare polyphenol oxidase (PPO) specific activities in various milling fractions of a variety of wheat cultivars and determine the levels of activities in a number of cultivars from different localities and harvesting seasons. Substrate specificities were also investigated. Bran was singled out as the richest source of PPO activity, which may also influence the activity in the other milling fractions that are known to have some proportion of bran content. We showed by gel electrophoresis and spectrophotometrically that the protein responsible for PPO activity apparently exists as a single isoform in bran and that the observed enzyme activity is likely to be a tyrosinase type, not a laccase or peroxidase. The specific activity was not significantly different between the reduction shorts and break shorts from the same cultivar, indicating a

similar level of bran contamination in these fractions. Very low levels of PPO activity were recorded in the flour of all cultivars studied. Bran was used, therefore, to determine the varietal differences in the PPO activities in a number of cultivars from different localities and seasons of harvest. Results showed that the most significant determinant of PPO activity was the genotype, and this may be influenced by seasonality. We also determined that, apart from substrate preferences by the PPO enzyme, some phenolic acids actually inhibit PPO. Furthermore, we found that bran of some cultivars extracted with acidified methanol inhibited PPO activity substantially, whereas other extracts had less inhibitory properties. Thus, these unknown compounds in wheat may inhibit endogenous PPO activity.

The enzyme with the common trivial name polyphenol oxidase (PPO) is generally accepted to be a *o*-diphenol: oxygen-oxidoreductase (EC 1.14.18.1). Because of the nature of its substrate specificity, PPO has a whole host of acronyms like phenol oxidase, catecholase, phenolase, catechol oxidase, and even tyrosinase. It has a broad phenolic substrate specificity ranging from monophenols to polyphenols of varying structural complexity. The enzyme uses copper ions as a prosthetic group that participates in the redox reactions it catalyzes. Oxygen is required for catalytic processes that lead to hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and oxidation of *o*-diphenols to *o*-quinones (diphenolase activity). The *o*-quinones formed are highly reactive and may nonenzymatically polymerize, oxidize other compounds, or undergo nucleophilic attack by protein amino acids, thiol compounds, and sugars, etc., to form melanoid-colored products (Pierpoint 1969; Taylor and Clydesdale 1987). Polyphenol oxidase is ubiquitous in microorganisms, plants, and animals. It plays a role in the physiology of these organisms. Yet, because of the nature of its activity, it may produce undesirable colored products that negatively influence flavor and the aesthetic attribute of some food products obtained from raw materials rich in the enzyme.

It is the ability of PPO to generate melanoid-colored products that has attracted enormous interest in the food industry. Its activity in a number of plant-derived food products may cause oxidative browning as in fruits and vegetables (Vamos-Vigyazo 1981). Polyphenol oxidase is present in wheat as well (Milner and Gould 1951; Abrol and Uprety 1970; Tikoo et al 1973; Taneja et al 1974; Kruger et al 1976). Several studies have investigated not only variations of PPO activity with quality characteristics (Hatcher and Kruger 1997; Park et al 1997) or with cultivars and growing locations (Lamkin et al 1981; Baik et al 1994; Park et al 1997), but also milling fractions (Marsh and Galliard 1986; McCallum and Walker 1990; Hatcher and Kruger 1997).

Since previous studies have suggested that the highest activity of PPO is localized in the bran fraction (Milner and Gould 1951; Marsh and Galliard 1986), we have focused our investigations on the levels of PPO in the bran from a variety of wheat cultivars grown in several locations in the United States in the consecutive harvests of 1998 and 1999. We also correlated those activities with what is

found in the milling fractions including the flour of some representative cultivars. Because of higher activities in the bran, a by-product of milling, we expected that using bran as the enzyme source would provide a better picture of varietal differences in the PPO levels than using low level sources like flour fractions or whole seed extracts. Moreover, flour fractions appear to acquire their share of the enzyme from being contaminated with bran during milling, as in the study on PPO distribution in flour millstreams by Hatcher and Kruger (1993).

Our preliminary data showed that there are unknown extractable compounds in wheat flour that may interfere with PPO activity. Similarly, some PPO inhibitory components were also detected in bran extracts but the effect they elicited was much less pronounced, allowing a more reliable determination of PPO activity. It was, therefore, justifiable to investigate the relationship between PPO activity in various milling fractions and that of flour fractions for comparison, but with a focus on the bran fraction as a good indicator of differences in PPO activity between wheat cultivars.

Direct evidence illustrating the role played by endogenous phenolic acids as PPO substrates is scant. Some studies have identified a variety of phenolic acids in either whole wheat grain or in millstreams (Maga and Lorenz 1974; Krygier et al 1982; Sosulski et al 1982; Kuninori and Nishiyama 1986; Pussayanawin and Wetzel 1987). McCallum and Walker (1990) found a correlation between total soluble phenolics in wheat flour with phenol oxidase activity in New Zealand millstreams. Hatcher and Kruger (1993, 1997) demonstrated correlations between some phenolic acids, polyphenol oxidase activity, as well as color and ash content for a number of Canadian wheat cultivars. Most of these reports are circumstantial evidence. However, Lamkin et al (1981) and Interesse et al (1982) produced some direct evidence documenting different levels of PPO activity using various phenolic acids as substrates. Furthermore, there has been no reported study showing that some phenolic acids actually inhibit PPO activity in wheat. Indeed, there are some phenolic acids and extractable components from wheat that inhibit PPO, as we will demonstrate in the present article.

Spectrophotometric measurement of product color change resulting from PPO oxidation of a phenolic substrate or oxygen consumption monitoring during the oxidation process have been used to assay PPO activity (Honold and Stahmann 1968; Lamkin et al 1981; McCallum and Walker 1990; Hatcher and Kruger 1997; Park et al 1997). Both of these methods measure relative PPO activity in sample extracts. We have, therefore, used a spectrophotometer in the present study because of its higher throughput and the ability to use smaller samples of standardized extractable protein content to more accurately measure the specific activity of the enzyme.

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MATERIALS AND METHODS

Wheat Samples

The wheat samples included experimental lines, some of which are now released, and some check cultivars submitted by public breeding programs in participating Midwest states (Colorado, Kansas, Nebraska, South Dakota, and Oklahoma) for quality testing under the auspices of Wheat Quality Council. The bagged samples of wheat were transported to Manhattan, KS. The fractions used in the study were collected during the milling test done at Kansas State University in the Department of Grain Science and Industry by the Hard Winter Wheat Quality Laboratory and the USDA Wheat Research Center, Wheat Quality Laboratory, Manhattan, KS.

Bran fractions were obtained from 18 (1998) and 14 (1999) hard red winter wheat cultivars and five (1998) and two (1999) hard white winter wheat cultivars harvested in 1998 and 1999. Flour, reduction shorts, and break shorts were obtained from the 1999 harvest only. The wheat samples were milled on a Miag Multomat Mill. Samples of bran were sieved using a 20 stainless steel bolting sieve (1,041- μ m screen opening) to remove flour or middlings contaminants. What went through the screen was discarded and the large clean bran particles retained were saved. Break shorts and reduction shorts were from pools as each fraction category flowed through the mill, and the straight-grade flour fraction ($\approx 72\%$ extraction) were collected and immediately put into polyethylene bags for cold storage until used.

Reagents

L-Dihydroxyphenylalanine (L-DOPA) and tropolone were purchased from Sigma (St. Louis, MO). All other reagents were of the highest grade available and purchased either from Sigma, Fisher (Pittsburgh, PA) or Biorad (Hercules, CA).

Extraction of Proteins from Wheat Milling Fractions

Each milling fraction (0.5 g) was weighed and soaked overnight in 5 mL of 100 mM phosphate buffer, pH 6.5, in a refrigerated cabinet (8°C). Subsequent homogenization was done with a Power Gen 700 homogenizer at speed setting 5 for 1 min with the sample tube immersed in ice. The samples were then gently shaken in a wrist-arm shaker at 8°C for 1.5 hr, followed by centrifugation at 4,800 $\times g$ in a swinging bucket rotor for 30 min. The clear supernatants were collected, and aliquots with known amounts of protein were used for the determination of polyphenol oxidase activity.

Identification of PPO by Gel Electrophoresis

Bran extracts of 10 representative cultivars, seven hard red and three hard white winter wheat from Colorado, Kansas, Nebraska, South Dakota, and Oklahoma were prepared. The extraction was accomplished by weighing 500 mg of each bran sample then homogenizing in 5 mL of 100 mM phosphate buffer containing 1% Triton x-114 and 4% polyvinylpyrrolidone to remove phenolics from the extracts. The extracts were subsequently processed as described above to isolate the supernatants. An aliquot of each sample containing $\approx 100 \mu$ g of proteins were processed and subjected to 12% SDS-PAGE essentially as described by Laemmli (1970), except under nonreducing conditions by omitting a reducing agent in the sample buffer and without heating. SDS was added to the sample buffer to activate latent PPO that may be present in the extract (preliminary studies had shown at least a threefold increase in PPO activity following activation with SDS). The gels were then run on Biorad Protean II electrophoretic apparatus under constant voltage (100 V) overnight with cooling or for ≈ 4 hr on the Hoefer apparatus. For each preparation, two sets of gels were run; one was stained with Coomassie Blue R 250 and a gel with corresponding samples was equilibrated in 50 mM phosphate buffer, pH 6.5, containing 6M urea for 10 min before staining with 10 mM DOPA in the phosphate buffer. Destained Coomassie blue gels and DOPA-developed gels were photographed or scanned into a computer for documentation.

Quantitation of PPO activity

PPO activity of the milling fractions was spectrophotometrically determined using DOPA as a substrate. Activity was monitored with a diode array spectrophotometer (Beckman DU 4700) equipped with molecular biology and enzyme kinetics software. An increase in absorbance at 480 nm as a result of DOPA converting into dopachrome by PPO in the extract was monitored. The reaction occurred in a 1-cm light path cuvette containing a volume of an extract equivalent to 500 μ g of protein, the amount predetermined to give a linear initial velocity of the reaction. The reaction temperature was kept constant at 30°C using Beckman Peltier temperature controller attached to the cell-holder of the spectrophotometer. The reaction was started by addition of 1 mL of 10 mM DOPA in 0.1M phosphate buffer, pH 6.5, to a final reaction volume of ≈ 1.3 mL and rapidly mixing, blanking then starting the recording of the increase in absorbance. Enzyme activity was monitored over a 3-min period, during which initial rate could easily be determined, or a 5–10 min

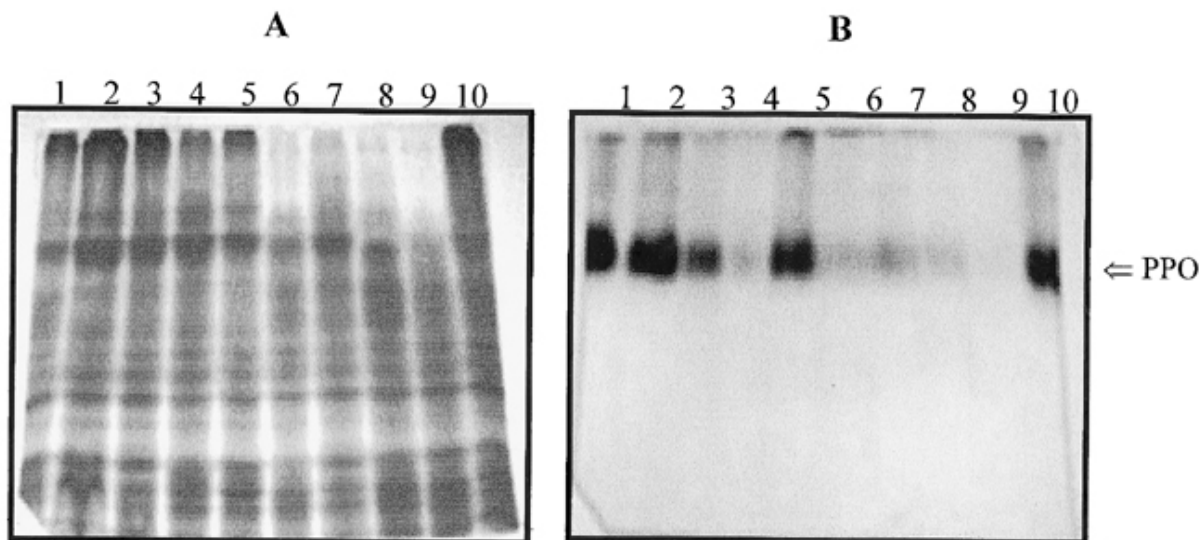


Fig. 1. Electropherograms showing SDS-PAGE (12% gel) analysis of proteins extracted from a bran fraction from hard winter wheat cultivars run under nondenaturing conditions. Each lane was loaded with $\approx 100 \mu$ g of bran protein extract. Samples: 1 Prowers, 2 Larned, 3 Trigo, 4 Lakin, 5 Heyne, 6 Cougar, 7 N94L205, 8 Tandem, 9 SD94149, and 10 Karl92. **A**, profiles of Coomassie blue stained proteins. **B**, polyphenol oxidase (PPO) bands revealed by staining with 10 mM DOPA in phosphate buffer after PPO activation with 6M urea in the gel.

period for flour extracts that exhibited rather slow absorbance changes. The spectrophotometer kinetics software package estimated the enzyme activity by measuring the slope of the reaction at the initial rate. One unit of PPO activity was defined as the amount of the enzyme giving a change in absorbance of 0.001/min as described above. Assay conditions were verified using a commercial preparation of mushroom tyrosinase purchased from Sigma. PPO activity was discriminated from that of peroxidase, if any, using tropolone as a selective inhibitor for PPO (Kahn and Andrawis 1985).

Activity and Inhibition of PPO Using Other Phenolic Acids and Methanolic Extracts of Bran

To test for substrate affinity, we determined PPO activity in bran extract from Prowers assayed as described above but using representative phenolic acids that occur naturally in wheat. Phenolic acids (10 mM) previously detected in wheat bran extracts (*unpublished data*) included caffeic acid, chlorogenic acid, ferulic acid, protocatechuic acid, and 3-phenyl-2-propenoic acid. They were prepared in 50 mM phosphate buffer, pH 6.8, and assayed individually. Maximum absorbance for the quinone product for each compound was predetermined by incubating each substrate with mushroom tyrosinase for 0.5 hr and scanning absorbance across the full spectral range using diode array spectrophotometry. PPO activity was monitored at predetermined absorbance maxima. The potential of the phenolics as inhibitors of extracted PPO from bran was investigated by preincubation and using DOPA as substrate. To determine the potential of natural phenolic extracts as PPO substrates, bran from durum and Heyne cultivars was extracted with acidified (0.1N HCl) methanol.

These extracts were used as substrates or used in preincubations with PPO extracts before DOPA addition to determine potential inhibitory effects on enzyme activity. To prepare the extracts, 500 mg of durum or Heyne bran was homogenized in 5 mL of acidified methanol. The extracts were heated under nitrogen at 100°C in a heating block for 10 min, cooled, and centrifuged at 10,000 × g for 30 min. Supernatants were collected and lyophilized. The lyophilizates were reconstituted to the same volume with 50 mM phosphate buffer, pH 6.5, ultrafiltered using Centricon 3 (MW cutoff of 3,000), and aliquots of the filtrates were used in the PPO assay or the filtrates were stored frozen at -18°C in vials under nitrogen until used.

Estimating Protein Concentration

Protein concentrations in the extracts used for PPO assays were determined by the Coomassie blue dye method of Bradford (1976). Coomassie blue protein assay dye and bovine serum albumin standard were purchased from Pierce Chemical Co. (Rockford, IL).

RESULTS

Extractability of Proteins from Wheat Milling Fractions

The extractability of proteins from milling fractions of wheat using phosphate buffer varied according to the fraction being extracted. The extraction level was highest in the reduction shorts fraction with ≈36 mg of protein extracted/g of sample followed by the bran fraction (≈28 mg/g of bran), whereas the extractable protein in the break shorts was only ≈20 mg/g of sample; the lowest amount of extractable proteins was from flour (≈8.5 mg/g of sample) as assessed by Coomassie blue protein assay (Bradford 1976). These measure-

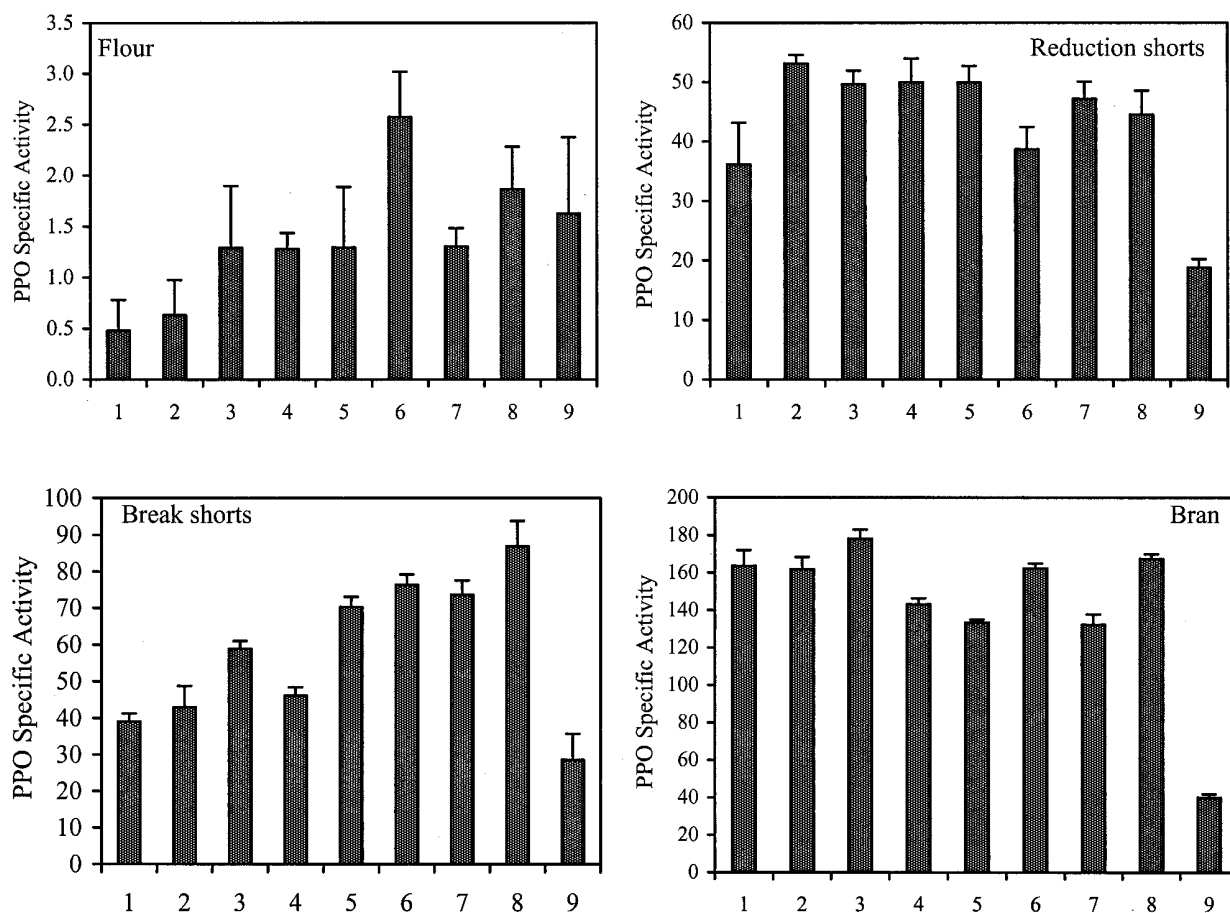


Fig. 2. Comparison of polyphenol oxidase (PPO) specific activities in extracts of flour, reduction shorts, break shorts, and bran from nine hard winter wheat cultivars. Extracts from milling fractions obtained from cultivars: 1 Prowers, 2 CO960026, 3 Jagger, 4 KS89180B-2-1-2, 5 KS98PO630-4-5, 6 2174 (Oklahoma check sample), 7 OK95571(LA), 8 Intrada, and 9 OK96717. Samples were extracted with 0.1M phosphate buffer, pH 6.5. Aliquots containing 500 µg of protein were assayed for PPO activity using 10 mM DOPA substrate. Each sample was replicated at least three times. Vertical bars ± standard error of the mean.

ments represent ≈ 3.6 , 2.8, 2., and 0.9% of the dry weight extractable as protein from the reduction shorts, bran, break shorts, and flour, respectively.

Identifying PPO by Gel Electrophoresis and Specificity

To ascertain the presence of PPO and possible isozyme variants in wheat, proteins were extracted from bran of representative cultivars and subjected to SDS-PAGE under nondenaturing conditions. The results show that the banding patterns of proteins as revealed by Coomassie Blue staining (Fig. 1A) were similar, except for Cougar, NuPlains, Tandem, and SD94149, where the slow mobility proteins were less abundant. When samples were subjected to electrophoresis under the same conditions as the Coomassie Blue stained gel, and the gel was impregnated with DOPA, positive dark-brown staining bands appeared. The bands representing PPO appear black in Fig. 1B. Similar electrophoretic mobilities were observed in all of the cultivars and single broad bands were discernible. The same results were obtained when we used 10 mM 4-methyl catechol as a staining substrate. Five of the 10 cultivars showed very intense staining (Prowers, Larned, Trigo, Heyne, and Karl 92) and four were rather weak (Lakin, Cougar, NuPlains, and Tandem), whereas SD94149 exhibited the weakest intensity despite the heavy protein loading. When the gels were preincubated with even as little as 0.1 mM tropolone or phenylthiourea, PPO staining was abolished.

Quantitation of PPO Activity

The extracts of milling fractions were assayed for PPO activity and the results are presented in Fig. 2. The data show that bran had the highest specific activity of polyphenols oxidase (40–180 units/mg of protein). Of all nine cultivars studied, the lowest PPO specific activity was observed in flour extracts (0.5–2.7 units/mg of protein) followed by reduction shorts (20–55 units/mg of protein); break shorts had the second highest specific activity (30–90 units/mg of protein).

Having determined that bran was the milling fraction with the highest overall PPO activity, subsequent studies were focused on this fraction. The specific activity of PPO was investigated in the

bran obtained from wheat harvested in both 1998 and 1999 growing seasons. The results show that of the 24 cultivars investigated in the 1998 harvest (Fig. 3), the highest PPO specific activity was found in Kansas Karl 92 and Colorado Prowers extracts (≈ 280 units/mg of protein). Other cultivars exhibiting relatively high PPO activity were CO950379, KS96HW94, Betty (≈ 260 units/mg of protein) and CO950043, Larned, Trigo, Heyne, and SD93267 (≈ 200 units/mg of protein). Other moderately low PPO cultivars predominantly grown in Nebraska were Scout, Cougar, NE93613, NuPlains, Millennium, NE94482, Wesley. Also South Dakota Tandem, SD94241, and Oklahoma OK95571 grown in two localities, Lahoma and Stillwater, had moderately low PPO (≈ 150 units/mg of protein). The lowest activities were registered in Lakin and SD94149 (50–70 units/mg of protein).

The 16 bran samples from the 1999 harvest were similarly analyzed and the results are presented in Fig. 4. Prowers, CO960026, Jagger, NE95473, Tandem, SD95218, SD check 2174, and Intrada all had high PPO activity (≈ 180 units/mg of protein). Moderately high activities were noted in CO9550043, KS89180B-2-1-2, KS98PO630-4-5, Culver, NE94654, SD95203 and OK95571 (≈ 130 units/mg of protein). The lowest specific activity was registered in Oklahoma cultivar OK96717 (≈ 40 units/mg of protein).

A comparison of PPO specific activities was made among five cultivars grown in four localities during the two seasons. The results are presented in Fig. 5. The data followed the same patterns as in Figs. 3 and 4, but it becomes evident that there was a seasonal influence on the levels of PPO activity, at least in cultivars grown in Colorado and Kansas. The 1998 harvest of Prowers, CO9550043, and Jagger from these two states showed higher activities than the 1999 harvest from the same localities. There were no differences in the enzyme activity from Tandem and OK95571 grown in the respective localities during the two seasons.

Activity or Inhibition of PPO Using Some Phenolic Acids and Methanolic Extracts of Bran

We observed major differences in the PPO activity when various phenolic acids were used as substrates (Table I). These substrates generated much lower PPO specific activities than when the com-

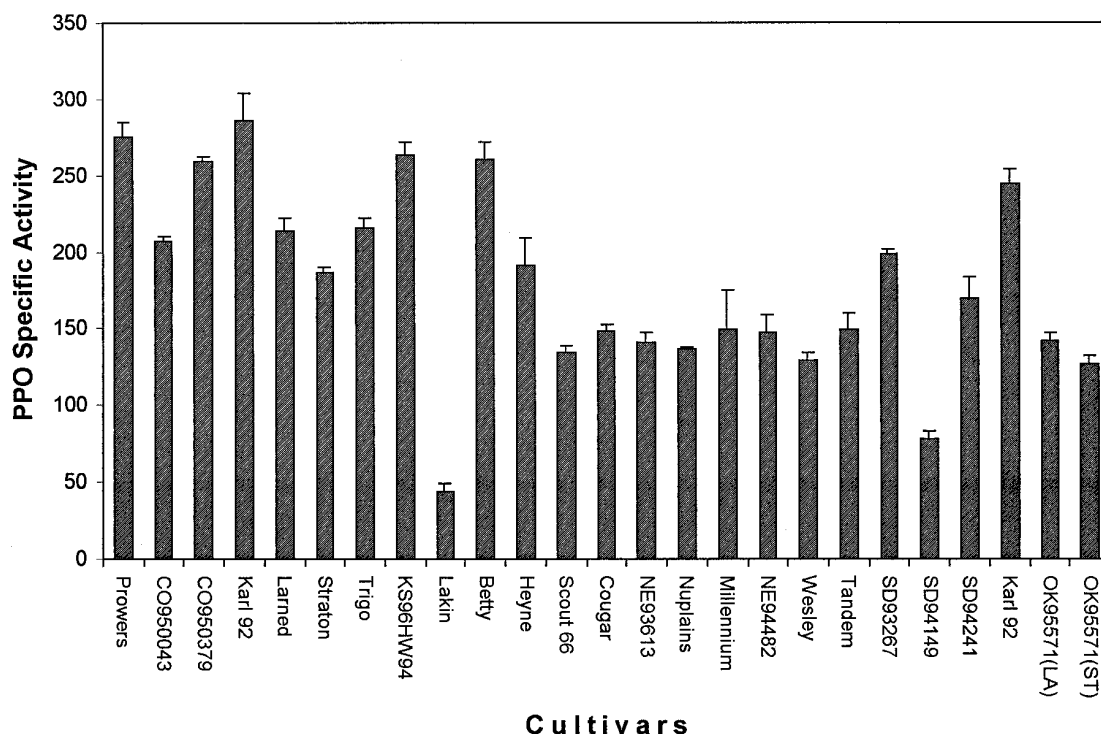


Fig. 3. Polyphenol oxidase (PPO) specific activities in bran milling fraction from 25 cultivars of hard winter wheat of 1998 harvest extracted with 0.1M phosphate buffer, pH 6.5. Aliquots from each cultivar containing 500 μ g of protein were assayed for PPO activity using 10 mM DOPA as substrate. Each sample was replicated at least four times. Vertical bars \pm standard error of the mean.

monly used substrates for PPO determinations, DOPA or 4-methylcatechol, were used.

3-Phenyl-2-propenoic acid was the worst substrate for the PPO extract as a zero reading was recorded with this substrate. Ferulic acid displayed the second lowest rate of conversion. Protocatechuic and caffeic acids had moderately low rates. The highest specific activity was obtained with chlorogenic acid. The rates were $\approx 1\%$ for ferulic acid, 5 and 6% for protocatechuic and caffeic acids, respectively, relative to that of DOPA conversion. The rate of DOPA oxidation was reduced at least twofold in the presence of equimolar concentrations of these poor substrates. However, when the extract was incubated with DOPA following preincubation with 3-phenyl-2-propenoic acid, the enzyme activity was eliminated altogether. Preincubation of a Prowers bran extract with durum bran acid-methanolic extract lowered PPO activity three- to fourfold, whereas the same quantity of a similar extract from Heyne lowered the enzyme activity only by 50%. Similarly, preincubation of Prowers extract with 2 mM tropolone, a specific PPO inhibitor, prevented DOPA oxidation.

To test whether Prowers bran extract had monophenolase or catecholase activity, the enzyme assay was conducted with 10 mM tyrosine as substrate at 480 nm and the results showed an initial lag period that lasted $\approx 2-3$ min, followed by an increase in absorbance typical of that observed with DOPA as substrate.

DISCUSSION

Data on phosphate buffer extractable proteins from milling fractions indicate that relatively more soluble proteins were extracted from reduction shorts than from any other fractions analyzed. Reduction shorts are composed of finer ground bran contaminated with some germ and flour. The higher extraction rate may be due to more extractable proteins that might have been contributed by the germ component with high levels of protein. Also, because the reduction shorts fraction was finer than other fractions, it is conceivable that the larger surface area allowed for better extractability than was observed in bran and the break shorts fractions. Flour could have contributed minimally because the extractable proteins from flour were the least. This is not surprising because storage proteins, glutenins, and gliadins that predominate in the endosperm (flour) are more extractable in acid-base solution or alcohols according

to the Osborne extraction scheme (Osborne 1924) than other aqueous media.

To conduct the investigations on PPO activity, we wanted to ascertain that what we were investigating in the extracts was PPO. We also wanted to determine if there were variants of this enzyme in the bran fraction. Specific staining with DOPA of electrophoretically separated protein bands in acrylamide gel under nonreducing conditions showed that there is only one isoform of PPO present in the bran extracts from a variety of cultivars that has an affinity for DOPA. Other reports previously showed varying numbers of multiple forms of PPO from wheat (Kruger 1976; Interesse et al 1981, 1982). It is unclear whether the differences in the numbers are due to differences in the seed tissue distribution, substrate specificity, or methodology employed for sample preparation. It is known, however, that some endogenous phenolic substrates of PPO have the potential of modifying the molecular structure of plant proteins during extraction (Loomis and Battaille 1966; Pierpoint 1969). This phenomenon may cause heterogeneity in the proteins being studied, including PPO. We purified PPO under stringent conditions that would minimize generation of highly reactive *o*-quinones that are notorious for spontaneously reacting with nucleophiles provided by protein molecules (Mason 1965; Singleton 1981). We hope this will shed some light as to the multiplicity of PPO variants in wheat bran, as the answer remains obscure.

Because PPO activity could be inhibited by tropolone, a known specific inhibitor of tyrosinase (Kahn and Andrawis 1985), in the spectrophotometric assay and by preincubation of the gels with tropolone in the electrophoretic assay, the PPO activity may have been attributable to a tyrosinase-like protein in the extracts of bran. This was further confirmed by hydroxylation of tyrosine into DOPA with subsequent oxidation into the colored product, DOPA-chrome, a process that can be achieved only by tyrosinase, not laccase. Finally, failure of bran extracts to oxidize syringaldazine, a known laccase substrate, supports the notion that PPO in bran is not a laccase type.

Extractability of proteins from the milling fractions varied with the fraction in question, so we standardized the amount of the extractable proteins used for PPO activity determinations. In preliminary studies, bran had 500 μg of protein in ≈ 1.3 mL of reaction mixture. The PPO activity from the milling fractions shown in Fig. 2 indi-

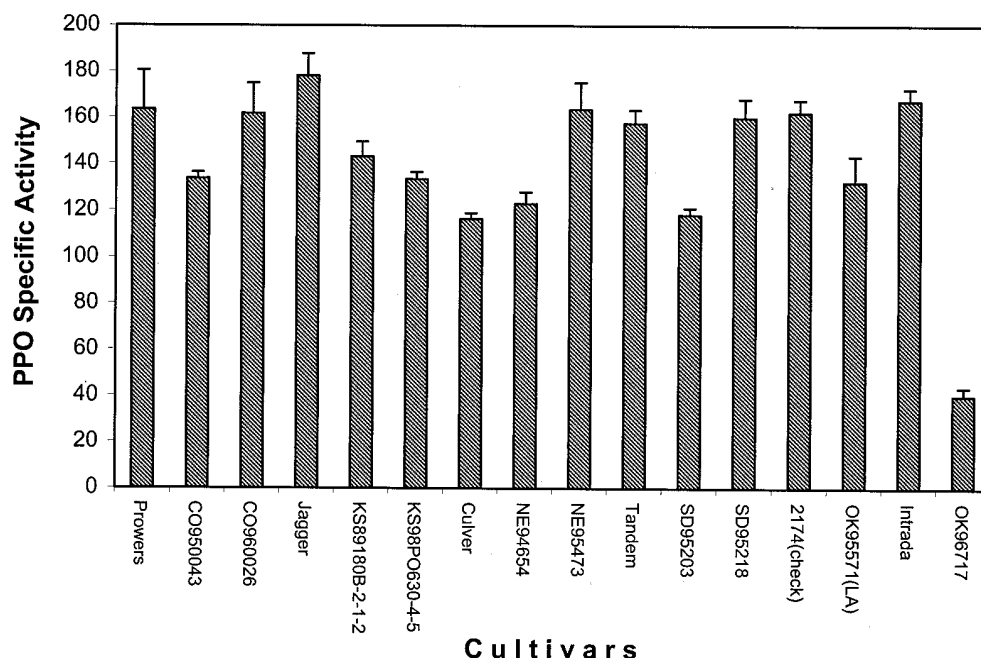


Fig. 4. Polyphenol oxidase (PPO) specific activities in bran milling fraction from 16 cultivars of hard winter wheat of 1999 harvest extracted with 0.1M phosphate buffer, pH 6.5. Aliquots from each cultivar containing 500 μg of protein were assayed for PPO activity using 10 mM DOPA as substrate. Each sample was replicated at least four times. Vertical bars \pm standard error of the mean.

cate that the highest PPO activity is extractable from the bran fraction, followed by break shorts, then reduction shorts, and finally in the flour. Earlier studies (Milner and Gould 1951; Marsh and Galliard 1986) also found that bran fraction had the highest PPO activity, although Marsh and Galliard (1986) used a polarographic method and compared bran with whole meal and flour only. Nonetheless, the two methods correlated well as shown by Kruger et al (1994), who also supported the notion that PPO is closely associated with the bran layer (Hatcher and Kruger 1993). PPO activity of flour was relatively low (Hatcher and Kruger 1993; Baik et al 1994; Park et al 1997), but the activities in the break and reduction shorts from the various cultivars followed the trends found in the bran (higher in the break shorts than reduction shorts). The reduction shorts fraction contains more flour contamination. It appears, therefore, that bran is the major contributor to PPO activity in the other milling fractions. There were only specks of bran in the flour fractions, so we presume that the observed low PPO activity in the flour (straight-grade flour) is attributable predominantly to bran contamination. This is further supported by the observation that the reduction shorts fraction had more flour contamination than break shorts and had a lower PPO activity.

Investigations of bran PPO from a number of cultivars grown in various regions during different seasons showed differences in PPO levels according to the cultivars, growing location, and the year of harvest. There was no apparent trend in the levels of PPO activity relative to whether the cultivar was a white or red wheat. These data suggest that the variation in the PPO activity was more dependent on the breeding strategy. Some hard white winter wheat cultivars grown in Kansas, such as Lakin, had very low levels of bran PPO activity, as did a hard red winter wheat like OK96717 grown in Oklahoma. Similarly, some hard white winter wheat cultivars like Betty and hard red winter wheat cultivars such as Prowers had high bran PPO activity. What appeared to be significant was the influence of the year of harvest (Fig. 5). Prowers and CO950043, the two cultivars grown in Fort Collins, CO, in 1998 and 1999 had much higher PPO activity in the 1998 harvest than in 1999, although it was reported to the Wheat Quality Council that these cultivars were grown under the same irrigated growing conditions with no diseases or

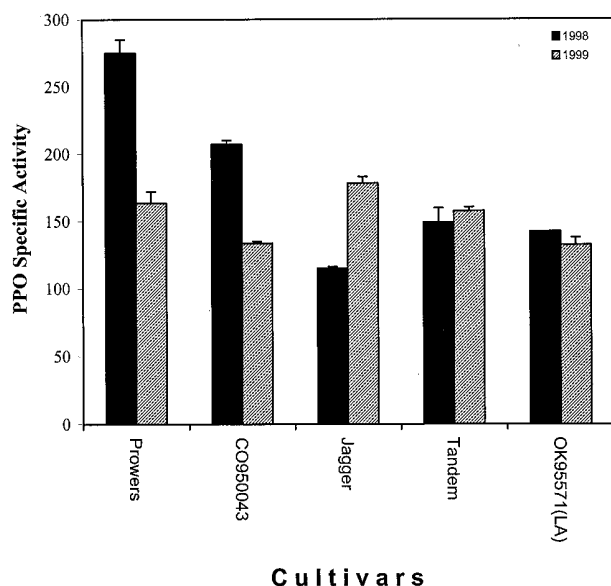


Fig. 5. Comparison of polyphenol oxidase (PPO) specific activities in bran milling fraction from five cultivars of hard winter wheat from the same four localities during consecutive harvesting seasons (1998 and 1999) extracted with 0.1M phosphate buffer, pH 6.5. Aliquots from each cultivar containing 500 µg of protein were assayed for PPO activity using 10 mM DOPA as substrate. Each sample was replicated at least four times. Vertical bars ± standard error of the mean.

insect impact. There is, therefore, no apparent explanation as to what might have caused the differences in the PPO activity in the bran of these two cultivars during this period. On the other hand, the Jagger cultivar grown in Hays, KS, had more PPO activity (≈30%) in the bran from 1999 harvest than from the 1998 harvest. It is likely that the weather conditions may have contributed to this difference; the 1999 harvest was reportedly delayed by rain. Extended wet weather conditions at harvest may have caused some low level sprouting. Sprouting causes a significant increase in the level of PPO activity (Kruger 1976). Therefore, the reported wet weather conditions might have induced higher enzyme activity in the 1999 harvest. The Tandem composite grown in South Dakota during the two seasons showed no differences in the bran PPO specific activities. Similarly, cultivar OK95571 grown in Oklahoma during the same seasons had no differences in the bran PPO specific activities, suggesting that the weather conditions were more stable in those states in the two growing seasons. Indeed, that was the case according to Reports 49 and 50 on Wheat Quality Hard Winter Wheat Technical Board of the Wheat Quality Council (1998, 1999). It appears, therefore, that PPO activity in wheat bran may be influenced, not only by genotype considerations, but also by locality, as in studies with either whole seed or flour (Baik et al 1993; Park et al 1997) and by weather conditions as noted in this study. Further investigations are needed to specifically confirm the impact of weather conditions on PPO activity.

One of the other factors that may affect PPO activity is the chemical nature of the substrate. Different phenolic substrates may be oxidized at different rates (Lamkin et al 1981; Interesse et al 1982). Phenolic acids that are potential substrates for PPO have been described from wheat flour extracts (Maga and Lorenz 1974; McCallum and Walker 1986; Pussayanawin and Wetzel 1987; Hatcher and Kruger 1997). In the present study, we found that not only are there differences in the rate at which various phenolic substrates are oxidized by wheat bran PPO extract, but that some of the slowly oxidizable phenolics and those that are unoxidizable may inhibit the oxidation of the phenolic acids that are normally readily oxidized, such as DOPA. We further showed that an acidic methanolic extract of bran from several wheat cultivars had inhibitory effects on bran PPO activity. The extract from durum bran was the most effective, suggesting that it either has the most potent inhibitor or the amount of the inhibitory factor is greatest from this source. The potency of the factor was present in the acidic methanolic extract that was boiled and recovered in the ultrafiltered supernatant fraction, which would suggest that the component is not proteinaceous in nature.

Studies are underway to isolate and more critically characterize the tyrosinase-like PPO protein found in bran. We are also conducting further studies geared toward identifying the components that are responsible for the PPO inhibitory activity from the wheat cultivars.

TABLE I
Polyphenol Oxidase (PPO) Specific Activities in Extracts of Prowers Bran^a

Substrate	λ Max (nm) ^b	PPO Specific Activity	Relative Activity (%) ^c
Dihydroxyphenylalanine	480	312	100
4-Methylcatechol	310	345	110
Chlorogenic acid	390	31	10
Caffeic acid	390	19	6
Protocatechuic acid	360	15	5
Ferulic acid	360	2	1
3-Phenyl-2-propenoic acid	330	0	0

^a Phenolic substrates (10 mM) in 0.1M phosphate buffer, pH 6.5.

^b Predetermined absorbance maxima.

^c PPO activity obtained using a given substrate expressed as % of specific activity obtained with the standard substrate, DOPA.

CONCLUSIONS

PPO activity was extracted from various milling fractions of wheat and was highest in the bran fraction. This is in agreement with previous reports. Bran offers a reliable source of PPO for the determination of improved wheat quality in the low PPO breeding program. One detectable PPO variant was found in bran, which has affinity for DOPA but not syringaldazine and can hydroxylate tyrosine and is inhibited by tropolone. Therefore, it is likely that the wheat bran PPO is a tyrosinase type, not a laccase or peroxidase. It is not known whether there are other isoforms in other anatomical parts of the wheat cultivars reported here, which have varying affinities to various phenolic acid substrates. It appears that durum bran has a potent PPO-inhibiting component; some phenolic acids are able to inhibit PPO activity significantly. These compounds have the potential of being used to control PPO induced browning in wheat-based products.

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