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Polyphenol Oxidase in Wheat Grain: Whole Kernel and Bran Assays for Total and Soluble Activity

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ABSTRACT

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Color is a key quality trait of wheat products, and polyphenol oxidase (PPO) is implicated as playing a significant role in darkening and discoloration. In this study, total and soluble PPO activities were characterized in whole kernel assays and bran extracts. In whole kernel assays similar to AACC Approved Method 22-85, four wheat cultivars were ranked the same for both total and soluble (leached) PPO activity with L-DOPA (diphenol) as the substrate. Total kernel PPO activity was much greater than soluble PPO activity in three hexaploid wheat cultivars, indicating that insoluble PPO was the major contributor to kernel PPO measurements. Tyrosine (monophenol) was an excellent PPO substrate in kernel assays as expected but had no activity as a substrate for soluble PPO. However, soluble PPO activity with tyrosine was activated by the addition of the diphenols chlorogenic acid and caffeic acid. When PPO

was assayed in homogenized bran, 89–95% of total PPO activity remained insoluble, associated with the bran particles. The kernel assay detected <2% of PPO measured in an equivalent amount of homogenized bran. However, total PPO activity was 2-fold higher in Klasic than in ID377s, both when measured in the kernel assay and in homogenized bran, indicating that the kernel assay was an accurate predictor of relative total extracted PPO activity in these two cultivars. Adding detergents (0.1% SDS plus 0.2% NP-40) to the bran extraction buffer increased both soluble and insoluble PPO activity. Results indicate that relative PPO activities among wheat cultivars are similar in whole kernel and kernel leachate assays, and that the predominant insoluble fraction of PPO, which is relatively uncharacterized, may be largely responsible for wheat product discoloration.

Color is a key quality trait of hexaploid (*Triticum aestivum* L.) and durum (*T. turgidum* ssp. *durum* [Desf.] Husn.) wheat products. In this regard, the prevention or minimization of discoloration is an important component to attaining good color in consumer products. Polyphenol oxidase (PPO) has been implicated as a leading cause of discoloration of Asian wheat noodles and other wheat products (Kruger et al 1994; Morris 1995). Considerable effort has been made to reduce levels of PPO in wheat germplasm through the use of whole kernel assays such as Approved Method 22-85 (AACC International 2000) (Anderson and Morris 2001; Bettge 2004). In this study, we explore PPO characteristics in these kernel assays and compare them with PPO characteristics in extracted bran.

PPO is enzymatically complex. PPO catalyzes the hydroxylation of monophenols to *o*-diphenols (monophenolase activity; E.C. 1.14.18.1) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity; E.C. 1.10.3.2) (Steffens et al 1994; Lerch 1995). The quinone products of PPO covalently modify nucleophiles such as amines, thiols, and phenolics, forming complex colored polymers (melanins) (Whitaker and Lee 1995). These reactions are the cause of discoloration of many food products by PPO. Monophenolase activity often shows a lag period that is reduced or eliminated by catalytic concentrations of *o*-diphenolic compounds (Steffens et al 1994; Perez-Gilabert and Garcia-Carmona 2000). Furthermore, PPO is present in latent forms in many plant tissues. Many reagents, including ammonium sulfate, detergents, proteolytic enzymes, and chaotropes, can activate latent PPO in wheat (Okot-Kotber et al 2002; Jukanti et al 2003) and other species (Steffens et al 1994).

Both PPO and phenolics are localized primarily in the bran layer of the wheat kernel (Okot-Kotber et al 2001). Thus, flour PPO activity and phenolic content increase with flour extraction rate (Hatcher and Kruger 1993, 1997). Very low levels of PPO are associated with the germ and flour (Marsh and Galliard 1986). Marsh and Galliard (1986) reported that 53–88% of total PPO activity in whole kernel wheat meal was insoluble. Few additional studies have compared soluble and insoluble forms of PPO in wheat.

Although whole kernel PPO assays are widely used in screening wheat germplasm, these assays have only been characterized to a limited extent. Kruger et al (1994) developed a kernel PPO assay in which kernels were steeped 16 hr in water; then PPO was measured using catechol as the substrate. This total kernel PPO activity was approximately twice that observed when the steep-water was discarded and replaced with substrate solution. This result implies the presence of both soluble and insoluble PPO in this whole kernel assay. Also, based on data presented earlier (Kruger et al 1994), the total kernel PPO activity can be estimated to comprise ≈4% of that measured in ground whole meal. Despite the small fraction of PPO measured in the whole kernel assay, results were well correlated ($r = 0.85$) with the ground whole meal assay among 22 wheat lines. Assays of shorter duration, such as Approved Method 22-85 (AACC International 2000), have been developed recently but have not been thoroughly characterized (Anderson and Morris 2001; Bettge 2004).

The objectives of this research were to 1) determine the extent to which a whole kernel PPO assay (similar to AACC Approved Method 22-85) measures soluble and insoluble PPO using a monophenolic and a diphenolic substrate; 2) characterize the rate of soluble PPO leakage from wheat kernels; 3) evaluate diphenolic compounds as activators of soluble wheat monophenolase activity with tyrosine as the substrate; 4) evaluate soluble and insoluble PPO from bran extracted using different extraction buffers (mild and detergent); and 5) compare the activity of PPO measured in the Approved Method 22-85 whole kernel assay versus the activity extracted from bran.

METHODS AND MATERIALS

Genetically pure, single-origin grain lots of the durum cultivar Renville (PI 510696) and the hexaploid wheat cultivars ID377s hard white spring (IDO377s, PI 591045), Klasic hard white spring

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(PI 486139), and Penawawa soft white spring (PI 495916) were used throughout this research as indicated. Mean kernel weights were 33.6 mg for Renville, 37.9 mg for ID377s, 43.3 mg for Klasic, and 34.9 mg for Penawawa.

Whole Kernel Total and Soluble PPO Assays

All whole kernel assays were based on the general method of Anderson and Morris (2001) given in Approved Method 22-85 (AACC International 2000; Bettge 2004). In brief, five kernels were placed in a 2-mL microcentrifuge tube (T-3531, Sigma) with 1.5 mL of substrate-buffer or buffer alone (depending on the assay) containing 0.02%, v/v, Tween-20. All reagents were from Sigma. Incubations of kernels and kernel leachates were conducted at room temperature (21°C) with constant mixing on an end-over-end rotating (8 rpm) shaker (Labquake model 415110, Barnstead/ThermoLyne). PPO activity was measured as the change in absorbance at 475 nm (ΔA_{475}) on a Shimadzu BioSpec-1601 spectrophotometer using Shimadzu UVProbe v. 2.00 software. Each experiment was replicated twice (two complete blocks) and results were expressed as $\Delta A_{475} \cdot \text{g bran}^{-1} \cdot \text{min}^{-1}$ or $\Delta A_{475} \cdot \text{g kernel}^{-1} \cdot \text{min}^{-1}$.

The whole kernel total PPO activity assay was similar to AACC Approved Method 22-85 except that substrate concentrations were reduced to 5 mM to allow comparisons with the soluble PPO assay. Renville, ID377s, Klasic, and Penawawa kernels were incubated in 5 mM L-DOPA (3,4-dihydroxy-L-phenylalanine), 50 mM MOPS (3-[N-morpholino] propane sulfonic acid) pH 6.5, or 5 mM L-tyrosine (disodium salt), 20 mM Tris [Tris(hydroxyethyl) aminoethane] base pH 9. The ΔA_{475} was measured after 1 hr of incubation and assays were replicated four times.

The whole kernel soluble PPO activity assay entailed preincubating kernels of each of the four wheat cultivars in 1.5 mL of either MOPS buffer pH 6.5 (for L-DOPA assay) or Tris buffer pH 9 (for tyrosine assay) for 1 hr. After preincubation, 0.75 mL of the leachate was removed and diluted 2-fold with either 10 mM L-DOPA in MOPS buffer or 10 mM tyrosine in Tris buffer (5mM final substrate concentration). The ΔA_{475} was measured after 1 hr of incubation. The limited solubility of tyrosine prevented the use of 10 mM final concentrations as in AACC Approved Method 22-85. Soluble PPO activity values reported here were multiplied by two to correct for the 2-fold dilution of enzyme in these assays. Separate studies demonstrated that PPO activity was directly proportional to leachate concentration (data not shown), thus justifying the correction for dilution. Each assay was replicated four times. "Soluble PPO assay activity" is referred to as "soluble activity" and "total PPO assay activity" is referred to as "total activity" subsequently in the text. However, it should be noted that total PPO in the kernel assay underestimates the total extractable PPO and that each assay represents a different experimental procedure.

Whole Kernel Soluble PPO Leakage Time-Course

For leakage time-course studies, kernels of Renville, ID377s, Klasic, and Penawawa were preincubated in 1.5 mL of MOPS buffer, pH 6.5. Leachate solution was removed after 0.5, 1, 2, and 3 hr for PPO activity assays. Fresh buffer solution was added to the kernels, and samples were returned to the shaker. Thus, sequential leachates were obtained from each five-kernel sample. Leachate solution (0.75 mL) was added to 0.75 mL of 10 mM L-DOPA (5 mM final concentration) and ΔA_{475} was measured after 1 hr of incubation. Each assay was replicated four times.

Diphenolic Activation of Soluble Monophenolase PPO Activity

Penawawa (100 kernels) was incubated in 30 mL of 20 mM Tris buffer, pH 9, for 1 hr and the leachate was recovered. For each assay, 0.75 mL of leachate was added to 0.75 mL of substrate solution and ΔA_{475} was measured after 1 hr of incubation. Substrate solutions were a complete factorial of two concentrations of tyrosine (0 and 10 mM) and two diphenolic activators

(caffeic acid and chlorogenic acid) each at four concentrations (0, 50, 100, and 200 μM) in Tris buffer. Final assay substrate concentrations were half the values indicated. The ΔA_{475} value for each assay was corrected for the absorbance of the corresponding substrate solution control (no leachate blank). In a subsequent experiment, the time-course for kernel leakage of soluble PPO was conducted in the same manner as described for L-DOPA substrate, except that kernels were preincubated in Tris buffer, pH 9, and assayed in 5 mM tyrosine plus 50 μM chlorogenic acid (final concentrations) in Tris buffer.

Milling and Bran Extraction

The two hard white spring wheat cultivars (ID377s and Klasic) were tempered to 16% moisture content and milled on a Quadrumat flour mill (C.W. Brabender, Hackensack, NJ) following the method of Jeffers and Rubenthaler (1977). This system produces break flour, middlings, and bran; regrinding the middlings produces reduction flour and shorts. Bran yields were 25.6 and 24.4% (w/w, as-is moisture basis) for ID377s and Klasic, respectively. Milling fractions were stored at -20°C and used as is.

In preliminary experiments, bran was subjected to two sequential extractions and centrifugations. However, we considered it impractical to wash the pellet thoroughly enough to eliminate soluble PPO from this fraction. The alternative, reported here, involved making a single, relatively thorough extraction followed by measurement of total PPO in the ground suspension and soluble PPO in the centrifuged supernatant. Unextracted PPO was calculated as the difference.

Bran (2 g) was added to 20 mL of either a mild extraction buffer containing MOPS, pH 6.5, with 0.2 mM Pefabloc protease inhibitor or a detergent extraction buffer composed of the mild buffer plus 100 mM KCl, 0.1%, w/v, SDS and 0.2%, v/v, NP-40. Milder extraction conditions prevent activation of PPO (Escribano et al 1997) and thus may allow for more appropriate comparisons with whole kernel assays. The bran sample was extracted at room temperature by vacuum infiltration while vortexing at 10-min intervals, homogenizing at high speed for 1 min on a polytron (model GLH, Omni International, Marietta, GA) at 30 min and then repeating the aforementioned process. Total extraction time was 1 hr. Aliquots of the suspension (total PPO) were withdrawn while vortexing and stored at -80°C . The remainder of the suspension was centrifuged at $20,000 \times g$ for 30 min at 4°C . The supernatant was passed through a 0.45- μm PVDF (polyvinylidene difluoride) filter (Durapore, Sigma) and aliquots (soluble PPO) were stored at -80°C . Each cultivar extraction was replicated two times.

Protein content in the soluble PPO extracts was assayed using bicinchoninic acid (BCA protein assay kit, Pierce). PPO activity was subsequently assayed within 1.5 hr of thawing the frozen samples.

Bran Total and Soluble PPO Assays

Soluble bran PPO was assayed using 75 μL of extract (375–525 μg of protein) in 1.5 mL of 10 mM L-DOPA in MOPS, pH 6.5, buffer. The ΔA_{475} was measured from 30 to 60 sec and results were expressed as $\Delta A_{475} \cdot \text{g bran}^{-1} \cdot \text{min}^{-1}$. There were two assays per extraction replicate.

Total bran PPO was measured using the bran suspension samples by diluting 4-fold in MOPS buffer, withdrawing 0.2 mL of the diluted suspension while vortexing, and transferring the aliquot to a 2-mL microcentrifuge tube with 1.3 mL of MOPS buffer and L-DOPA (10 mM final concentration). Samples were incubated on the shaker for 3 min (primary reaction) and then filtered (0.45 μm PVDF). The ΔA_{475} of the filtered sample was measured from 30 to 180 sec. The rate of change in absorbance was used to back-calculate the absorbance of the sample at the time of filtration (i.e., at the end of the 3-min primary reaction). The initial (time zero) absorbance contributed by the filtered, diluted suspension itself was determined by measuring the absorbance value of two filtered no-substrate controls for each sample. The mean of these

RESULTS

Whole Kernel Total and Soluble PPO Assays

Using L-DOPA as the substrate, the four wheat cultivars showed marked variation in total PPO activity, ranging from 0.0043 to 0.1300 $\Delta A_{475} \cdot \text{g kernel}^{-1} \cdot \text{min}^{-1}$ for Renville durum and Penawawa, respectively (Fig. 1). ID377s had the lowest L-DOPA PPO activity of the three hexaploid cultivars, Klasic was intermediate to ID377s and Penawawa. The $\text{LSD}_{\alpha=0.05}$ using the four-replicate data was 0.0106 $\Delta A_{475} \cdot \text{g kernel}^{-1} \cdot \text{min}^{-1}$; DMRT indicated that all four cultivar grain lots had significantly different PPO activities.

Total PPO activity using tyrosine as the substrate was also markedly different among the four wheat cultivars (Fig. 1). Activity ranged from 0.0040 to 0.0715 $\Delta A_{475} \cdot \text{g kernel}^{-1} \cdot \text{min}^{-1}$. The ranking of the four cultivars with tyrosine was the same as that obtained with L-DOPA. The $\text{LSD}_{\alpha=0.05}$ was 0.0101, indicating that this substrate also differentiated the cultivars similarly. However the total PPO activity means of Renville and ID377s with tyrosine as the substrate were not significantly different (LSD) at $P = 0.05$. Although assay values for Renville were very low, they were well above the signal-to-noise ratio because the coefficient of variation for PPO activity was <10% for both total and soluble kernel PPO (Figs. 1 and 2). For both the L-DOPA and tyrosine total PPO assays, the ANOVA models were robust with R^2 values of 0.985 and 0.96, respectively.

The L-DOPA-to-tyrosine activity ratios of total PPO activity using L-DOPA and tyrosine substrates, respectively, varied widely among the four cultivars (Fig. 1). For Renville durum, the ratio was 1.1, indicating that the total PPO of this cultivar grain lot was similar in activity against both the diphenol and the monophenol substrates. The L-DOPA-to-tyrosine activity ratio for the three hexaploid wheat cultivars ranged from 1.7 to 2.4. Of these, ID377s had the overall lowest PPO activity for both substrates but the highest L-DOPA-to-tyrosine ratio (2.4). Penawawa had the highest PPO activity but its substrate activity ratio (1.8) was similar to that of Klasic (1.7).

Soluble L-DOPA PPO activity was lowest in Renville (0.0036 $\Delta A_{475} \cdot \text{g kernel}^{-1} \cdot \text{min}^{-1}$) (Fig. 2). Soluble PPO activity among the hexaploid cultivars ranged from 0.0057 to 0.0300 $\Delta A_{475} \cdot \text{g kernel}^{-1} \cdot \text{min}^{-1}$ for ID377s and Penawawa, respectively. ANOVA showed that, as before, the model was highly significant ($R^2 = 0.94$) with a small mean square error. The LSD for soluble PPO activity was 0.0046. Penawawa and Klasic were distinctly different, whereas ID377s and Renville were not (DMRT).

The ratios of soluble-to-total PPO activity using L-DOPA appeared to fall into two distinct groups (Fig. 2). The ratio for Renville was 0.84, indicating that soluble and total PPO activity levels were similar. Ratios of soluble-to-total PPO for the hexaploid wheat cultivars ID377s, Klasic, and Penawawa were ≈ 0.2 (Fig. 2). When comparing these results, it should be kept in mind that assays for soluble PPO measured all PPO that leached from the kernel and accumulated *during* a 1-hr preincubation period, whereas the total PPO assay measured PPO during the period of accumulation. Presumably the total PPO assay starts with a zero soluble component of PPO activity, which increases as PPO leaches out of the kernel during incubation. Thus, these ratios may overestimate the true ratio of soluble-to-total PPO activity using L-DOPA. Nevertheless, these results demonstrate that the total PPO assay includes significant soluble and insoluble PPO components.

Experiments using tyrosine as the substrate and the same protocol for soluble PPO activity revealed zero PPO activity for all cultivars when absorbance of leachate alone was subtracted (data not shown). It is unlikely that tyrosine was oxidized to an uncolored product because PPO would initially oxidize tyrosine to its *o*-diphenol, L-DOPA, a good colorimetric reagent. This lack of monophenolase activity in the soluble fraction was examined further in a subsequent experiment involving monophenolase activation using diphenolic compounds.

two controls, which represented time zero absorbance, was subtracted from the sample absorbance value at the time of filtration (3 min) for the duplicate reaction assays. The difference was used to calculate the primary reaction rate (total PPO activity [$\Delta A_{475} \cdot \text{g bran}^{-1} \cdot \text{min}^{-1}$]). There were four duplicate assays per extraction replicate. Total PPO activity was also calculated on a per gram of kernels basis using values for bran yield for the purpose of comparing total PPO in extracted bran (above) with total PPO in AACC Approved Method 22-85 whole kernel assay.

AACC Approved Method 22-85

Additional whole kernel PPO assays were conducted with ID377s and Klasic using AACC Approved Method 22-85 (four replicates), which uses 10 mM L-DOPA. Results were expressed as $\Delta A_{475} \cdot \text{g kernel}^{-1} \cdot \text{min}^{-1}$.

Proximate and Statistical Analyses

Bran protein ($N \times 5.7$) was determined following Approved Method 46-30 (AACC International 2000) on a Dumas combustion nitrogen instrument (model FP-428, Leco Corp., St. Joseph, MI). Bran moisture was determined in a Leco TGA-601 thermogravimetric analyzer.

All statistical analyses were conducted using SAS v. 9.0 (SAS Institute, Cary, NC). Duplicate PPO assay values were averaged to produce a single observation for each replicate in assays of total and soluble PPO from bran. Replicates were analyzed for analysis of variance (ANOVA) and mean separation using the general linear models (GLM) procedure in SAS with $\alpha = 0.05$. Mean separation was evaluated using least significant differences (LSD) (two means) and Duncan's multiple range test (DMRT) (more than two means).

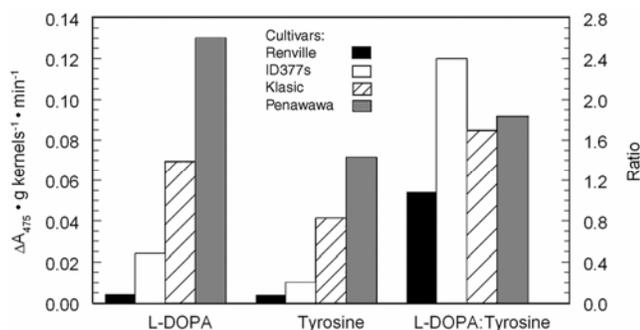


Fig. 1. Total PPO activity using L-DOPA and tyrosine as substrates and ratio of L-DOPA to tyrosine PPO activity in whole kernels of four wheat cultivars. Activity was measured by incubating kernels in buffered 5 mM L-DOPA or tyrosine for 1 hr.

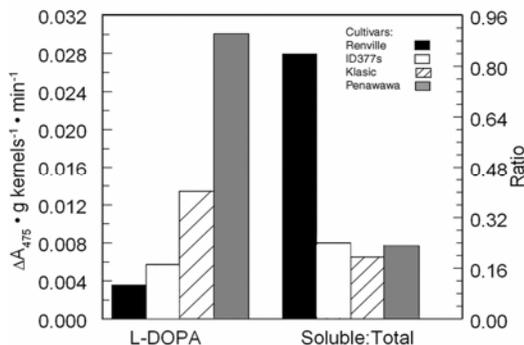


Fig. 2. Soluble PPO activity and ratio of soluble to total PPO activity using L-DOPA as substrate in whole kernels of four wheat cultivars. Activity was measured by preincubating kernels in buffer for 1 hr and measuring PPO activity of the leachate in 5 mM L-DOPA.

Whole Kernel Soluble PPO Leakage Time-Course

The rate of leakage of PPO activity from wheat kernels over time was evaluated using L-DOPA as substrate. Leakage of soluble PPO from whole kernels was most rapid during the first 0.5 hr and slowed thereafter (Fig. 3). The relative activity of PPO in the leachate of these four cultivars at 1 hr was similar to that seen in the soluble whole kernel assays (Fig. 2) and the cultivars were ranked the same. Parallel experiments with tyrosine again showed no activity, indicating that PPO in the leachate was specific to the diphenolic L-DOPA, consistent with the soluble assay described above (Fig. 2).

Diphenolic Activation of Monophenolase PPO Activity

We observed that soluble wheat PPO, whether leached from kernels (as previously noted) or extracted from bran (data not shown), had no activity with tyrosine (a monophenol substrate). It is well documented that *o*-diphenolic compounds at catalytic concentrations can eliminate the lag phase of monophenolase activity (Steffens et al 1994; Perez-Gilbert and Garcia-Carmona 2000). We evaluated eight potential diphenolic activators of monophenolase activity including caffeic acid, chlorogenic acid, L-DOPA, catechol, 4-methyl-catechol, protocatechuic acid, hydrocaffeic acid, and (+)-catechin (results not shown for the latter six compounds). Only caffeic acid and chlorogenic acid exhibited three properties: 1) effective activators of soluble monophenolase PPO activity when tyrosine was used as the substrate; 2) minimal absorbance at 475 nm of the PPO-catalyzed products of the diphenolic compounds alone; and 3) minimal auto-oxidation rate at pH 9. Therefore chlorogenic and caffeic acids were selected as diphenolic activators of PPO monophenolase activity using tyrosine as the substrate in Penawawa kernel leachate. The average assay value for the no-substrate control (zero concentrations of tyrosine and diphenolic compounds) was $0.0054 \Delta A_{475} \cdot \text{g kernel}^{-1} \cdot \text{min}^{-1}$ (Fig. 4), indicating that the leachate itself had a low level of absorbance at 475 nm. This value is only relevant in assays with very low activity levels such as these. The average absorbance value for tyrosine alone (5 mM tyrosine, 0 μM diphenolic compounds) was $0.0052 \Delta A_{475} \cdot \text{g kernel}^{-1} \cdot \text{min}^{-1}$ (Fig. 4), essentially the same as the no-substrate control, demonstrating that soluble wheat PPO has no activity with added tyrosine alone.

Both diphenolic acids activated PPO monophenolase activity with tyrosine to a similar degree (Fig. 4). PPO activity of the leachate with the diphenolic acids alone was only 8–15% of the activity with tyrosine plus the diphenolic acid activators (Fig. 4) when data were corrected for leachate absorbance. Chlorogenic acid (50 μM) was chosen as the diphenolic activator of PPO using tyrosine as the monophenolic substrate for the subsequent study, but this choice was arbitrary based upon available information.

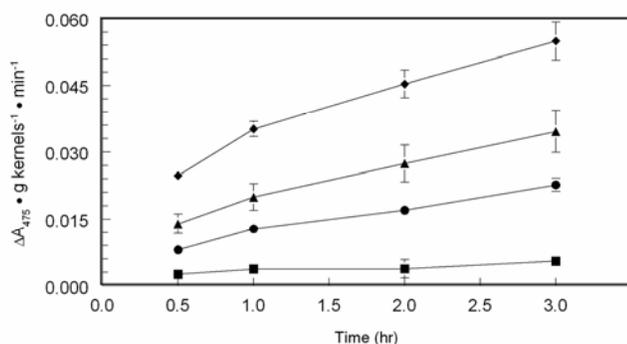


Fig. 3. Cumulative leaching of soluble PPO activity measured with 5 mM L-DOPA substrate from kernels of four wheat cultivars: Renville (■), ID377s (●), Klasic (▲), and Penawawa (◆). Kernels were sequentially preincubated in buffer for 0–0.5, 0.5–1.0, 1.0–2.0, and 2.0–3.0 hr and PPO activity of each leachate was assayed in 5 mM L-DOPA for 1 hr. Error bars represent standard deviations for four replicates.

Leakage of soluble PPO activity was greatest during the first 0.5 hr of incubation and slowed rapidly thereafter with 5 mM tyrosine plus 50 μM chlorogenic acid (Fig. 5). The general pattern of leakage was similar to that observed with L-DOPA substrate (Fig. 3). The soluble PPO activity levels with tyrosine plus chlorogenic acid (Fig. 5) were much less than the kernel leakage assay with L-DOPA (Fig. 3), and less than half the values of total kernel PPO with tyrosine (Fig. 1). The relative activities of PPO among the four lines (Fig. 5) were ranked the same as previously (Figs. 1–3), with Penawawa having the greatest activity and Renville the least.

Bran Total and Soluble PPO Assays

Wheat kernel PPO is primarily localized in the bran which constitutes an enriched source of enzyme (Hatcher and Kruger 1993, 1997). Two hard white spring hexaploid wheat cultivars (ID377s and Klasic) with contrasting PPO levels were chosen for these experiments. Total PPO activity in the homogenized bran suspension, as determined using L-DOPA and a mild extraction buffer, was 2.1-fold higher in the bran of Klasic compared with ID377s (Table I). Soluble PPO activity represented ≈ 5 –6% of the total activity. Therefore, the majority of activity was considered unextracted and remained associated with the homogenized bran particles. Using a buffer containing KCl and the detergents SDS and NP-40, soluble PPO activity increased to 11% of the total (Table I).

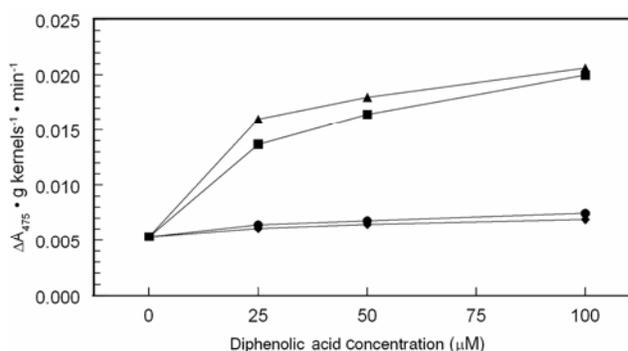


Fig. 4. Effect of caffeic acid and chlorogenic acid concentration on PPO activity with or without tyrosine (5 mM) in Penawawa kernel leachate: chlorogenic acid alone (◆), caffeic acid alone (●), chlorogenic acid plus tyrosine (▲), and caffeic acid plus tyrosine (■). Each data value was corrected for the particular substrate solution (assay buffer control). Data were not corrected for absorbance of kernel leachate alone. All four lines converge at zero concentration, where some symbols are obscured. Error bars representing standard deviations are smaller than the symbols; the greatest was 0.00026.

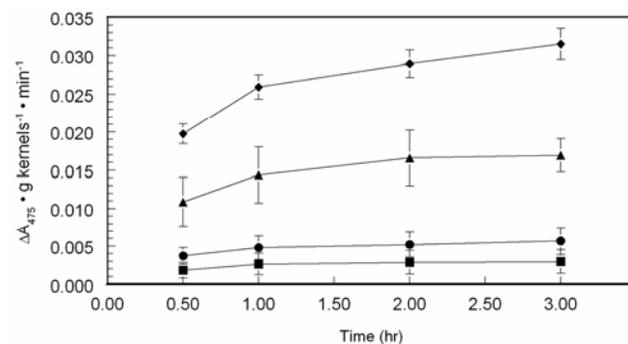


Fig. 5. Cumulative leaching of soluble PPO activity measured with 5 mM tyrosine plus 50 μM chlorogenic acid substrates from kernels of four wheat cultivars: Renville (■), ID377s (●), Klasic (▲), and Penawawa (◆). Kernels were sequentially preincubated in buffer for 0–0.5, 0.5–1.0, 1.0–2.0, and 2.0–3.0 hr and PPO activity of each leachate was assayed in 5 mM tyrosine plus 50 μM chlorogenic acid for 1 hr. Error bars represent standard deviations for four replicates.

DISCUSSION

Total soluble protein extracted from the bran was fairly similar for the two cultivars, which was $\approx 34\text{--}40 \text{ mg} \cdot \text{g bran}^{-1}$ (Table II). For comparison, Dumas combustion $\text{N} \times 5.7$ was $\approx 176 \text{ mg} \cdot \text{g bran}^{-1}$ (as is, $\approx 14.2\%$ moisture basis in both cultivars). With the detergent buffer, protein in the soluble fraction increased to $55 \text{ mg} \cdot \text{g bran}^{-1}$ for both cultivars. Specific activity of PPO in the soluble fraction was approximately 3-fold higher in Klasic than in ID377s using both extraction buffers (Table II).

The effect of detergent versus mild extraction buffers is summarized in Table III. Detergent extraction buffer increased total PPO activities 1.2-fold in ID377s and somewhat more (1.5-fold) in the high PPO activity cultivar Klasic. In comparison, the increase in soluble PPO activity was much greater (≈ 2.5 -fold) in both cultivars. This increase may have been due, in part, to greater protein solubility as the increase in soluble protein using the detergent buffer was on the order of 1.5-fold greater. In addition, detergent buffer increased PPO activity/unit of protein (specific activity) by 1.8-fold in ID377s and 1.6-fold in Klasic. On a percentage basis, detergent increased soluble PPO activity and decreased unextracted PPO activity (Table I). However, on an absolute basis, detergent increased not only soluble PPO, as previously noted, but also unextracted PPO 1.1-fold to 1.4-fold (Tables I and III).

AACC Approved Method 22-85

Whole kernel PPO activity measured using AACC Approved Method 22-85 was compared with total bran PPO activity using the same assay buffer, 10 mM L-DOPA , 50 mM MOPS , $\text{pH } 6.5$ (Table IV). Bran data were from extraction in mild buffer and were calculated on a per gram of kernels basis to facilitate the comparison. The PPO activities in the whole kernel assays were 1.6–1.7% of those in the total bran PPO assay and indicate the extraction efficiency associated with homogenizing the bran. However, the PPO activity in Klasic relative to that in ID377s was similar in both assays, being 2-fold higher in Klasic in both instances. The total bran PPO activities (Table IV) were ≈ 100 -fold higher than those obtained in the whole kernel assays (using 5 mM L-DOPA) (Fig. 1). The soluble PPO activity extracted from bran (Table I) recalculated on a per gram of kernels basis was 25-fold to 30-fold higher than that which freely leached from intact kernels (Fig. 2).

Polyphenol oxidase is enzymatically complex with latent and bound forms that have varied and often broad substrate specificity for both monophenols and diphenols (Steffens et al 1994). All of these phenomena were observed with PPO from wheat grain in the present study. The four most salient results of the research reported here are 1) most PPO activity in hexaploid wheat is insoluble (i.e., it does not freely leach from whole kernels nor is it readily extracted in buffer); 2) a more aggressive buffer that includes detergent extracts and activates more PPO compared with a mild buffer but this soluble PPO is nevertheless still a minor fraction of the total activity; 3) the soluble PPO that does leach from kernels does not oxidize the monophenol tyrosine in the absence of diphenolic activators; and 4) regardless of the specific assay, buffer system, grain fraction, or substrate that was used, the four wheat cultivars were ranked the same for PPO activity and generally were significantly different from one another. Consequently, the conclusion is that even though simple PPO kernel assays measure a minor fraction of kernel PPO, they are effective in differentiating PPO levels among cultivars and grain lots. These low PPO activity levels are more likely to produce more desirable consumer products (Morris 1995).

In our evaluation, the whole kernel assay using Approved Method 22-85 (AACC International 2000) measured only 1.6–1.7% of total wheat kernel PPO, as measured in a homogenized bran extract (Fig. 1, Tables I–IV). In another study, total PPO in a whole kernel assay was $\approx 4\%$ of the total PPO in extracted whole meal (Kruiger et al 1994); our value is similar in magnitude despite using different protocols. Yet, somewhat surprisingly, total PPO activity in whole kernel assays was much greater than the PPO activity which is freely soluble (leached) into the bathing buffer solutions in hexaploid wheat (Figs. 1–3). Therefore, there is a considerable amount of catalytically active PPO associated with wheat kernels that is not freely soluble. Consequently, exogenous substrates supplied in these whole kernel PPO assays must

TABLE I
Total, Soluble, and Unextracted^a PPO Activity^b ($\Delta A_{475} \cdot \text{g bran}^{-1} \cdot \text{min}^{-1}$)
from Wheat Bran Using Mild and Detergent Buffers

PPO Activity	Buffer	Cultivar	
		ID377s	Klasic
Total	Mild	10.9 (100) ^c	23.3 (100)
Soluble	Mild	0.5 (5)	1.5 (6)
Unextracted	Mild	10.3 (95)	21.8 (94)
Total	Detergent	12.7 (100)	34.7 (100)
Soluble	Detergent	1.4 (11)	3.9 (11)
Unextracted	Detergent	11.3 (89)	30.8 (89)

^a Unextracted PPO activity calculated by difference of total minus soluble.

^b All activities obtained using 10 mM L-DOPA substrate.

^c Percentage of total activity indicated in parentheses.

TABLE II
Total Protein and PPO Specific Activity^a in Soluble Fraction of Wheat Bran Extracted Using Mild and Detergent Buffers

Property	Buffer	Cultivar	
		ID377s	Klasic
Total protein ($\text{mg} \cdot \text{g bran}^{-1}$)	Mild	40	34
	Detergent	55	55
PPO specific activity ($\Delta A_{475} \cdot \text{g protein}^{-1} \cdot \text{min}^{-1}$)	Mild	13	43
	Detergent	25	70

^a Activities obtained using 10 mM L-DOPA substrate.

TABLE III
Effect of Detergent vs. Mild Extraction Buffer on PPO Activity^a
Parameters and Soluble Protein in Wheat Bran

Property	Cultivar	
	ID377s	Klasic
Total PPO activity	1.2 ^b	1.5
Soluble PPO activity	2.5	2.6
Unextracted PPO activity	1.1	1.4
Soluble protein	1.4	1.6
PPO specific activity	1.8	1.6

^a Activities obtained using 10 mM L-DOPA substrate.

^b Fold-difference, detergent buffer activity relative to mild buffer activity.

TABLE IV
Comparison of Total PPO Activity from Wheat Bran^a
with Total PPO Activity Measured in Whole Wheat Kernels
Using AACC Approved Method 22-85^b

Assay	Cultivar	
	ID377s	Klasic
Bran total PPO Activity ($\Delta A_{475} \cdot \text{g kernel}^{-1} \cdot \text{min}^{-1}$)	2.72	5.53
Whole kernel assay PPO activity ($\Delta A_{475} \cdot \text{g kernel}^{-1} \cdot \text{min}^{-1}$)	0.044 (1.6) ^c	0.094 (1.7)

^a PPO activity in ground bran suspension obtained using mild extraction buffer; activity measured with 10 mM L-DOPA substrate; activity was calculated on a gram of kernels basis using milling bran yield.

^b Method 22-85 uses 10 mM L-DOPA substrate.

^c Whole kernel PPO activity as a percentage of bran PPO activity indicated in parentheses.

first diffuse into the kernel surface where they react with immobilized or bound PPO, and then the reaction products must diffuse back out into the bathing solution where their absorbance is detected. This model is speculative, however.

Homogenizing the bran milling fraction in a mild buffer similar to that used in Approved Method 22-85 increased PPO activity \approx 60-fold compared with the activity obtained in whole kernels using Approved Method 22-85 (Table IV). Homogenizing the bran presumably provided greater physical access to the PPO that is bound and constitutes a far greater proportion of kernel PPO. In contrast with this result, Demeke et al (2001a) found that physically abrading the bran on the outside of intact kernels produced only a small initial increase in PPO L-DOPA assay activity (similar to Approved Method 22-85), whereas further abrasion decreased PPO activity as bran tissue was removed. In our study, homogenizing the bran did not substantively change the fact that most PPO remained bound and associated with the bran pellet.

Elaborating further on the observed substrate specificity of wheat kernel PPO showed that activity levels were significantly higher with L-DOPA than with tyrosine (or tyrosine plus chlorogenic acid) in the total and soluble (leachate) whole kernel enzyme assays (Figs. 1–5). For example, the whole kernel PPO activity of Penawawa was 1.8-fold higher with L-DOPA compared with tyrosine (Fig. 1). Even when soluble kernel PPO in Penawawa was activated with chlorogenic acid in the tyrosine assays, L-DOPA activity was 1.3-fold and 1.7-fold higher than the tyrosine activity at the 1 and 3 hr time points, respectively (Figs. 3 and 5). Assay pH may explain part of this difference because the pH optimum of plant PPO is typically close to pH 7, with significantly lower activity at pH 9 (Perez-Gilabert and Garcia-Carmona 2000). Our L-DOPA assays and Approved Method 22-85 were conducted at pH 6.5, while the tyrosine assays were conducted at pH 9. It was not possible to conduct these studies at a common pH when using 5–10 mM substrates because tyrosine is minimally soluble at the lower pH and L-DOPA auto-oxidizes rapidly at higher pH (Anderson and Morris 2001). Even when pH can be held constant, PPO activities with monophenolic substrates are often lower than those with diphenolic substrates (Anderson and Morris 2001). For example, the diphenolase/monophenolase activity ratio for eggplant (*Solanum melongena* L.) PPO was 41 when using 4-methyl catechol and *p*-cresol as substrates (Perez-Gilabert and Garcia-Carmona 2000).

It is noteworthy that the small amount of PPO that is freely soluble from intact wheat kernels (or solubilized from bran) is nonreactive with the monophenol tyrosine in the absence of low concentrations of diphenolic activators. This lack of monophenolase activity when using tyrosine as substrate was unexpected because high levels of PPO activity were observed with tyrosine in whole kernel assays (Fig. 1) (Anderson and Morris 2001). It is possible that bound PPO has monophenolase activity, or that endogenous phenolics or altered pH promote monophenolase activity in the microenvironment of the kernel surface. One may conclude from the evidence at hand that any diphenols that leach from the kernel during the assay are insufficient to activate soluble PPO when tyrosine is used as the exogenous substrate.

Further studies are needed to optimize the soluble monophenolase assay using tyrosine plus chlorogenic or caffeic acid. Also, the contribution of the diphenolic compounds alone to reaction rates should be evaluated in cultivars other than Penawawa. It may be possible to further reduce interfering absorbance from PPO products of the diphenolic compounds by finding alternative compounds or by measuring products at a longer wavelength (e.g., 550 nm). Longer wavelengths may be more selective because tyrosine PPO products absorb at longer wavelengths than the products of caffeic acid and chlorogenic acid (results not shown). In addition, detergents or chaotropes could be evaluated as soluble PPO activators (Okot-Kotber et al 2002; Jukanti et al 2003) when tyrosine is used as the sole substrate. Alternative mono-

phenol substrates should also be evaluated, particularly those with higher solubility at neutral pH.

Detergent buffer increased total, soluble, and unextracted PPO activities to varying degrees (Tables I and III). The 2.5-fold to 2.6-fold increase in soluble PPO may be attributed to both PPO activation, as indicated by increased specific activity (1.6-fold to 1.8-fold) and increased protein extraction (1.4-fold to 1.6-fold) (Tables II and III). Even though detergent buffer increased soluble PPO, it is clear that the vast majority of PPO is not readily solubilized using the extraction protocol and buffers reported here. Reports utilizing alternative extraction protocols indicated that SDS in the range of 0.1–5.6% increased soluble PPO activity from 2-fold to 7-fold (Marsh and Galliard 1986; Okot-Kotber et al 2002; Jukanti et al 2003). If such increases were applied to our protocol, in which only 5–6% of total bran PPO was extracted without detergent (Table I), soluble PPO activity would still be less than half of the total. In addition, insoluble PPO activity appears to increase, not decrease, with addition of detergent. Unextracted PPO activity increased 1.1-fold to 1.4-fold with our detergent buffer (Table I) and 2.2-fold to 3.8-fold with 0.1–5.6% SDS, respectively (Marsh and Galliard 1986). These increases in unextracted PPO activity may be due to PPO activation or to increased substrate-enzyme accessibility. We observed that 89–95% of PPO was unextracted (Table I), whereas Marsh and Galliard (1986) reported that 53–88% of PPO was unextracted. Several differences in protocols may have contributed to these differences, possibly including increased homogenization of the bran particles (greater surface area) with the polytron in our protocol. Nonetheless, both studies indicated that greater than half of the total PPO is insoluble. These observations collectively indicate that 1) detergents are clearly but nonetheless only moderately effective in extracting and activating wheat kernel PPO (Tables I–IV) (Marsh and Galliard 1986; Okot-Kotber et al 2002; Jukanti et al 2003); 2) it is not possible to assess PPO extraction (solubilization) efficiency without also measuring insoluble PPO levels because detergents can increase both soluble and insoluble PPO; and 3) inferences as to the total PPO activity in wheat kernels are equivocal because the degree of latency of soluble and insoluble PPO varies, substrate activity and specificity vary, and the bran milling fraction probably contains most but not all of the kernel PPO.

Although the AACC Approved Method 22-85 whole kernel PPO assay apparently detected <2% of the total PPO present in the bran, the assay was an accurate indicator of the relative quantities of PPO in our study. Twice as much PPO activity was present in Klasic than ID377s, both when measured in whole kernels and as total PPO in extracted bran (Table IV). Kruger et al (1994) also concluded that their whole kernel PPO assay was well correlated with the PPO assay of whole meal.

Some genetic inferences may be drawn from the results. The L-DOPA soluble-to-total PPO activity ratio in whole kernel assays was >0.8 in Renville durum versus \approx 0.2 in the hexaploid wheat cultivars (Fig. 2). In addition, the L-DOPA-to-tyrosine activity ratio was \approx 1.0 for Renville versus 1.7–2.4 for the hexaploid cultivars (Fig. 1). These differences in PPO solubility and substrate specificity may partly result from different PPO isoforms (soluble vs. bound) present in the kernels and may be related to the absence of PPO activity associated with the D-genome in Renville, specifically chromosome 2D (Jimenez and Dubcovsky 1999; Anderson and Morris 2001; Demeke et al 2001b; Mares and Campbell 2001). However, more extensive comparisons of cultivars and controlled genetic studies are required to substantiate this hypothesis. Total PPO activity ranges overlap among durum and hexaploid wheat lines (Bernier and Howes 1994) and activity was not significantly different between Renville and ID377s using tyrosine in the whole kernel assay (Fig. 1).

In conclusion, our results indicate that whole kernel PPO assays similar to AACC Approved Method 22-85 measure both soluble and insoluble PPO components and the contribution of these two

components varied among the four wheat cultivars evaluated. The insoluble component predominated in the whole kernel assay in the three hexaploid cultivars. When PPO was extracted from homogenized bran using both mild and more aggressive detergent buffers, a maximum of only 11% of the total activity was recovered in the soluble fraction. Further characterization of the insoluble PPO component is needed because this activity predominates and may be more important than soluble activity in the discoloration of wheat products. Nevertheless, these results indicate that for screening hexaploid wheat germplasm and grain lots, whole kernel PPO assays such as AACC Approved Method 22-85 are effective in delineating differences and identifying lines with lower PPO activity levels.

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