JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Delineating the Role of Polyphenol Oxidase in the Darkening of Alkaline Wheat Noodles

E. PATRICK FUERST,[†,‡] JAMES V. ANDERSON,[§] AND CRAIG F. MORRIS*[,‡]

Department of Crop & Soil Sciences, Washington State University, and Western Wheat Quality Laboratory, Agricultural Research Service, U.S. Department of Agriculture, E-202 Food Science & Human Nutrition Facility East, P.O. Box 646394, Washington State University, Pullman, Washington 99164-6394, and Biosciences Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Fargo, North Dakota 58105-5674

This study evaluated the effects of inhibitors on polyphenol oxidase (PPO) activity, the effect of the PPO inhibitor tropolone on noodle darkening, and the correlation of PPO activity with darkening of alkaline noodles. The PPO inhibitors tropolone and salicylhydroxamic acid (each at 1 μM) reduced kernel PPO activity by approximately 50% in three hexaploid wheat cultivars but did not inhibit PPO activity in the two very low PPO cultivars, durum Langdon, and the synthetic hexaploid-derived ID580. Tropolone (100 μg/g flour) inhibited alkaline noodle darkening (ΔL^*) by 13−25% in the low PPO wheat cultivar, ID377s, and by 39−54% in the high PPO wheat cultivar, Klasic. Alkaline noodle darkening among 502 wheat samples was correlated with kernel PPO activity ($r = 0.64$). Results substantiate the hypothesis that PPO plays a major role in darkening of alkaline noodles. However, results also indicate that substantial darkening would occur even at zero PPO activity, as measured in the kernel PPO assay. Therefore, darkening of alkaline noodles is probably due to the cultivar-specific level of PPO activity and the presence of at least one additional darkening mechanism. Further investigation is required to identify the phenolic discoloration agent(s) and to determine the potential roles of non-PPO discoloration mechanisms, both enzymatic and nonenzymatic, in wheat products.

KEYWORDS: Wheat; *Triticum aestivum*; polyphenol oxidase; alkaline noodles; noodle darkening; discoloration; wheat quality; darkening mechanisms; inhibitors

INTRODUCTION

Polyphenol oxidase (PPO) has been implicated as a leading cause of discoloration in raw Asian noodles and other wheat products (*1*, *2*). Darkened wheat products are unacceptable to consumers, and there has been a considerable effort to reduce genetic levels of PPO activity in wheat germ plasm. Kernel PPO activity assays, such as AACC Approved Method 22-85 (*3*,*4*), have been useful for eliminating high PPO lines in wheat breeding programs.

Both PPO and phenolic compounds are found primarily in the bran layer of wheat; hence, flour PPO activity and flour phenolic content increase with flour extraction rate (*5*, *6*). PPO catalyzes the hydroxylation of monophenols to *o*-diphenols ("monophenolase" activity) and the oxidation of *o*-diphenols to *o*-quinones ("diphenolase" activity) (*7*). The quinone products of PPO react with a number of functional groups, such as amines, thiols, and phenolics, and form complex colored products (melanins) (*8*). This is the basis for PPO-mediated discoloration of many food products. The extent to which discoloration of wheat products is due to PPO, and whether PPO is the sole mechanism of discoloration, is not entirely clear. One report indicated that significant discoloration could occur at near-zero PPO levels (*9*).

There is limited information available pertaining to the effects of PPO inhibitors on discoloration of wheat products and inhibition of PPO. Many reducing compounds, such as ascorbate, sodium bisulfite, and thiol compounds, inhibit darkening of food products both by inhibiting PPO and by reducing the quinone products of PPO to the original diphenolic compound (*10*). As a result of the dual mode of action of these compounds, it is not possible to know the extent to which inhibition of darkening is due to PPO inhibition vs termination of polymerization reactions. A number of alternative inhibitors are available. Tropolone (2-hydroxy-2,4,6-cycloheptatriene), L-mimosine {α-amino-β-(N-[3-hydroxy-4-pyridone])propionic acid}, and kojic acid (5-hydroxy-2-hydroxymethyl-4-pyranone) have adjacent ring-hydroxyl and -keto groups and thus are half-product analogues. Tropolone and 4-hexylresorcinol (4-hexyl-1,3-dihydroxybenzene) were competitive inhibitors of eggplant (*Solanum melongena* L.) PPO (*11*). Cinnamic acid (3-phenyl-2-propenoic acid) and salicylhydroxamic acid (SHAM) are also PPO

* To whom correspondence should be addressed. Tel: +1-509-335-4062. Fax: +1-509-335-8573. E-mail: morrisc@wsu.edu.
[†] Department of Crop & Soil Sciences, Washington State University.
[‡] Western Wheat Quality Laboratory, U.S. Department of Agriculture.
[§] Biosciences Research Laboratory, U.S. Department of Agriculture.

10.1021/jf0526386 CCC: $33.50
Published on Web 02/23/2006

inhibitors (*12*, *13*). The inhibition of Asian noodle darkening by PPO inhibitors tropolone and SHAM was previously indicated but not quantified (*14*).

Several reports have evaluated the relationship between PPO and darkening of raw Asian wheat noodles. Correlations between kernel PPO and ΔL^* (0−24 or 0−75 h) in alkaline (Cantonese) and/or white-salted (udon) noodles ranged from $r = 0.41$ to 0.87 (*1*, *15*−*18*). Martin et al. (*19*) selected for high and low PPO groups within seven wheat populations. The PPO activity in flour from high PPO population groups was 3−5 times greater than flour from low PPO population groups. However, the difference in darkening was relatively small, only 0.7 units on average (ΔL^*, 0−24 h), between alkaline noodles from high vs low PPO population groups. These studies indicate that kernel PPO assays have been only partially effective in predicting noodle darkening.

The goal of our research is to characterize and minimize the biochemical mechanism(s) that contributes to darkening of wheat products, with the initial purpose of delineating the contribution of PPO in alkaline noodle darkening. The specific objectives of this study were to (i) evaluate the activity of known PPO inhibitors on PPO activity in kernel assays, (ii) evaluate the effect of the highly active PPO inhibitor tropolone on darkening of alkaline noodles, and (iii) conduct a retrospective study on the correlation of alkaline noodle color parameters with kernel PPO activity, flour ash, and flour protein, using samples from standard wheat quality evaluations at the U.S. Department of Agriculture Western Wheat Quality Laboratory in Pullman, WA.

METHODS AND MATERIALS

Genetically pure, single-origin dryland grain lots of durum Langdon (CItr 13165), hard white spring hexaploid ID580 (aka IDO580, PI 620638), and soft white spring hexaploid wheat cultivar Penawawa (PI 495916) were used throughout this research as indicated. ID580 has near-null kernel PPO activity and was derived from a cross that included the near-null PPO synthetic hexaploid, M2 [*Triticum turgidum* subsp. *durum* (Desf.) × *Aegilops tauschii*; these synthetics are also referred to as *X Aegilotriticum* sp.] from CIMMYT (*20*). Experiments using hard white spring hexaploids ID377s (aka IDO377s, PI 591045) and Klasic (PI 486139) were produced in Pullman, WA, in 2003, unless otherwise indicated. One experiment compared ID377s and Klasic grain lots produced in 2003 with grain lots produced in 2000 in Aberdeen, ID. Kernels of all grain lots were plump and well-filled; mean kernel weights were 42.8 mg for Langdon, 33.9 mg for ID580, 38.7 mg for Penawawa, 37.9 mg for ID377s 2000, 34.3 mg for ID377s 2003, 43.3 mg for Klasic 2000, and 36 mg for Klasic 2003. Grain lots were tempered, in accordance with their hardness, to final moisture contents of 13% for Penawawa, 16% for Langdon, and 14.5% for ID377s, ID580, and Klasic. Tempered wheat was milled on a Quadrumat flour mill (C. W. Brabender Instr., Inc., Hackensack, NJ) following the method of Jeffers and Rubenthaler (*21*). This system produced break flour, middlings, and bran; regrinding the middlings produced reduction flour and shorts. Noodles were made from blended break and reduction flours (straight-grade).

Grain lots were evaluated for kernel PPO, flour PPO, ash, and protein. Kernel PPO was determined according to Approved Method 22-85 (*3*, *4*) with 10 mM L-DOPA, five kernels per replicate and four replications per grain lot. The PPO activity of 200 mg of flour was measured in a 2 mL microcentrifuge tube by adding 1.5 mL of 10 mM L-DOPA, 50 mM MOPS (3-[*N*-morpholino]propane sulfonic acid), pH 6.5, plus 0.02% Tween-20. Samples were incubated for 1 h on an end-over-end rotating (8 rpm) mixer (Labquake model 415110, Barnstead/Thermolyne) and centrifuged for 3 min at 10000*g*. The PPO activity was measured as absorbance at 475 nm (A_{475}) on a Shimadzu BioSpec-1601 spectrophotometer using Shimadzu UVProbe v. 2.00 software. Control flour samples were assayed as described above but without L-DOPA substrate. The absorbance of control flour samples was subtracted from the absorbance of L-DOPA flour samples for each specific grain lot. There were two replicates each of control and L-DOPA substrate samples per experiment. Results of two experiments were combined and expressed as ΔA_{475}/min g flour. Flour ash was determined using a Leco TGA-601 Thermogravimetric Analyzer, and flour protein ($N \times 5.7$) was determined following Approved Method 46-30 (*3*) on a Dumas combustion nitrogen instrument (model FP-428, Leco Corp., St. Joseph, MI). Both ash and protein were expressed on a 14% flour moisture basis.

Inhibitors of Kernel PPO Activity. The procedure for studying inhibitors of kernel PPO activity was based on AACC Approved Method 22-85 (*3*). For each sample, five kernels were placed in a 2 mL microcentrifuge tube and preincubated for 30 min in 1.35 mL of a given inhibitor in 50 mM MOPS, pH 6.5, plus 0.02% (v/v) Tween-20 at room temperature (21 °C) with constant mechanical mixing as previously described. This preincubation assured that PPO would be inhibited to the maximum extent before adding substrate. Following preincubation, 0.15 mL of 10 mM L-DOPA was added. The final L-DOPA concentration, 1 mM, was 1/10 the concentration in Approved Method 22-85 (*3*). The limited solubility of L-DOPA precluded obtaining a final concentration of 10 mM with this protocol. Final inhibitor concentrations are reported. The PPO activity was measured as previously described. The kernel leachate itself had a measurable A_{475} value, which can affect results with very low PPO samples (*22*). Thus, the A_{475} of the kernel leachate was determined using the protocol described above, omitting inhibitors and substrate; leachate absorbance was subtracted as background for each grain lot. Each assay was replicated four times, each experiment was replicated (two complete blocks), and the results of combined experiments were expressed as ΔA_{475}/min g kernels. All reagents were from Sigma.

Effect of Tropolone on Alkaline Noodle Darkening. The hard white spring cultivars ID377s and Klasic were selected for this study based on their contrasting properties: Klasic had a higher PPO activity and greater noodle darkening than ID377s (*23*). Alkaline noodles were produced as previously described (*23*), with modifications. The noodle formula consisted of 15 g (14% moisture) of flour, 36% (v/w, flour weight basis) water absorption, 0.5% (w/w) sodium carbonate, 2% (w/w) sodium chloride, and a range of tropolone concentrations. The dough was mixed without resting using a planetary pin type mixer (National Manufacturing Co., Lincoln, NE; bowl volume, 48 mL) with a head speed of 220 rpm. The dough was scraped off the head pins, bowl side, and bottom at 1.5 min; the total mixing time was 4 min. The dough was compressed into a rectangular block by hand, and noodle sheets (approximately 10 cm × 10 cm) were produced by progressively reducing the gap setting in a pasta maker (model No. 150, Imperia Trading S.r.l., Andt' Ambrogio di Torino, Italy), with a 1.5 mm final noodle thickness. A previous study compared small-scale noodles made with a pasta maker with larger- scale noodles (*24*). Results indicated that color parameters of small- and large-scale noodles varied quantitatively but not qualitatively; however, there was little effect of noodle sheet size on ΔL^*.

Noodle sheet color values (L^*, a^*, and b^*; Commission International l'Eclairage, CIE, *25*) were measured with a chromometer (model 310, Minolta Camera Co., Ltd., Osaka, Japan) with a 50 mm (diameter) measuring tube, using a white tile background as previously described (*23*). L^*, a^*, and b^* values denote lightness (white-black), red-green, and yellow-blue scales, respectively. Three color readings per noodle sheet were made at 0, 2, 4, 6, 8, and 24 h. Noodle sheets were stored in plastic bags at 21 °C between color readings. Changes in color values (ΔL^*, Δa^*, and Δb^*) were calculated by subtracting readings at 2, 4, 6, 8, or 24 h from zero-time readings. There were two experiments. The first experiment evaluated the effect of tropolone concentration on noodle color in ID377s 2003 and Klasic 2003. The second experiment evaluated the effect of crop year (2000 vs 2003) on tropolone response in ID377s and Klasic. There were two replicate noodle sheets for each treatment, and each experiment was replicated (two complete blocks).

Retrospective Analysis of Wheat Quality Parameters. A retrospective study on alkaline noodle darkening was conducted on data accrued from 502 hexaploid wheat samples subjected to standard wheat quality testing at the U.S. Department of Agriculture Western Wheat

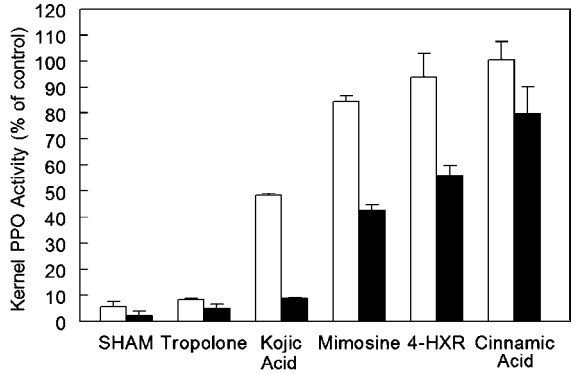

Figure 1. Effect of six PPO inhibitors on kernel PPO activity of hard white spring wheat cultivar Klasic 2003. Abbreviation: 4-HXR, 4-hexylresorcinol. Kernels were preincubated for 30 min with an inhibitor, and then, L-DOPA substrate (1 mM final concentration) was added for 60 min. The final inhibitor concentrations were 0.1 (open bars) or 1 mM (closed bars). Error bars indicate standard deviations. "Control" samples (not shown) had no added inhibitor, and the mean control activity was 0.026 ΔA_{475}/min g kernels. PPO activities are expressed as a percentage of control.

Quality Laboratory. Samples came from wheat breeding nurseries grown at numerous locations in the Pacific Northwest and harvested in 2003 (12% of samples) and 2004 (88% of samples). In total, the wheat samples were comprised of 53% hard white spring, 25% soft white winter, 14% hard white winter, and 8% other wheat classes. The kernel PPO was measured according to Approved Method 22-85 (*3*) using 10 mM L-DOPA substrate and two replications. Samples were milled on the Quadrumat flour mill (previously described) and subjected to the following analyses: alkaline noodle color parameters (L^*, a^*, and b^*) at 0 and 24 h (*23*), flour ash by the oven method according to Approved Method 08-01 (*3*), and flour protein by NIR according to Approved Method 39-11 (*3*).

Statistical Analyses. All statistical analyses were conducted using SAS Version 9.0 (SAS Institute, Cary, NC). In the retrospective data analysis, duplicate PPO assay values were averaged to produce a single observation for each wheat sample. Results of two or more combined experiments (blocks) are presented, except for the retrospective data analysis. Standard deviations were calculated for each experiment, and the average experimental sample standard deviation is reported. Replicates were analyzed for analysis of variance and mean separation using the general linear models procedure in SAS with α set equal to 0.05. Mean separation was evaluated using Duncan's multiple range test.

RESULTS AND DISCUSSION

Inhibitors of Kernel PPO Activity. The effects of six PPO inhibitors at 0.1 or 1 mM on kernel PPO activity were evaluated in Klasic wheat (**Figure 1**). SHAM and tropolone were clearly the most active, followed by kojic acid, mimosine, 4-hexylresorcinol, and cinnamic acid. The inhibitory activity of SHAM and tropolone was also evaluated at lower concentrations. These two compounds had very similar levels of activity and caused approximately 50% inhibition at 1 μM (**Figure 2**). In comparison, Osuga and Whittaker (*10*) reported that reducing agents, such as ascorbate, sodium bisulfite, and thiol reagents, required concentrations of $\geq$0.1 mM to inhibit mushroom PPO by 50% and that onset of inhibition took place over 1−3 h.

The response of kernel PPO activity to 1 μM SHAM and tropolone was compared among five wheat cultivars (**Figure 3**). There was a relatively wide range of kernel PPO activity among the three hexaploid wheat cultivars, ID377s, Klasic, and Penawawa, in the absence of inhibitors (open bars); kernel PPO activities were reduced approximately 50% by each inhibitor

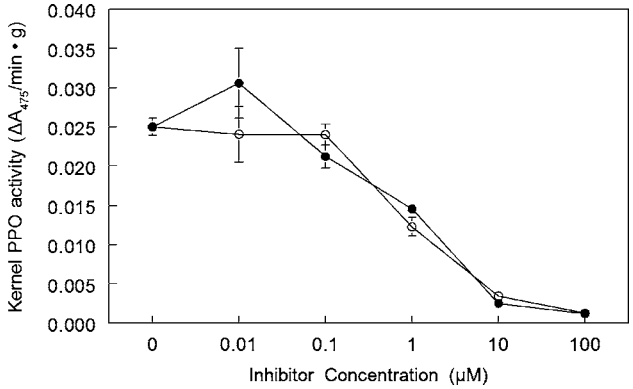

Figure 2. Effect of SHAM (open circles) and tropolone (closed circles) concentrations on kernel PPO activity in Klasic 2003. Assays were conducted as in **Figure 1**. Error bars indicate standard deviations; where no error bars are visible, the standard deviation is less than the size of the symbol.

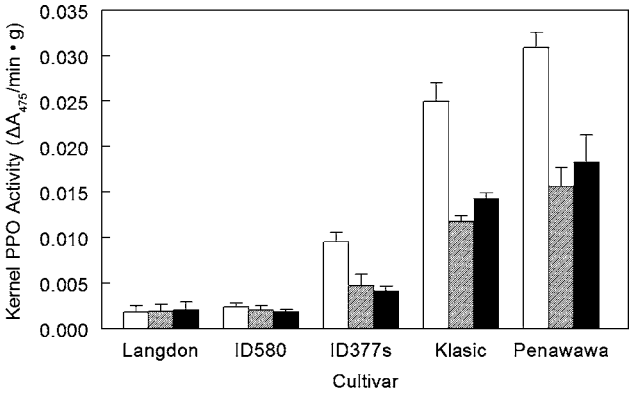

Figure 3. PPO activity with no inhibitor (open bars), with 1 μM tropolone (striated bars), and with 1 μM SHAM (closed bars) in five wheat cultivars. Assays were conducted as described in **Figure 1**. Error bars indicate standard deviations.

in these cultivars. However, there was no significant effect of either inhibitor on the very low PPO activity levels of ID580 and Langdon.

Effect of Tropolone on Alkaline Noodle Darkening. Studies were conducted with small-scale noodle sheets (15 g of flour) rather than larger (80−100 g) noodle sheets that are standard in our laboratory (*23*) (see Methods and Materials). Darkening, measured as ΔL^* (0−24 h), was similar in the 15 and 80 g noodle sheets of ID377s 2003 and Klasic 2003 (**Table 1**), indicating that the two procedures provided similar results.

Tropolone was selected to test the role of PPO in darkening of alkaline noodles. This selection was somewhat arbitrary since neither tropolone nor SHAM can be considered monospecific inhibitors. Tropolone chelates metals (*26*) while SHAM inhibits a broader known array of oxidative enzymes (*27*−*29*). As the tropolone concentration increased, noodle darkening measured as ΔL^* (0−24 h) decreased (**Figure 4A**) and L^* (24 h) increased (data not shown) in a log-linear fashion in Klasic 2003. At the highest tropolone concentration, ΔL^* (0−24 h) values were similar for Klasic and the low PPO cultivar ID377s (**Figure 4A**). In ID377s, ΔL^* (0−24 h) was relatively unresponsive to tropolone. Visual noodle speckiness was inhibited by 100−316 μg/g tropolone (data not shown). Klasic showed much greater visual speckiness than ID377s, and likewise, inhibition of speckiness by tropolone was more dramatic in Klasic than in

Table 1. PPO Activity, Alkaline Noodle Color, and Flour PPO, Ash, and Protein of Wheat Grain Lots[a]

cultivar/ grain lot	kernel PPO[b] (ΔA_{475}/min g)	noodle ΔL^* (0–24 h) 80 g flour	noodle ΔL^* (0–24 h) 15 g flour	flour PPO (ΔA_{475}/min g)	flour ash (%)	flour protein (%)
ID377s 2000	0.051 c		7.8 c	0.0029 cd	0.43 b	12.4 b
Klasic 2000	0.103 b		16.3 a	0.0083 a	0.39 c	13.0 a
ID377s 2003	0.041 c	6.4 c	5.8 d	0.0035 c	0.41 bc	11.4 c
Klasic 2003	0.110 b	10.2 a	10.7 b	0.0089 a	0.31 d	10.9 de
Penawawa	0.121 a	10.7 a		0.0069 b	0.44 b	8.1 f
ID580	0.008 d	6.7 c		0.0018 e	0.44 b	10.6 e
Langdon	0.008 d	8.9 b		0.0022 de	0.61 a	11.7 c

[a] Numbers within columns followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple range test. [b] Determined by Approved Method 22-85 (*3*) with 10 mM L-DOPA.

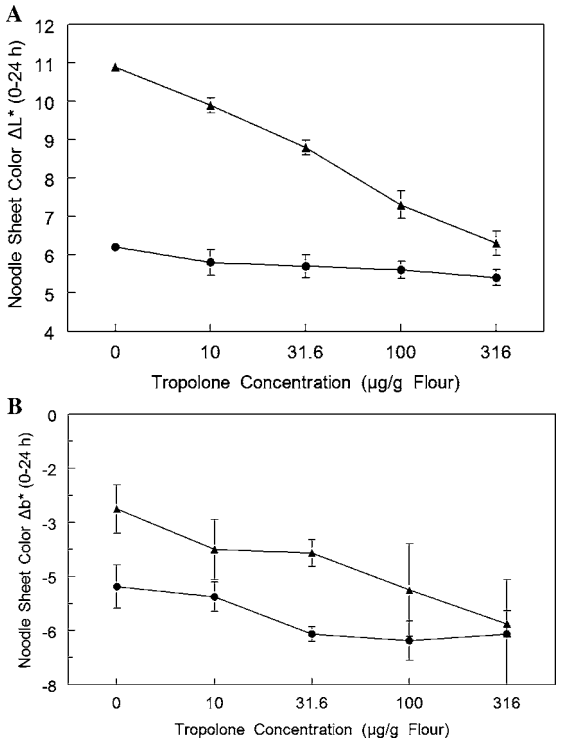

Figure 4. Effect of tropolone concentration on (**A**) ΔL^* (0–24 h) and (**B**) Δb^* (0–24 h) in 15 g alkaline noodle sheets; ●, ID377s; ▲, Klasic. For reference, the values of L^* and b^* were, respectively, 86.2 and 22.8 for ID377s (2003) and 85.9 and 22.7 for Klasic 2003, at zero time with zero tropolone. Error bars indicate standard deviations; where no error bars are visible, the standard deviation is less than the size of the symbol. Note that the abscissa is scaled log-linear.

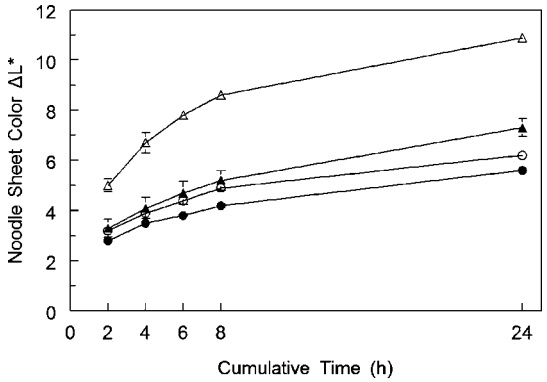

Figure 5. Effect of tropolone (100 μg/g flour) on ΔL^* over a range of time periods in alkaline noodles made from wheat cultivars Klasic (△, control; ▲, tropolone) and ID377s (○, control; ●, tropolone). Data points quantify darkening (decrease in brightness) at 0–2, 0–4, 0–6, 0–8, and 0–24 h. Error bars indicate standard deviations; where no error bars are visible, the standard deviation is less than the size of the symbol.

ID377s. Results indicate that PPO plays a major role in the darkening of noodles made from Klasic and a lesser role in ID377s.

Tropolone had relatively small effects on Δa^* (0−24 h) (data not shown). Negative Δa^* (0−24 h) values ranged from about −1.5 to −2.5, indicating a gradual shift toward reduced green (or increased red) in both cultivars, independent of tropolone concentration. For unknown reasons, concentrations of tropolone ≥100 μg/g flour caused significant red color development by 48 h (data not shown).

As the tropolone concentration increased, Δb^* (0−24 h) decreased (**Figure 4B**) and b^* increased (data not shown) in both ID377s and Klasic. The decrease of Δb^* (0−24 h) in Klasic in response to tropolone concentration (**Figure 4B**) resembled the decrease in ΔL^* (0−24 h) (**Figure 4A**). Decreased Δb^* implies increased yellowness. In a previous report, however, b^* measurements were directly affected by L^* measurements (but not the reverse), such that addition of India ink to noodle dough caused L^* and b^* to decrease by 6 and 5 units, respectively (*14*). Therefore, Δb^* (0−24 h) responses to tropolone can be partially attributed to the direct effect of darkening (decreased L^*) on b^* measurements. However, in ID377s, the response of Δb^* (0−24 h) (**Figure 4B**) to tropolone concentration was much greater than the response of ΔL^* (0−24 h) (**Figure 4A**). Therefore, tropolone apparently increased yellowness in ID377s. Perhaps PPO initiated polymerization reactions, which ultimately oxidized and bleached carotenoids, while tropolone inhibited such reactions.

All noodles darkened over time regardless of cultivar or tropolone treatment (**Figure 5**). Darkening was most rapid and the tropolone response was greatest during the 0−2 h period in Klasic (**Figure 5**). The rate of darkening in the absence of tropolone continued to be somewhat greater in Klasic than in ID377s after 2 h. Tropolone reduced ΔL^* and the rate of darkening in Klasic to levels near those of ID377s. However, tropolone had a minimal effect in ID377s. In Klasic, the greater response to tropolone and the greater rate of darkening in the absence of tropolone are probably due to higher kernel and flour PPO activity relative to ID377s (**Table 1** and **Figure 3**). These observations again suggest that PPO is a greater factor in alkaline noodle darkening of Klasic than ID377s and also suggest that a non-PPO darkening mechanism is operative regardless of the PPO activity level.

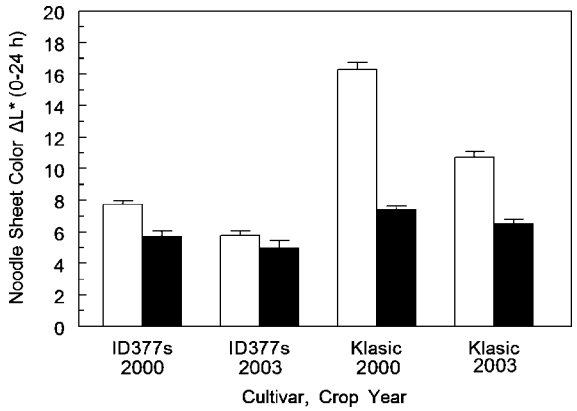

Figure 6. Effect of no inhibitor control (open bars) vs tropolone (100 μg/g flour) (solid bars) on alkaline noodle ΔL^* (0–24 h) of ID377s and Klasic grain lots from crop years 2000 (Aberdeen, ID) and 2003 (Pullman, WA). Error bars indicate standard deviations.

Baik et al. (*15*) previously reported that noodle discoloration rate and water activity were greatest immediately after mixing, and both declined rapidly thereafter. It seems unlikely that decreased water activity has a substantive direct effect on color, although this hypothesis was not tested. However, it is probable that tropolone and PPO activities are dependent on water activity. Thus, greater darkening and tropolone responses in Klasic during the 0−2 h time period (**Figure 5**) are probably due to a higher water activity during that period. The reduced activity of tropolone in noodles is further indicated by the concentration required to inhibit discoloration: A tropolone concentration of 100 μg/g flour is equivalent to a 2.3 mM solution, based on the water added to make noodles with 36% moisture. Thus, ΔL^* (0−24 h) of noodle sheets (**Figure 4A**) was far less sensitive to tropolone than kernel PPO activity (**Figure 3**) in these cultivars.

The effect of environment, as proxied by crop year, and tropolone (100 μg/g flour) on alkaline noodle darkening was evaluated in ID377s and Klasic (**Figure 6**). Darkening and tropolone response were greater in Klasic than in ID377s in both crop years. Similarly, darkening and tropolone response were greater in 2000 than in 2003 in both cultivars. Responses of Δb^* (0−24 h) (data not shown) were very similar to those of ΔL^* (0−24 h), as previously observed (**Figure 4A**,**B**). Tropolone reduced ΔL^* (0−24 h) by 25 and 13% in ID377s and by 54 and 39% in Klasic, in 2000 and 2003, respectively (**Figure 6**).

Kernel and flour PPO activities were positively correlated with noodle darkening ($r = 0.82$ and 0.79, respectively) among the seven grain lots (**Table 1**). Flour PPO had somewhat higher correlations than kernel PPO with noodle darkening in other studies (*1*, *17*). Flour PPO activity was completely inhibited by 1 mM tropolone (data not shown). Noodle darkening was positively correlated, but to a lesser extent, with flour ash and flour protein ($r = 0.31$ and 0.55, respectively) among the seven grain lots (**Table 1**), consistent with previous reports (*15*, *30*−*32*). These relationships were examined in a much larger set of germ plasm in the following retrospective study.

Retrospective Analysis of Noodle Color, PPO, and Wheat Quality Parameters. Statistically significant correlations were observed between kernel PPO activity and all noodle color parameters (**Table 2**). The correlation between kernel PPO and ΔL^* (0−24 h) in this large data set was similar but somewhat less than that observed among the previous seven grain lots (above). The correlations of kernel PPO with the changes in color, i.e., ΔL^* (0−24 h), Δa^* (0−24 h), and Δb^* (0−24 h), were higher than with the absolute color values, L^* (24 h), a^* (24 h), and b^* (24 h), respectively. Correlations of alkaline noodle ΔL^* (0−24 h) with flour ash ($r = 0.00$) and flour protein ($r = 0.14$) were much lower than with kernel PPO activity ($r = 0.64$), indicating that kernel PPO activity was the best single predictor of alkaline noodle darkening. For unknown reasons, the correlations of noodle darkening with flour ash and flour protein were much lower than previously reported (*15*, *30*−*32*) and observed among the seven grain lots (**Table 2**).

The positive correlation between alkaline noodle ΔL^* (0−24 h) and kernel PPO activity (**Table 2**) was evaluated by linear regression. Considerable variation around the regression line (**Figure 7**) was observed even though the PPO assays (two replications) and noodle ΔL^* (0−24 h) measurements were reasonably precise and repeatable (*4*; Engle, D. A.; Morris, C. F. Unpublished data). Other studies have reported correlations of kernel PPO activity with ΔL^* (0−24 h) in raw Asian wheat noodles ranging from $r = 0.41$ to 0.87 (*1*, *15*−*18*), as previously discussed. Thus, our correlation was near the middle of this range of values. Genetics and environment are the main parameters influencing PPO activity of a grain lot (*15*). However, a number of factors may contribute to the variable relationship between kernel PPO activity and ΔL^* (0−24 h): (i) experimental variability; (ii) the AACC standard PPO assay (similar to other kernel PPO assays) is a rather indirect indicator of PPO activity in wheat products because the assay uses kernels, not flour, and an exogenous substrate (L-DOPA); (iii) milling, since bran content of flour affects both PPO and phenolic content of flour (*5*, *6*); (iv) the potentially variable contribution of a non-PPO darkening mechanism(s); and (v) the potentially variable concentration of unidentified phenolic discoloration substrates.

The extrapolated regression line for ΔL^* (0−24 h) vs kernel PPO activity suggests that, at zero kernel PPO activity, ΔL^* (0−24 h) would be 4.4 ± 0.18 (**Figure 7**). A similar relationship

Table 2. Pearson Correlation Coefficients[a] for Kernel PPO, Alkaline Noodle Color Parameters, Flour Protein, and Flour Ash among 502 Hexaploid Wheat Samples from 2003 and 2004 Analyzed with Standard Procedures at the U.S. Department of Agriculture Western Wheat Quality Laboratory

	kernel PPO[b]	24 h			0–24 h			flour protein
		L^*	a^*	b^*	ΔL^*	Δa^*	Δb^*	
L^* (24 h)	–0.56**							
a^* (24 h)	0.25**	–0.64**						
b^* (24 h)	–0.28**	0.01	–0.35**					
ΔL^* (0–24 h)	0.64**	–0.94**	0.57**	–0.17*				
Δa^* (0–24 h)	–0.41**	0.64**	–0.62**	0.17*	–0.59**			
Δb^* (0–24 h)	0.50**	–0.21**	0.17*	–0.69**	0.27**	–0.49**		
flour protein	–0.01	–0.17**	0.51**	–0.15*	0.14*	0.17*	–0.17*	
flour ash	–0.01	–0.07	0.35**	–0.07	0.00	–0.17*	0.06	0.31**

[a] * indicates $0.01 \geq P \geq 0.0001$; ** indicates $P < 0.0001$. [b] Determined by Approved Method 22-85 (*3*) with 10 mM L-DOPA and calculated as ΔA_{475}/min g kernels.

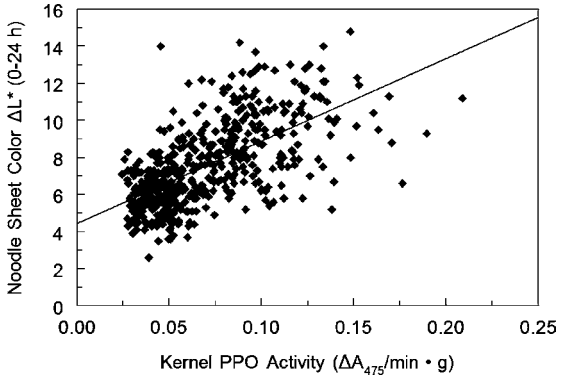

Figure 7. Correlation of kernel PPO activity with noodle darkening measured as ΔL^* (0–24 h) in 502 hexaploid wheat samples from the 2003 and 2004 crop years analyzed with standard procedures at the U.S. Department of Agriculture Western Wheat Quality Laboratory. Overall mean values of L^* were 88.3 and 80.7 at 0 and 24 h, respectively. The kernel PPO activity is shown as the average of two replications, assayed according to AACC Approved Method 22-85 (*3*), with 10 mM L-DOPA and five kernels per replication. The least squares regression equation is $y = 44.6x + 4.4$; $r = 0.64$.

was reported for recombinant inbred wheat lines (*9*). This implies that substantial darkening will occur even in the absence of PPO and that another discoloration mechanism is involved. All of the wheat samples involved in the retrospective analyses were hexaploid, and all samples had kernel PPO activities ≥ 0.024 ΔA_{475}/min g kernels (**Figure 7**). However, Langdon and ID580 both had the extremely low kernel PPO activity of 0.008 ΔA_{475}/min g kernels (**Table 1**), below the apparent threshold. Langdon and ID580 also had the lowest flour PPO activity among the grain lots tested (**Table 1**). Nonetheless, Langdon and ID580 had substantial alkaline noodle darkening, with ΔL^* (0−24 h) values of 8.9 and 6.7, respectively (**Table 1**), corroborating the hypothesis that, as PPO approaches zero, ΔL^* (0−24 h) does not approach zero, and that a non-PPO mechanism contributes to darkening. The extremely low PPO activity in Langdon and ID580 might be related to one or more loci on the D-genome, since Langdon, a durum, does not have the D-genome, and ID580 was derived from a synthetic hexaploid, which derived its D-genome from *Ae. tauschii*. A locus on the 2-D chromosome has been widely implicated as contributing to high kernel PPO activity and Asian noodle darkening in hexaploid wheat (*33*, *34*).

Non-PPO mechanisms of discoloration have not been extensively studied in hexaploid wheat. Peroxidase has been studied as a potential darkening agent in pasta, made from durum (*35*), and in strawberries (*36*). Hexaploid wheat contains peroxidase (*37*), but its role in darkening of wheat products remains obscure. It is also possible that other oxidative enzymes are involved in discoloration. Alternatively, nonenzymatic reactions are possible based on the auto-oxidation of some phenolic compounds at alkaline pH (*33*), reactions that could occur in alkaline noodles. Our lack of information on the identity of the phenolic compound(s) that contributes to discoloration hinders our understanding of the basis for both PPO- and non-PPO-based discoloration.

It is possible that PPO influences noodle color parameters other than L^* since there were significant correlations of kernel PPO with a^* and b^* parameters (**Table 2**). The correlation between kernel PPO and Δa^* (0−24 h) ($r = -0.41$; **Table 2**) was somewhat surprising. The Δa^* (0−24 h) values were much smaller (mean = −0.80) in magnitude than ΔL^* (0−24 h) (mean = 7.64) and Δb^* (0−24 h) (mean = −5.83). The positive correlation between Δb^* (0−24 h) and kernel PPO activity ($r = 0.50$; **Table 2**) was consistent with previous observations (**Figure 4A**,**B**). Part of this relationship is due to the direct effect of darkening (ΔL^*) on Δb^* measurements, as previously discussed. However, the correlations between b^* parameters and kernel PPO were greater than between b^* and L^* parameters, implying that PPO may influence b^* values (directly or indirectly), as previously suggested for ID377s (**Figure 4B**). In summary, the correlations of kernel PPO activity with a^* and b^* parameters suggest that PPO may influence these color parameters via its effects on phenolic compounds.

In conclusion, SHAM and tropolone were potent inhibitors of kernel PPO activity in the three hexaploid wheat cultivars, ID377s, Klasic, and Penawawa, but not in the very low PPO cultivars Langdon and ID580 (**Figure 3**). Several observations in this study indicate that there is a non-PPO component to alkaline noodle darkening: (i) Tropolone (1 μM) inhibited kernel PPO activity to a similar degree (~50%) in the high PPO cultivar Klasic and the low PPO cultivar ID377s (**Figure 3**), but it had far greater activity as an inhibitor of noodle darkening in Klasic than in ID377s (**Figures 4**−**6**); this implies the presence of a tropolone-insensitive, non-PPO noodle darkening mechanism in ID377s. (ii) Tropolone treatment, relative to the no inhibitor control, only slightly inhibited the rate of darkening in Klasic at times ≥4 h (**Figure 5**), implying that a non-PPO darkening mechanism may cause darkening in the later time periods. (iii) Extrapolation of the regression line for kernel PPO activity vs ΔL^* (0−24 h) among 502 wheat samples indicated that as kernel PPO activity approached zero, ΔL^* (0−24 h) approached a value of 4.4 (**Figure 7**), implying that darkening would occur even with zero PPO activity (as measured here with L-DOPA). (iv) Langdon and ID580 had lower kernel and flour PPO activity than the other (hexaploid) wheats evaluated (**Table 1** and **Figure 7**); yet, both Langdon and ID580 had substantial noodle darkening (**Table 1**). Together, these observations suggest that although PPO contributes substantially to darkening of alkaline noodles made from high PPO wheat cultivars, an additional unknown mechanism of darkening is probably also involved. Results indicate the need for research to identify non-PPO mechanisms of darkening in wheat products. We suggest that further investigation is required to identify the phenolic discoloration agent(s) and to determine the potential roles of non-PPO discoloration mechanisms, both enzymatic and nonenzymatic, in wheat products.

ACKNOWLEDGMENT

We appreciate the statistical analysis by Arthur D. Bettge, the technical assistance of Doug A. Engle, Gail Jacobson, Rebecca Wallace, and the staff of the Western Wheat Quality Laboratory, and the provision of ID377s, Klasic, and ID580 seed by Edward Souza.

LITERATURE CITED

(1) Kruger, J. E.; Hatcher, D. W.; DePauw, R. A whole seed assay for polyphenol oxidase in Canadian prairie spring wheats and its usefulness as a measure of noodle darkening. *Cereal Chem.* **1994**, *71*, 324−326.

(2) Morris, C. F. Breeding for end-use quality in the western U.S.: A cereal chemist's view. In *Proceedings of the. 45th Australian Cereal Chemistry Conference*; Williams, Y. A., Wrigley, C. W., Eds.; Royal Australian Chemical Institute: North Melbourne, 1995; pp 238−241.

(3) AACC. Methods 08-01, 22-85, 39-11, and 46-30. In *Approved Methods of the American Association of Cereal Chemists*, 10th ed.; The Association: St. Paul, MN, 2000.

(4) Bettge, A. D. Collaborative study on L-DOPA−Wheat polyphenol oxidase assay (AACC Method 22-85). *Cereal Foods World* **2004**, *49*, 338−342.
(5) Hatcher, D. W.; Kruger, J. E. Distribution of polyphenol oxidase in flour millstreams of Canadian common wheat classes milled to three extraction rates. *Cereal Chem.* **1993**, *70*, 51−55.
(6) Hatcher, D. W.; Kruger, J. E. Simple phenolic acids in flours prepared from Canadian wheat: Relationship to ash content, color, and polyphenol oxidase activity. *Cereal Chem.* **1997**, *74*, 337−343.
(7) Steffens, J. C.; Harel, E.; Hunt, M. D. Polyphenol oxidase. In *Recent Advances in Phytochemistry*; Ellis, B. E., Kuroki, G. W., Stafford, H. A., Eds.; Genetic Engineering of Plant Secondary Metabolism; Plenum Press: New York, 1994; Vol. 28, pp 275−313.
(8) Whitaker, J. R.; Lee, C. Y. Recent advances in chemistry of enzymatic browning: an overview. In *Enzymatic Browning and Its Prevention*; Lee, C. Y., Whitaker, J. R., Eds.; American Chemical Society: Washington, DC, 1995; pp 2−7.
(9) Mares, D. J.; Panozzo, J. F. Impact of selection for low grain polyphenol oxidase activity on darkening in Asian noodles. In *Proceedings of the Ninth Assembly*, Williamson, P., Banks, P., Haak, I., Thompson, J., Campbell, A., Eds.; Wheat Breeding Society of Australia: Toowoomba, Queensland; 1999; pp 32−34.
(10) Osuga, D. T.; Whitaker, J. R. Mechanisms of some reducing compounds that inactivate polyphenol oxidases. In *Enzymatic Browning and Its Prevention*; Lee, C. Y., Whitaker, J. R., Eds.; American Chemical Society: Washington, DC, 1995; pp 210−222.
(11) Perez-Gilabert, M.; Garcia-Carmona, F. Characterization of catecholase and cresolase activities of eggplant polyphenol oxidase. *J. Agric. Food Chem.* **2000**, *48*, 695−700.
(12) Mazzafera, P.; Robinson, S. P. Characterization of polyphenol oxidase in coffee. *Phytochemistry* **2000**, *55*, 285−296.
(13) Okot-Kotber, M.; Liavoga, A.; Yong, K.-J.; Bagorogoza, K. Activity and inhibition of polyphenol oxidase in extracts of bran and other milling fractions from a variety of wheat cultivars. *Cereal Chem.* **2001**, *78*, 514−520.
(14) Mares, D. J.; Wang, Y.; Baydoun, M. Colour of Asian noodles: stability of xanthophylls and flavonoids and interaction with darkening. In *Cereals 2000*: *Proceedings of the 11th ICC Cereal and Bread Congress and of the 50th Australian Cereal Chemistry Conference*; Wootton, M., Batey, L., Wriglely, C. W., Eds.; Royal Australian Chemical Institute; North Melbourne, Victoria, 2001; pp 320−322.
(15) Baik, B.-K.; Czuchajowska, Z.; Pomeranz, J. Discoloration of dough for oriental noodles. *Cereal Chem.* **1995**, *72*, 198−205.
(16) Davies, J.; Berzonsky, W. A. Evaluation of spring wheat quality traits and genotypes for production of Cantonese Asian noodles. *Crop Sci.* **2003**, *43*, 1313−1319.
(17) Ge, X.; He, Z. H.; Yang, J.; Zhang, Q. J. Polyphenol oxidase activities of Chinese winter wheat cultivars and correlations with quality characteristics. *Acta Agron. Sin.* **2003**, *29*, 481−485.
(18) Jukanti, A. K.; Bruckner, P. L.; Habernicht, D. K.; Foster, C. R.; Martin, J. M.; Fischer, A. M. Extraction and activation of wheat polyphenol oxidase by detergents: biochemistry and applications. *Cereal Chem.* **2003**, *80*, 712−716.
(19) Martin, J. M.; Berg, J. E.; Fischer, A. M.; Jukanti, A. K.; Kephart, K. D.; Kushnak, G. D.; Nash, D.; Bruckner, P. L. Divergent selection for polyphenol oxidase and its influence on agronomic, milling, bread, and Chinese raw noodle quality traits. *Crop Sci.* **2005**, *45*, 85−91.
(20) Souza, E. J.; Guttieri, M. J.; Udall, J. A. Registration of "IDO580" spring wheat germplasm. *Crop Sci.* **2005**, *45*, 429−430.
(21) Jeffers, H. C.; Rubenthaler, G. L. Effects of roll temperature on flour yield with the Brabender Quadrumat experimental mills. *Cereal Chem.* **1977**, *54*, 1018−1025.
(22) Fuerst, E. P.; Anderson, J. V.; Morris, C. F. Polyphenol oxidase in wheat grain: Whole-kernel and bran assays for total and soluble activity. *Cereal Chem.* **2006**, *83*, 10−16.
(23) Morris, C. F.; Jeffers, H. C.; Engle, D. E. Effect of processing, formula and measurement variables on alkaline noodle color—Toward an optimized laboratory system. *Cereal Chem.* **2000**, *77*, 77−85.
(24) Kruger, J. E.; Matsuo, R. R.; Preston, K. A comparison of methods for the prediction of Cantonese noodle colour. *Can. J. Plant Sci.* **1992**, *72*, 1021−1029.
(25) Wyzecki, G.; Stiles, W. S. *Color Science. Concepts and Methods, Quantitative Data and Formulae*, 2nd ed.; John Wiley & Sons: New York, 2000.
(26) Espin, J. C.; Wichers, H. J. Slow-binding inhibition of mushroom (*Agaricus bisporus*) tyrosinase isoforms by tropolone. *J. Agric. Food Chem.* **1999**, *47*, 2638−2644.
(27) Gessler, N. N.; Gomboeva, S. B.; Shumaev, K. B.; Bykhovskii, V. Y.; Lankin, V. Z. Free radical lipid peroxidation inhibits enzymatic conversion of β-carotene into vitamin A. *Bull. Exp. Biol. Med.* **2000**, *131*, 451−453.
(28) Vanlerberghe, G. C.; McIntosh, L. Signals regulating the expression of the nuclear gene encoding alternative oxidase of plant mitochondria. *Plant Physiol.* **1996**, *111*, 589−595.
(29) Goyal, A. Glycolate metabolism in algal chloroplasts: Inhibition by salicylhydroxamic acid (SHAM). *Physiol. Plant.* **2002**, *116*, 264−270.
(30) Miskelly, D. M.; Moss, H. J. Flour quality requirements for Chinese noodle manufacture. *J. Cereal Sci.* **1985**, *3*, 379−387.
(31) Kruger, J. E.; Anderson, M. H.; Dexter, J. E. Effect of flour refinement on raw Cantonese noodle color and texture. *Cereal Chem.* **1994**, *71*, 177−182.
(32) Zhang, Y.; Nagamine, T.; He, Z. H.; Yoshida, H.; Penewawa, R. J. Variation in quality traits in common wheat as related to Chinese fresh white noodle quality. *Euphytica* **2005**, *141*, 113−120.
(33) Anderson, J. V.; Morris, C. F. An improved whole-seed assay for screening wheat germplasm for polyphenol oxidase activity. *Crop Sci.* **2001**, *41*, 1697−1705.
(34) Mares, D. J.; Campbell, A. W. Mapping components of flour and noodle colour in Australian wheat. *Aust. J. Agric. Res.* **2001**, *52*, 1297−1309.
(35) Feillet, P.; Autran, J.-C.; Verniere, C.-I. Pasta brownness: An assessment. *J. Cereal Sci.* **2000**, *32*, 215−233.
(36) Lopez-Serrano, M.; Ros Barcelo, A. Histochemical localization and developmental expression of peroxidase and polyphenol oxidase in strawberries. *J. Am. Soc. Hortic. Sci.* **2001**, *126*, 27−32.
(37) Hatcher, D. W.; Barker, W. A rapid microassay for peroxidase in wheat and flour. *Cereal Chem.* **2005**, *82*, 233−237.

Received for review October 24, 2005. Revised manuscript received January 13, 2006. Accepted January 26, 2006. This research was supported in part by U.S. Department of Agriculture-NRI Grant Award 2002-35503-12670. Mention of trademark or proprietary products does not constitute a guarantee or warranty of a product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

JF0526386