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ARTICLE

Physical−Chemical Analysis of Non-Polyphenol Oxidase (Non-PPO) Darkening in Yellow Alkaline Noodles

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Darkening in yellow alkaline noodles (YAN) was measured over 24 h in a high polyphenol oxidase (PPO) bread wheat (*Triticum aestivum* L. cv. Tasman) and a very low PPO durum wheat (*Triticum durum* cv. Kamilaroi). Over 24 h non-PPO darkening occurred across a range of pH 3.5−10.5, and in Tasman this was overlaid by darkening from PPO activity. The rate of darkening in YAN was separated into two main time periods, 0−4 and 4−24 h. The first 4 h of darkening was further divided into two stages using a composite first-order rate equation. Several specific inhibitors that partially inhibited non-PPO darkening were identified. These inhibitors, as well as the PPO inhibitors SHAM and tropolone, were used to analyze YAN darkening. The rate of the early stage of darkening was not altered by any inhibitors used; however, the magnitude of darkening was reduced by inhibitors specific for non-PPO darkening. Both the rate and extent of non-PPO darkening of the second stage of darkening were decreased in Tasman and Kamilaroi by inhibitors specific for non-PPO darkening, whereas both PPO inhibitors only decreased darkening in Tasman. The second and third stages of darkening showed similar characteristics. The third stage of darkening was examined in YAN made from Kamilaroi over a temperature range from −4 to 65 °C. It followed an Arrhenius relationship indicating non-PPO darkening during this stage was nonenzymatic. The inhibitor data suggested that the reactive component(s) was/were present in a reasonably high concentration(s) and that the soluble protein fraction was involved in the non-PPO darkening process.

KEYWORDS: Yellow alkaline noodle; polyphenol oxidase; non-PPO; darkening; inhibitor

INTRODUCTION

Yellow alkaline noodles (YAN) are a major end-product of Australian hard wheat. They are made by treating wheat flour with alkaline salts consisting primarily of potassium carbonate and/or sodium carbonate (*1*). The exact mixture of salts depends on the flour used and consumer preference. Noodle color is a major determinant of noodle marketability. Yellow is a positive attribute, whereas dark or gray colors are negative attributes. A common fault is the tendency for fresh noodles to darken over 24 h. Marketability declines with an increase in darkening. To improve shelf life it is desirable to halt or at least slow the darkening.

It is well established that polyphenol oxidase (PPO) contributes to darkening in YAN (*2*−*4*). Black or dark brown pigments produced by PPO are associated with bran specks (*5*). Germplasm with very low or near-zero PPO has been identified (*6*), along with effective screening technologies, including molecular markers, providing the basis for wheat breeders to develop new cultivars with lower rates of darkening (i.e., improved color stability). However, darkening also occurs in the absence of PPO activity (*4*, *7*). It has been estimated that PPO contributes < 50% of total darkening of YAN (*3*, *4*, *8*). Further improvements to minimize darkening in YAN will be dependent on minimizing non-PPO darkening. There is little or no known genetic variation known for non-PPO darkening (*9*), and this has been one of the main limitations in both in the study of non-PPO darkening and varietal improvement.

The bread wheat cultivar Tasman has high PPO activity and the durum cultivar Kamilaroi has very low PPO activity (effectively zero) as determined using a standard seed assay (*2*, *6*, *10*), which makes it possible to study the effects of different treatments on both PPO and non-PPO darkening. Using the standard seed assay, a number of different inhibitors have been tested for inhibition of PPO and the most effective were salicylhydroxamic acid (SHAM) and tropolone, giving total inhibition (*11*). Furthermore, a competitive assay in YAN involving both inhibitor, either SHAM or tropolone, and the PPO substrate, tyrosine, indicated that both of these inhibitors completely inhibited PPO activity in YAN (*11*). Thus, the inhibitors SHAM and tropolone make it possible to differentiate between PPO and non-PPO darkening in YAN.

A previous study of YAN has already suggested that non-PPO darkening may be the result of both enzymatic and nonenzymatic mechanisms (*9*). The effect of pH and temperature on YAN

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darkening can help establish whether enzymes are involved in non-PPO darkening. Furthermore, the identification of inhibitors specific for non-PPO darkening, whether partial or complete, can also increase our understanding of the mechanisms of non-PPO darkening and the role of enzymes, if any.

MATERIALS AND METHODS

Materials. A high-PPO bread wheat (*Triticum aestivum* L. cv. Tasman) and a near-zero-PPO durum wheat (*Triticum durum* cv. Kamilaroi) were selected on the basis of surveys of variation in grain PPO activity in Australian germplasm (*2*, *6*, *10*). In the absence of PPO (Kamilaroi) or in the presence of specific PPO inhibitors (Tasman) a large amount of darkening in noodle sheets was still evident and is referred to as non-PPO darkening. Grain of both cultivars was produced in field trials at the Waite Campus of the University of Adelaide.

Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich. Aminoguanidine was used as the bicarbonate salt, whereas guanidine and semicarbazide were both used as the hydrochlorides.

Flour Milling and Noodle Sheet Preparation. Grain was conditioned overnight to 15% moisture and milled on a Buhler experimental mill, and the break and reduction flour streams were combined. The pollard stream was resieved on a shaker and recovered flour added to the combined streams from the mill. Different batches gave slightly different color responses, and therefore the same batch was used for each experiment and, when possible, for different experiments.

Flour (10 g), with or without inhibitors, and 3.6 mL of 2% sodium carbonate solution were mixed into dough in a cylindrical mixing bowl using a drill press with a modified mixing paddle. The dough was mixed for 105 s with intermittent pauses to clean dough adhering to the paddle. The dough was rolled into a ball and made into a noodle sheet using a domestic pasta maker (Atlas 150, Marcato S.P.A., Campodarego, Italy). The noodle sheet was then placed in a resealable plastic bag to prevent drying and make noodle handling easier. CIE-Lab color measurements of raw noodles (except the boiled treatment) were made using a Dataflash 100 (Datacolor International, Lawrenceville, NJ) reflectance spectrophotometer. Except for the temperature experiment, the noodles were made and stored at room temperature (22 ± 1 °C).

To examine the effect of pH on noodle darkening, noodles were made with a series of chemical solutions and buffers: 2% HCl, 0.2 mol L^{-1} sodium citrate (pH 4.0, 5.0, 6.0), 0.2 mol L^{-1} Tris (pH 7.0, 8.0, 9.0, 10.0), 0.2 mol L^{-1} $NaCO_3$, 1% NaOH, 1.5% NaOH, and 2% NaOH. The noodles (1 g) were dispersed in 2 mL of water and centrifuged, and the pH was measured.

Extraction of 50% Propanol Fraction. After some experimentation, it was found that a 50% crude propanol extract contained reactants specific for non-PPO darkening. The crude product was obtained by extracting 100 g of flour with 400 mL of 50% propanol/water. The suspension was centrifuged and the supernatant dried. The crude extract was added to the noodle at a rate of 1 g/10 g of flour.

Determination of Inhibitor Concentration Optima. The optimum concentration of the inhibitors specific for non-PPO darkening was estimated by adding an increasing concentration of the inhibitor, and the 24 h ΔL^* response was recorded. To estimate the concentration of 50% inhibition (I_{50}), logistic curves were fitted to the data derived from the addition of aminoguanidine, $ZnSO_4$, $Al_2(SO_4)_3$, and semicarbazide, whereas a rectangular hyperbola was fitted to guanidine data. Curves were fitted using WinCurveFit (*12*).

For the inhibitor assays, SHAM and tropolone were used at a concentration of 0.01 mol L^{-1} (*3*), $ZnSO_4$ and $Al_2(SO_4)_3$ at 0.1 mol L^{-1}, and aminoguanidine, guanidine, and semicarbazide at 2 mol L^{-1}. The pH of all inhibitors except semicarbazide was adjusted to pH 10.5−11.0. For the boiling treatment, noodles were placed in plastic bags, boiled for 2 min, and then allowed to cool at ambient temperature (22 ± 1 °C).

Darkening Rates. The rate of darkening for the two time periods was determined by measuring the L^* value of the noodle sheets at 5, 10, 15, 20, 30, 40, 50, 60, 90, 120, 150, 180, 210, 240, and 1440 min. Curves were fitted and standard errors estimated using WinCurveFit (*12*).

Sedimentation Assay. The sedimentation assay was developed as a sensitive method of measuring changes in protein tertiary and quaternary structure. The sedimentation assay was modified from that of Axford et al. (*13*). The section of noodle sheet (1.25 g) was homogenized (homogenizer model GLH-220; Omni International, Marietta, GA) in 12.5 mL of water, the slurry poured into a 25 mL measuring cylinder, and 12.5 mL of SDS (30 g/L)/lactic acid buffer (0.025 mol L^{-1}) added. The solution was mixed by inversion four times, and the height was measured every 5 min for the first 30 min and then every 10 min until 90 min. The experiment was conducted at 22 ± 1 °C. The rate of sedimentation (k_s mm/min) and final height (mm) was calculated using a first-order rate equation. A linear curve was fitted to the initial phase of sedimentation and a quadratic curve fitted to the second phase.

Temperature Analysis. The temperature analysis was executed to determine whether noodle darkening between the 4 and 24 h time period deviated from an Arrhenius type relationship whereby any deviation may indicate the activity of enzymes. The k'_{4-24} at −4, 4, 10, 20, 30, 37, 45, 54, 66, and 75 °C was obtained for noodles made from Tasman and Kamilaroi with and without inhibitors. Noodle color (L^*) was measured at 0, 2, 4, and 24 h.

The mechanisms of inhibition by the inhibitors specific for non-PPO darkening inhibitors was examined using the Arrhenius equation (eq 1), whereby the natural log (ln) of the Kamilaroi rate data was plotted against inverse of temperature (K)

$$\ln(k'_{4-24}) = -\frac{E_a}{RT} + \ln A \quad (1)$$

where k'_{4-24} is ΔL^*/s, T is the absolute temperature (K), R is the gas constant, E_a is the activation energy, and A is the pre-exponential factor. It was then possible to compare the activation energies using E_a/R and the pre-exponential factors (A) of the different inhibitors. The molarities of the reactants were unknown, and therefore the actual activation energy E_a could not be determined. All curves were fitted and errors estimated using WinCurveFit (*12*).

Size Exclusion High-Performance Liquid Chromatography (SE-HPLC). SE-HPLC was performed to observe whether the inhibitors $Al_2(SO_4)_3$ or 2 mol L^{-1} semicarbazide had any measurable effect on protein structure. The assay for the determination of extractable polymeric protein (% EPP) and unextractable polymeric protein (% UPP) was based on the methods of Singh et al. (*14*), Batey et al. (*15*), Gupta et al. (*16*), and Larroque et al. (*17*). The EPP and UPP of noodles containing 0.1 mol L^{-1} $Al_2(SO_4)_3$ or 2 mol L^{-1} semicarbazide were compared with control noodles. The samples were prepared by cutting 1 cm^2 of the noodle sheet into 1 mm^2 pieces and 0.1 g of sample added to 1 mL of phosphate extraction buffer (0.05 mol L^{-1} Na_2HPO_4/NaH_2PO_4, 0.173 mol L^{-1} sodium dodecyl sulfate; pH 6.9). The EPP was determined by homogenizing the sample by grinding with a pestle for 1 min and then mixing the samples for 15 s with a vortex mixer. The samples were placed in a 30 °C oven for 2 h and mixed using a vortex mixer at 15 min intervals. After incubation, the samples were centrifuged for 10 min (13000*g*) to pellet the insoluble fraction. The pellet was retained for UPP determination. The supernatant was filtered (0.45 μm) and incubated for 2 min in an 80 °C water bath prior analysis. The UPP was estimated by adding 1 mL of phosphate extraction buffer (pH 6.9) to each pellet. The samples were sonicated for 30 s and then centrifuged for 10 min to pellet the residue. The supernatant was filtered (0.45 μm) and incubated for 2 min in an 80 °C water bath prior to analysis.

Analysis of the extracted protein was performed using a HPLC system equipped with a Waters 717 plus autosampler (Waters Corp., Milford, MA), a Waters 486 detector, and a Waters 600 controller. Separation was achieved using a Protein-Pak 300 SW (7.8 mm × 30 cm; Waters Corp.) column. The mobile phase was 49.5% aqueous acetonitrile/1% trifluoroacetic acid at a flow rate of 0.5 mL/min, and the column was maintained at 40 °C. The run time was 40 min, and the detection was at 214 nm.

RESULTS AND DISCUSSION

Effect of pH on Noodle Darkening. In noodles made from Tasman wheat, the 0−24 h data showed an optimum pH for PPO darkening at approximately 8.2 (**Figure 1a**) above a high baseline of non-PPO darkening that occurred across the pH range of 3.5−10.5. Two PPOs have been isolated from wheat bran, both with pH optimums at approximately pH 5 (*18*, *19*). However, most

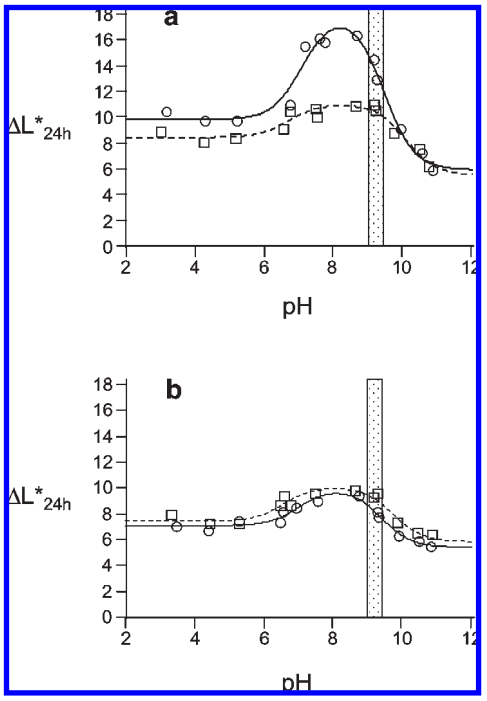

Figure 1. Effect of pH on darkening of YAN made from (**a**) Tasman and (**b**) Kamilaroi flour in the presence (□) and absence (○) of the PPO inhibitor SHAM. The shaded areas indicate the pH of YAN.

PPO is bound, and the optimum of PPO (pH 6−7) in commercial wholemeal is broad (*20*). This suggests that the matrix has a considerable effect on the pH optimum of the PPO enzyme.

SHAM is a well-known effective and selective noncompetitive inhibitor of polyphenol oxidase (*21*−*23*) and has been used to study PPO activity in alkaline noodles (*4*, *7*). In previous studies in wheat grain SHAM gave total inhibition of PPO, and, moreover, a competitive assay between SHAM and tyrosine, a PPO substrate, indicated that SHAM completely inhibited PPO activity in YAN (*11*). SHAM appears to increase darkening slightly in Kamilaroi; however, there is no statistical difference between the two curves. SHAM has little effect on darkening in noodles made from Kamilaroi flour, indicating that Kamilaroi has very low levels of PPO if any (**Figure 1b**). In the Tasman noodles made with the PPO inhibitor, non-PPO darkening had a broad peak with maximum darkening between pH 7 and 9. The small broad pH optimum above the baseline observed in the Tasman plus SHAM and the Kamilaroi data is suggestive of an enzyme contributing to non-PPO darkening. Although it is possible that SHAM did not completely inhibit PPO darkening in Tasman, SHAM had little effect in Kamilaroi, indicating the peak was not due to PPO. The high baseline observed in both Tasman and Kamilaroi (**Figure 1**) suggests a large proportion of darkening in YAN is pH independent and therefore nonenzymatic. Therefore, PPO darkening in YAN can be defined as darkening inhibited by SHAM, whereas non-PPO darkening is defined as darkening not inhibited by SHAM.

Study of Inhibitors Specific for Non-PPO Darkening. SHAM and tropolone have already been identified as PPO inhibitors (*4*, *24*), but there are no reports of specific non-PPO inhibitors. In preliminary studies, several compounds were identified as potential, although not perfect, specific inhibitors of non-PPO darkening. None of these inhibitors were specific enzyme inhibitors. The study of these inhibitors and their activity has the potential of improving the understanding of non-PPO darkening and perhaps identifying methods of decreasing this darkening in YAN.

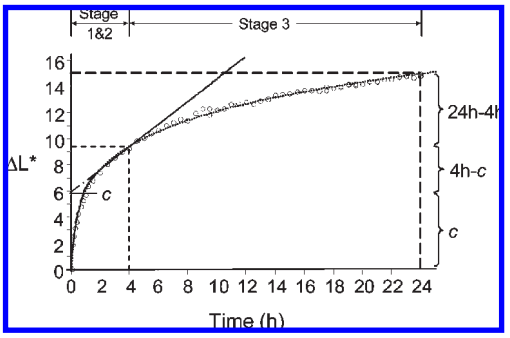

Figure 2. Noodle darkening showing rapid darkening in stage 1 and slowing to linear darkening during stage 3.

Several metal ions were tested (including copper, iron, cobalt, manganese, magnesium, calcium, gallium, barium, and sodium), but only zinc and aluminum showed any specificity toward preventing non-PPO darkening. Vadlamani and Seib first noted that zinc and aluminum ions improved initial noodle brightness without affecting PPO darkening (*25*); however, these authors did not identify these metal ions as potential non-PPO inhibitors. A logistic concentration response curve for both aluminum and zinc yielded equivalence points (I_{50}) of 0.087 and 0.069 mol L^{-1}, respectively. Differences in equivalence points may reflect the stoichiometry where zinc may form higher complexes than aluminum or the strength (binding constant) of the ligand. These differences may also result from differences in the metal ion equilibria. At high pH (>7) it is the aluminate ion that occurs in solution; however, it is the Al^{3+} ion that is involved in ligand formation (*26*). Aluminum and zinc have similar degrees of inhibition with $\Delta L^*_{(\text{treated-control})\max} = 5.8 \pm 0.5$ and 6.7 ± 0.8, respectively.

Aminoguanidine and semicarbazide, two known Maillard reaction inhibitors (*27*), had little activity on darkening at low concentration (*9*); however, they showed specific non-PPO inhibition at high concentration (>1 mol L^{-1}). Semicarbazide and aminoguanidine have a very similar ranges of activities with I_{50} of 0.55 and 0.52 mol L^{-1}, respectively. The I_{50} concentrations for aminoguanidine and semicarbazide are 9−10-fold higher than those of zinc and aluminum, indicating a different mode of action for these two groups of inhibitors. Whereas semicarbazide appeared to be a more efficient inhibitor, providing a greater reduction in the amount of darkening, $\Delta L^*_{(\text{treated-control})\max} = 15.6 \pm 2.5$, compared to aminoguanidine with $\Delta L^*_{(\text{treated-control})\max} = 4.2 \pm 0.5$, for semicarbazide to be an effective non-PPO inhibitor, an associated decrease in pH (3.1) was required. Although the effect of semicarbazide was confounded with pH, it was included in this study because it had a large effect on non-PPO darkening.

Guanidine is a known protein denaturant (*28*). The concentration of 50% inhibition for guanidine ($I_{50} = 0.78$ mol L^{-1}) was similar to those for aminoguanidine and semicarbazide. The magnitude of the reduction in darkening was similar to that of aminoguanidine; however, guanidine shows a different curve for inhibition with no lag prior to inhibition, indicating that a critical concentration is not required before inhibition begins.

Rate of Noodle Darkening. The rate of darkening is a measure of product formation. As has been observed previously (*29*), noodle darkening initially proceeds rapidly and then slows over the 24 h period (**Figure 2**). This is a mixed-order rate reaction.

Due to the complexity of studying mixed-order rate reactions, time constraints, and lack of automation, it was decided to split the data into two time periods: 0−4 and 4−24 h. The 0−4 h data

Table 1. Rates of Darkening (ΔL^*/min) in YAN Made from Kamilaroi and Tasman Flour during the First, Second, and Third Stages of Storage As Defined by k'_1, k'_2, and k'_{4-24}, Respectively, at 22 °C

	Kamilaroi			Tasman		
treatment	k'_1 ($\times 10^{-1}$)	k'_2 ($\times 10^{-2}$)	k'_{4-24} ($\times 10^{-3}$)	k'_1 ($\times 10^{-1}$)	k'_2 ($\times 10^{-2}$)	k'_{4-24} ($\times 10^{-3}$)
control	1.13 (±0.19)	1.12 (±0.09)	2.83 (±0.09)	0.87 (±0.16)	3.34 (±0.20)	5.20 (±0.07)
tropolone	1.12 (±0.16)	1.12 (±0.08)	2.21 (±0.04)	1.29 (±0.19)	2.00 (±0.14)	3.67 (±0.13)
SHAM	0.99 (±0.17)	1.11 (±0.09)	3.04 (±0.08)	0.85 (±0.11)	1.64 (±0.11)	3.16 (±0.13)
$ZnSO_4$	0.78 (±0.26)	0.56 (±0.11)	1.56 (±0.20)	0.85 (±0.15)	0.85 (±0.08)	2.17 (±0.21)
$Al_2(SO_4)_3$	0.71 (±0.13)	0.82 (±0.10)	1.78 (±0.02)	0.71 (±0.12)	0.88 (±0.07)	3.83 (±0.12)
aminoguanidine	1.27 (±0.17)	0.82 (±0.07)	1.56 (±0.01)	0.71 (±0.14)	2.60 (±0.17)	3.17 (±0.12)
guanidine	1.00 (±0.14)	0.90 (±0.06)	2.24 (±0.27)	0.52 (±0.08)	2.86 (±0.22)	3.56 (±0.11)
semicarbazide	1.06 (±0.14)	−0.04 (±0.03)	−0.24 (±0.02)	0.98 (±0.16)	2.01 (±0.06)	0.16 (±0.06)
boiled	1.44 (±0.35)	−0.04 (±0.03)	−0.08 (±0.09)	1.07 (±0.55)	0.05 (±0.05)	−0.01 (±0.10)

Table 2. Estimate of the Contribution (ΔL^*) of the First, Second, and Third Stages of YAN Darkening As Defined by c, 4 h − c, and 4−24 h, Respectively, at 22 °C

	Kamilaroi			Tasman		
treatment	c	4 h − c	4−24 h	c	4 h − c	4−24 h
control	3.11 (±0.13)	2.43 (±0.43)	3.40 (±0.54)	5.52 (±0.31)	5.12 (±0.48)	6.25 (±0.57)
tropolone	3.41 (±0.12)	2.51 (±0.22)	2.65 (±0.27)	5.38 (±0.20)	4.62 (±0.68)	4.41 (±0.84)
SHAM	3.00 (±0.14)	2.51 (±0.28)	3.64 (±0.38)	4.59 (±0.17)	3.13 (±0.31)	3.80 (±0.46)
$ZnSO_4$	1.63 (±0.11)	1.35 (±0.41)	1.88 (±0.61)	2.26 (±0.12)	2.03 (±0.24)	2.60 (±0.45)
$Al_2(SO_4)_3$	1.64 (±0.09)	1.98 (±0.19)	2.14 (±0.21)	1.97 (±0.10)	2.04 (±0.22)	4.60 (±0.36)
aminoguanidine	3.17 (±0.10)	1.85 (±0.24)	1.88 (±0.25)	4.12 (±0.26)	2.16 (±0.55)	3.81 (±0.69)
guanidine	2.70 (±0.09)	2.03 (±0.40)	2.76 (±0.73)	6.27 (±0.38)	6.53 (±0.65)	4.27 (±0.78)
semicarbazide	1.29 (±0.05)	−0.35 (±0.15)	−0.29 (±0.17)	1.81 (±0.08)	0.25 (±0.21)	0.10 (±0.28)
boiled	0.83 (±0.04)	−0.09 (±0.42)	−0.10 (±0.52)	1.21 (±0.23)	−0.55 (±0.33)	−0.01(±0.45)

were fitted with a composite first-order rate equation

$$y = a\,e^{-k'_1 x} + k'_2 x + c \qquad (2)$$

where k'_1 and k'_2 are rate constants (ΔL^*/min), c is a measure of the contribution of darkening by k'_1 (ΔL^*), and a is a constant (**Figure 2**). It was possible to obtain estimates of two reaction rates from eq 1: an initial rapid reaction, defined by k'_1, and a slower linear reaction, defined by k'_2, indicating two stages of darkening. The constant c can be considered a measure of the amount of darkening attributable to the first stage of darkening associated with k'_1, whereas the magnitude of darkening associated with second stage can be estimated by the amount of darkening at 4 h less the value c (4 h − c). The 4−24 h time period was considered the third stage of darkening. The 4−24 h darkening rate (k'_{4-24}) data were approximated as linear, and the rate of darkening was measured as ΔL^*/min.

The rates for the different stages of darkening in noodles made from Kamilaroi and Tasman flour in the presence of inhibitors were compared with the respective controls (**Table 1**). In Kamilaroi k'_1 is approximately 10-fold greater than k'_2 and 50-fold greater than k'_{4-24}, and the Tasman k'_1 is approximately 2.5-fold greater than k'_2 and 17-fold greater than k'_{4-24}. The difference in reaction rates between Tasman and Kamilaroi, especially k'_2 and k'_{4-24}, indicates the impact of PPO on darkening. In contrast, the similarity of k'_1 between Tasman and Kamilaroi indicates that PPO has minimal effect during the early darkening stage. The initial rate (k'_1) is surprisingly unresponsive to the different treatments (**Table 1**) including boiling, and as boiling has no effect on k'_1 it is unlikely that enzyme activity is important to darkening at this stage.

The initial darkening stage contributes approximately one-third of total darkening as measured by c in both Kamilaroi and Tasman (**Table 2**). Zinc sulfate, aluminum sulfate, semicarbazide, and boiling decrease the amount of darkening in the initial stage (**Table 2**), with boiling being the most effective treatment in both varieties. In Tasman SHAM and tropolone had minimal effect on c,

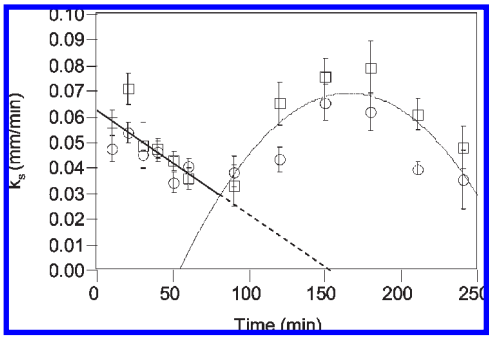

Figure 3. Sedimentation rate (k_s) of noodles made from Kamilaroi (○) and Tasman (□) measured during aging. A linear curve was fitted to the first phase of sedimentation, and a quadratic curve was fitted to the second phase of sedimentation.

indicating that PPO darkening is not a major factor during this stage of darkening.

The addition of crude 50% propanol Kamilaroi flour extracts to noodles made from the same flour had little effect on k'_1 ($0.74 \pm 0.12 \times 10^{-1}$) but increased the amount of darkening during this stage c (4.57 ± 0.23). This agrees with eq 2 that k'_1 is independent and c is dependent on the concentration of reactant(s). Therefore, the inhibitors of stage 1 darkening (c) may either react with the reactant(s) or limit the diffusion mobility of the reactant(s) by changing the matrix to decrease yield or react with the colored product(s) to render it/them colorless.

The lack of response of k'_1 to inhibitors suggests that it may be associated with physical changes in the flour. Often noodle dough is "rested" prior to cutting and cooking (*30*). It is thought that dough resting helps water penetrate the dough particles evenly, resulting in less streaky and smoother dough after sheeting. However, protein hydration conformational change and aggregation (*31*) are also expected during this time. To test whether changes in proteins could be related to early noodle

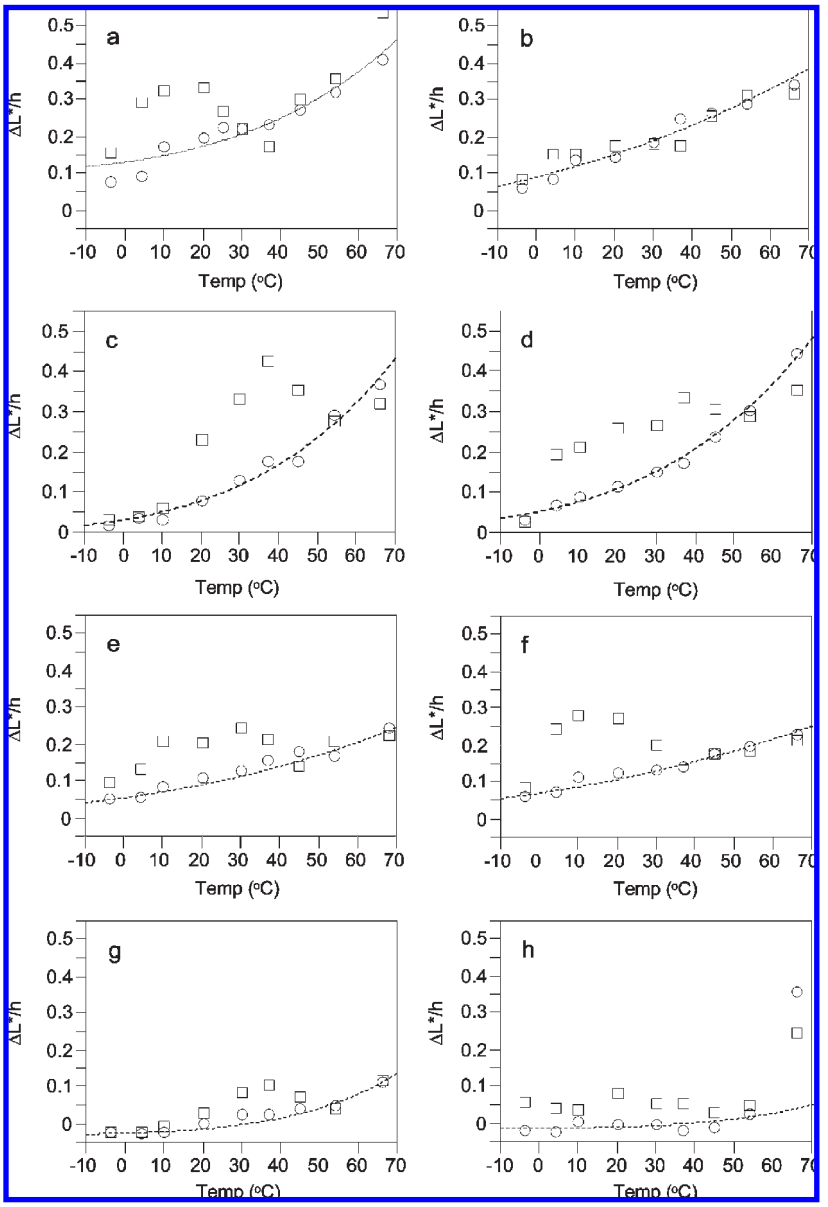

Figure 4. Responses to temperature in the rate k'_{4-24} of (**a**) control noodles compared with noodles made with the PPO inhibitor, (**b**) SHAM, the inhibitor specific for non-PPO darkening (**c**) aluminum sulfate, (**d**) zinc sulfate, (**e**) aminoguanidine, (**f**) guanidine, and (**g**) semicarbazide and (**h**) the boiled treatment. Noodles were made from Kamilaroi (○) and Tasman (□) flour. The dotted line indicates the approximate baseline of non-PPO darkening in Kamilaroi using the Arrhenius equation; $k'_{4-24} = a\, e^{-b/(T+273)} + c$, where k'_{4-24} is $\Delta L^*/h$, T is the temperature (°C), and a, b, and c are constants.

darkening, the SDS sedimentation assay was adapted to study changes in proteins during dough development. The sedimentation test shows two phases; an initial phase that is linear and a second phase that has a broad parabolic peak (**Figure 3**). There was a high degree of correlation ($r^2 = 0.53$) between initial darkening rate and SDS−lactic acid-induced flocculation, suggesting this phase of darkening was associated with changes in protein structure.

The PPO inhibitors, tropolone and SHAM, both slowed and decreased darkening in Tasman and had little effect in Kamilaroi during stages 2 and 3 of darkening. Zinc sulfate aluminum sulfate, aminoguanidine, and guanidine all partially inhibited k'_2 and k'_{4-24}, whereas semicarbazide and boiling completely inhibit both k'_2 and k'_{4-24} in Tasman and Kamilaroi. In noodles made from Kamilaroi using the semicarbazide and boiling treatments, the k'_2 and k'_{4-24} rates were slightly negative, indicating a decrease in darkness is occurring, albeit at a very low rate. The amount of darkening occurring during stages 2 and 3 (**Table 2**) agrees with the rate data (**Table 1**).

Effect of Temperature on Noodle Darkening. Although the rate data give information regarding at what stages the inhibitors are active, they give little information about their mode of action. In an attempt to bridge this gap, the k'_{4-24} rate was further studied by examining the effects of temperature and temperature/inhibitor interactions on darkening.

For the temperature study, it was not possible to analyze the noodles during the 0−4 h time period at the temperatures required, and therefore only the 4−24 h data were measured. In the control noodles, high temperatures (>66 °C) produced a substantial increase in darkening that was indicative of Maillard reaction, which requires a high activation energy. Tropolone was not used for this experiment because it increased the rate of darkening above expectation with increasing temperature.

Tasman and Kamilaroi showed distinct peaks coinciding with optimum temperature for PPO activity (**Figure 4a**), with the relative peak heights consistent with PPO activities in Tasman and Kamilaroi. The peak was confirmed to be due to PPO by its absence in the presence of SHAM (**Figure 4b**). To obtain the

Table 3. Temperature Optima of PPO in Noodles Made from Tasman Flour Comparing Inhibitors Specific for Non-PPO Darkening with the Control

treatment	temp (°C)	treatment	temp (°C)
control	14 (±10)	aminoguanidine	28 (±10)
aluminum sulfate	35 (±16)	guanidine	14 (±12)
zinc sulfate	19 (±15)	semicarbazide	35 (±17)

temperature of maximum PPO enzyme activity in noodles, the curve above the tangential baseline was fitted

$$k'_{4-24} = \frac{1}{aT^2 + bT + c} \quad (3)$$

where T is the temperature (°C) and a, b, and c are constants. The temperature of maximum enzyme activity was estimated using the real root of the differential equation (i.e., when $2aT + b = 0$).

The temperature optimum for Tasman PPO activity (14 ± 10 °C) in YAN is lower than expected (**Table 3**). PPO was less stable at high temperature than determined by Marsh and Galliard (*20*) at pH 6.6−7.0. A comparison of the temperature optima of PPO in the presence or absence of inhibitors indicates that most inhibitors help stabilize PPO at higher temperatures with aluminum sulfate and semicarbazide having the greatest effect and guanidine having no effect (**Figure 4**; **Table 3**).

Non-PPO darkening in Kamilaroi follows the Arrhenius curve (**Figure 4**), suggesting that the 4−24 h darkening is mainly due to chemical reaction(s). As the molarity of reactants was unknown, it was only possible to compare the activation energies using E_a/R and the pre-exponential factors (**Table 4**). It is reasonable to assume a multistep mechanism for the darkening reaction, and therefore the rate-limiting step controls the rate of product formation. However, inhibitors can control steps that are not normally limiting.

Aluminum sulfate, zinc sulfate, and semicarbazide act as noncompetitive inhibitors by slowing darkening by increasing activation energy. At high pH, aluminum forms coordination complexes with carboxyl and sulfhydryl groups of proteins, resulting in cross-linking (*32*). However, aluminum is also strongly bonded by the ester carbonyl groups in proteins such as collagen, an animal protein (*33*). Zinc has a high affinity for protein sulfhydryl groups. Semicarbazide is also known to bind with the protein fraction of wheat flour (*34*). The exact method by which the semicarbazide binds to the protein is unknown; however, it is well-known that semicarbazide reacts with free carbonyl groups. In collagen it is thought that the semicarbazide reacts with $\alpha\beta$-unsaturated carbonyls (*35*). Furthermore, SE-HPLC of flour extracts suggests that semicarbazide reacts with the soluble but not the insoluble polymeric protein (data not shown).

Guanidine slowed the reaction rate by decreasing the pre-exponential factor without increasing activation energy. Although aminoguanidine is not significantly different from the control, the Arrhenius data suggest that guanidine and aminoguanidine act by a similar mechanism. Guanidine at sub-denaturing concentrations stabilizes proteins (*28*) by lowering the conformational entropy of the protein, and this may provide a mechanism for inhibition of darkening. Urea, another chaotropic agent, used at 2 mol L^{-1} has little effect on darkening. Guanidine has a pK_a of 12.5, whereas urea has a pK_a of 0.08, and therefore in the alkaline noodle guanidine is predominantly cationic, whereas urea is neutral. At high pH the proportion of negatively charged species will increase and guanidine can interact strongly with these negatively charged species by direct electrostatic interaction.

Boiling appears to act as a chain breaker and almost completely stops non-PPO darkening. In boiled noodles, darkening occurs only if noodles are then stored at temperatures > 54 °C.

Table 4. Arrhenius Estimates of E_a/R and the Pre-exponential Factor, A, of Noodles Made from Kamilaroi Flour in the Presence and Absence of Non-PPO Inhibitors[a]

treatment	E_a/R ($\times 10^3$)	ln(A)
control	2.01 (±0.12)	−3.09 (±0.74)
SHAM	2.15 (±0.18)	−2.78 (±0.61)
aluminum sulfate	3.91 (±0.24)	2.52 (±0.79)
zinc sulfate	3.07 (±0.21)	0.04 (±0.69)
aminoguanidine	1.94 (±0.14)	−3.85 (±0.47)
guanidine	1.41 (±0.14)	−5.52 (±0.48)
semicarbazide	6.41 (±1.28)	16.74 (±4.10)
boiled	0	

[a] Boiled noodles showed rapid browning at >54 °C.

This darkening is rapid and therefore attributable to the Maillard reaction. Boiling denatures proteins and thereby stops enzyme activity. However, it changes the conformation of most if not all proteins and also affects the way protein interacts with starch (*36*). Thus, boiling has a great effect on the matrix and thereby on non-PPO darkening.

Non-PPO Darkening in YAN. The data in this paper suggest that the specific inhibitors of non-PPO darkening react with soluble protein precursors or products to inhibit darkening in YAN. There are two possible mechanisms by which darkening may proceed: (a) proteins containing aromatic amino acids participate in reactions that extend the chromophore and shift the absorbance maximum into the visible region or (b) low-temperature Maillard reaction (amine−carbonyl) resulting in a colored chromophore (*37*).

The minimization of PPO darkening in YAN can be easily achieved using cultivars with very low or near-zero PPO (*6*). However, the control of non-PPO darkening is more difficult. In the fresh product, the only method available is to make YAN with the minimum acceptable level of protein. It is unlikely that inhibitors specific for non-PPO darkening suitable to the food industry will be identified without further elucidation of the mechanism for protein darkening. In the absence of a satisfactory inhibitor, cooking appears to be the only method of limiting non-PPO darkening.

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