A Whole Seed Assay for Polyphenol Oxidase in Canadian Prairie Spring Wheats and Its Usefulness as a Measure of Noodle Darkening[1]

J. E. KRUGER,[2] D. W. HATCHER,[2] and R. DePAUW[3]

Cereal Chem. 71(4):324-326

ABSTRACT

A quantitative assay has been developed for assessing the polyphenol oxidase (PPO) levels in grain without the requirement for seed grinding. Seeds were steeped for 16 hr in water and then catechol substrate was added. The resulting color of the liquid was measured after 30 min using a kinetic microplate reader. The method correlated well ($r = 0.85$) for both the standard oxygen-electrode method for PPO assay performed on ground grain suspensions and the rate of decrease in brightness (L^*) or increase in yellowness (b^*) of a raw Cantonese noodle sheet after storage for 4 or 24 hr. A substantial amount of the enzyme is present in steep water and can also be used to assay for the relative PPO activity in the seed. The method should be particularly useful to plant breeders for screening of PPO levels in Canadian Prairie Spring wheats.

The enzyme polyphenol oxidase (PPO) can cause undesirable color changes in numerous wheat-based end products such as chapattis (Abrol et al 1971), Middle East flat breads (Faridi 1988), steamed bread (Dexter et al 1984), and noodles (Kruger et al 1992). The ongoing development by Canadian plant breeders of the Canadian Prairie Spring (CPS) wheat class (white and red seed coats) for utilization in these products requires development of a quantitative assay for PPO screening of large numbers of samples. Presently, the only quantitative assay involves measuring the change in oxygen consumption of a suspension of ground grain and mono- or diphenolic substrates (Lamkin et al 1981, Marsh and Galliard 1986, Hatcher and Kruger 1993). The assay is time-consuming and not suited to a plant-breeding program. A number of subjective assays exist in which the darkening of whole seeds treated by either phenol or tyrosine are visually assessed (Abrol et al 1971, Mahoney and Ramsay 1992). For CPS wheat, differences have been observed in the darkening of whole kernels during the phenol test, but subjective rankings between degrees of darkening have been hard to make. This article describes a method of measuring PPO in whole kernels that is suitable for large-scale screening of PPO in the CPS-white seed coat (CPS-W) wheat. In addition, the relationship of PPO to enzymatic darkening in Cantonese noodles is clarified.

[1]Contribution 712 of the Canadian Grain Commission, Grain Research Laboratory.
[2]Canadian Grain Commission, Grain Research Laboratory, 1404-303 Main Street, Winnipeg, MB.
[3]Agriculture Canada Research Station, PO Box 1030, Swift Current, SK.

MATERIALS AND METHODS

Wheat and Flour Samples

Wheat samples used in this study were from 22 individual breeder lines of CPS-W wheat grown at Swift Current, SK. The wheat samples had a test weight range of 79.1-84.1 kg/hl. The 1,000-kernel weight range was 32.6-42.9 g. Samples were milled into flours with an Allis Chalmers laboratory mill (Dexter and Tipples 1987). The ranges of wheat and flour characteristics were: flour yield 74.17-76.67%, particle size index 47.7-60.9% (Williams and Sobering 1986), flour protein 10.5-13.3% (Williams 1973), wet gluten 33.4-42.8% (ICC 1980), flour ash 0.46-0.56% (AACC 1983), and starch damage 16-37% (Farrand 1964).

Noodle Preparation

Laboratory preparation of noodles was similar to that described previously in this laboratory and others (Oh et al 1983, Miskelly and Moss 1985, Shelke et al 1990, Kruger et al 1992). A Hobart N50 mixer (Hobart, Canada, North York, ON) was used to mix the ingredients. An Ohtake laboratory noodle machine (Ohtake Noodle Machine Mfg. Co. Ltd., Tokyo, Japan) was used to prepare the noodles. A solution of 1% kansui (a mixture of 9 parts sodium carbonate and 1 part potassium carbonate) was added to 250 g of flour in a Hobart N50 mixer over 30 sec with slow speed mixing (setting 1) to a final water absorption of 32%. Mixing was continued for 30 sec at slow speed, followed by 1 min at high speed (setting 2), and an additional 3 min at low speed. Then the ingredients were passed through the Ohtake laboratory noodle machine with a 3.00-mm clearance between rollers and a roller temperature of 28°C. The sheet was passed through the machine twice more at this gap setting, once with folding and once without. The sheet was then processed through the noodle machine with the roller gap successively reduced 15% for each

of the next six passes (gaps of 2.55, 2.15, 1.85, 1.57, 1.33, and 1.10 mm). The final sheet was not cut, but retained for color measurement.

Color Measurement

Raw noodle sheet color was measured with a Labscan II spectrocolorimeter (HunterLab, Reston, VA) using the CIE 1976 *L**, *a**, *b** color scale with a D65 illuminant (Kruger et al 1992). Noodle sheets were stored in plastic bags at 24.5°C. Readings were made at 0, 4, and 24 hr. Measurements were made in triplicate at two surface locations near the center of the sheet. The differences in readings between 4 and 0 hr, as well as those between 24 and 0 hr, were calculated.

PPO on Ground Grain and Flour (Standard Method)

PPO activity was determined with a YSI model 5300 biological oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, OH) as described by Marsh and Galliard (1986), with the following modifications. The temperature of the assay was raised to 37°C, and assay medium consisted of 4 ml of air-saturated 0.01*M* McIlvaines buffer, pH 6.8, with catechol as the substrate (Kruger 1976). Results are expressed as nmol of O_2 consumed per minute per gram at 37°C. Analyses were performed in triplicate.

Whole Grain PPO Analyses

Five whole wheat seeds were added to 3 ml of distilled water and steeped overnight (16 hr) at room temperature. Freshly prepared 4% catechol (1 ml) was added to the combined steep water and seed. The mixture was then vortexed and incubated for 30 min at 37°C. A 350-μl portion of the liquid was removed and placed in a microtiter well. Color was measured with a Thermomax UV kinetic microplate reader (Molecular Devices, Palo Alto, CA). Results were expressed as the A_{405} per minute per gram of sample $\times 10^3$. Analyses were performed in triplicate.

Two variations of this method were also examined. In the first variation, the 16-hr steep water was discarded, and 4 ml of substrate consisting of 1% catechol was added to the seeds. This was followed by a 30-min incubation and color measurement as described above. In the second variation, a 100-μl aliquot of the 16-hr steep water was incubated with 200 μl of 1% catechol, and the kinetic rate of reaction was measured with the kinetic microplate reader.

RESULTS AND DISCUSSION

Preliminary Experiments on Whole Seeds

As PPO can utilize a variety of substrates, the monophenolic substrates, tyrosine and phenol, were compared with the diphenolic substrate, catechol, in preliminary experimentation and were found to be less satisfactory in terms of the low rate of color production. Furthermore, it was felt that results with the diphenolic substrate would relate better to the standard ground-grain oxygen-electrode method that employs catechol as substrate. Initial attempts were made to measure the PPO activity of whole seeds by adding the catechol solution directly to the dried seeds. Although a detectable amount of yellow color was observed in as short a time as 15 min, there was very little discernment between cultivars with different enzyme activities, even with reaction times as long as 60 min. After an overnight steep in distilled water, however, a reaction time of 30 min was sufficient for adequate discrimination of steeped seeds when substrate was added to seed and steep water. Furthermore, the increase in color production at A_{405} was linear, with reaction times between 0 and 60 min even for the wheat samples with the highest PPO activities in the sample set. This indicated that the reaction was not substrate-limiting. Further confirmation of this was the finding that increasing the substrate concentration from 1 to 3% did not affect the rate of color change with time. A noticeable (~50%) increase in reaction rate was observed when the water was saturated with oxygen. However, later studies indicated that employing oxygen-saturated water did not improve upon the relative activities of samples in PPO methods. For simplicity, this was not incorporated as a part of the method.

Relationship Between Whole Seed and Ground Grain PPO Assays

The relationship between the whole seed PPO assay (measuring the enzyme activity present in the whole seed and steep water) and that of the standard PPO method for 22 lines of CPS-W is shown in Figure 1A. The Pearson correlations of 0.85 ($P < 0.001$) indicated that the whole seed PPO assay could be used to give a good approximation of total PPO activity. Performing the assay on the whole seed, with the steep water discarded and replaced with substrate solution, gave a relationship ($r = 0.86$, $P < 0.0001$) that was equally as good as that of total ground grain PPO (Fig. 1B). The differences in the regression equations suggested that the amount of extractable PPO varied depending on the level of total activity. Calculations indicated that, as the PPO activity in the seed increased, a greater percentage became soluble in the steep water ($r = 0.72$, $P < 0.0001$). The close association between the PPO methods gives further credence to previous reports that the enzyme PPO must be closely associated with the bran layer, at least in ungerminated seeds (Marsh and Galliard 1986, Hatcher and Kruger 1993, Kruger and Hatcher 1993). The outer layer, rather than the inner part of the bran, likely contains the bulk of the seed PPO because permeability and consequent reaction of residual whole seed PPO with substrate occurred rapidly.

A considerable amount of the PPO activity was observed in the 16-hr steep water when measured alone. This residual steep water can also be used for an indication of PPO, which is advantageous if retention of the steeped seeds for planting is desirable. In preliminary experimentation, 200 μl of 1% catechol was added to 100 μl of 16-hr steep water. The reaction rate was monitored

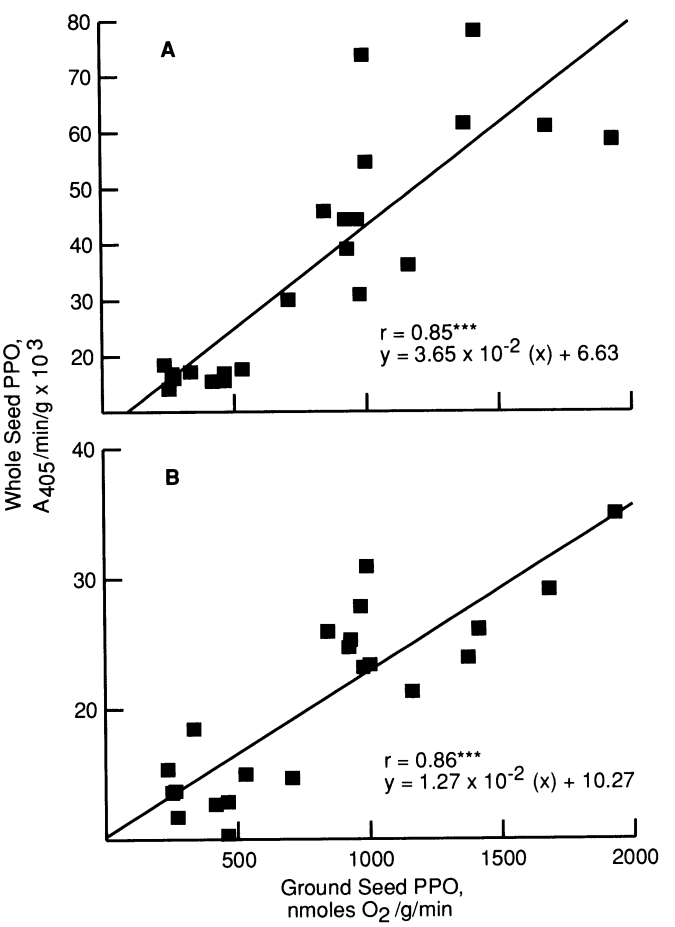

Fig. 1. Relationship between whole seed polyphenol oxidase (PPO) assay and ground seed PPO assay determined by the oxygen electrode method: **A,** with steep water; **B,** without steep water.

TABLE I
Pearson Correlation Coefficients Between Polyphenol Oxidase (PPO) Assays and Rate of Change in Noodle Color Parameters[a]

Change in Noodle Color Component Over Time	PPO (Standard Assay)		PPO (Whole Grain Assay)
	Wheat	Flour	
ΔL^* (0–4 hr)	0.84	0.84	0.72
ΔL^* (0–24 hr)	0.87	0.81	0.77
Δb^* (0–4 hr)	0.87	0.77	0.63
Δb^* (0–24 hr)	0.88	0.75	0.74

[a] All correlations were significant at $P < 0.001$.

at 37°C over 10 min at 405 nm, using the kinetic microplate reader. A correlation of $r = 0.81$ ($P < 0.001$) was obtained between the V_{max} and PPO activity determined by the oxygen-electrode method for the 22 lines of CPS-W used in this study.

Relationship of PPO Assays to Noodle Discoloration

Wheat PPO levels, determined by the whole grain or ground-grain oxygen-electrode method, correlated with flour PPO levels determined by the oxygen-electrode method ($r = 0.70$ and 0.91, respectively, at $P < 0.0001$). It was of interest, therefore, to see whether the PPO assays described in this article were practical for ranking flours in order of their abilities to cause noodle darkening in raw Cantonese noodles. Such noodles are prepared with kansui, a mixture of alkaline carbonates, and they are frequently stored raw for up to 24 hr before cooking. Both the alkali conditions and the long storage time may cause noodle darkening (Miskelly 1984), which is aggravated by elevated levels of PPO enzymes (Kruger et al 1992). Previous research in this laboratory has indicated that the rate of change in brightness (L^*) of a noodle sheet, measured with a HunterLab colorimeter, can be a measure of such enzymatic darkening (Kruger et al 1992). Therefore, Cantonese noodle sheets were prepared from straight-grade flours of 22 CPS-W lines. Color changes (L^* and b^*) were monitored at 0, 4, and 24 hr. The difference in color between the 0 hr and 4 or 24 hr was plotted against the standard oxygen-electrode method activities for both the wheats and the flour, as well as the activity determined by whole grain assay. As the PPO activity increased, the rate of change in brightness (L^*) and yellowness (b^*) increased. The resulting Pearson correlation coefficients for the relationships between the polyphenol oxidase assays and rate of change in noodle color are shown in Table I. The rate of change for both L^* and b^* correlated fairly well with the PPO level of wheat and flour determined using the standard oxygen-electrode method at both time periods. Correlation of the rate changes in L^* and b^* by the whole seed PPO assay were lower than those obtained by the oxygen-electrode method at both time periods, but they were still very suitable for screening purposes. Correlations existed with the a^* values at 4 hr, but they were nonsignificant after 24 hr.

CONCLUSIONS

The PPO enzyme that is leached out of the wheat kernel, or is near the surface of the bran and accessible to substrate, provides for a convenient quantitative assay that correlates well with the standard assay. It is particularly relevant for plant-breeding purposes, where throughput is important. It should be kept in mind that the test has been evaluated with the CPS class of wheat. Methods for other classes of wheat might dictate modifications to the operational conditions. The present assay was performed on five kernel portions, but it should be possible to analyze single kernels when necessary. Although activity was performed on a per gram basis, elimination of the weighing step provided an acceptable, although lower, correlation with the standard method. This compromise in accuracy is probably warranted in the interests of increasing throughput.

In most cases, the whole kernel assay using both the whole seed and steep water will be the most convenient and sensitive because a better approximation of total PPO will be measured. If a laboratory has access to a kinetic microplate reader, however, analyses of the extractable enzyme may provide increased throughput; 96 samples can be run simultaneously and reaction rates are calculated automatically by the computer software. Moreover, as only the steep water is utilized, further germination and plant growth can be conducted on those steeped seeds that are low in PPO activity.

The practicality of linking increased PPO activity to increased noodle darkening emphasizes the relevance and practical value of plant-breeding efforts in monitoring for this enzyme in CPS wheats and the value of the current whole-grain seed test for rapid quantitative screening.

LITERATURE CITED

ABROL, Y. P., UPRETY, D. C., RAM, A., and TIKOO, S. 1971. Phenol color reaction as an indicator of chapatti quality in wheat. SABRAO Newsl. 3:17-21.

AMERICAN ASSOCIATION OF CEREAL CHEMISTS. 1983. Approved Methods of the AACC, 8th ed. Method 08-01, approved April 1961, revised October 1981; Method 54-10, approved April 1961, revised October 1982. The Association: St. Paul, MN.

DEXTER, J. E., and TIPPLES, K. H. 1987. Wheat milling at the Grain Research Laboratory. Part 2. Equipment and procedures. Milling 180(6):16,18-20.

DEXTER, J. E., PRESTON, K. R., MATSUO, R. R., and TIPPLES, K. H. 1984. Development of a high extraction flow for the GRL Pilot Mill to evaluate Canadian wheat potential for the Chinese market. Can. Inst. Food Sci. Technol. 14:253-259.

FARIDI, H. 1988. Flat breads. Pages 457-506 in: Wheat: Chemistry and Technology, 3rd ed., Vol. 2. Y. Pomeranz, ed. Am. Assoc. Cereal Chem.: St. Paul, MN.

FARRAND, E. A. 1964. Flour properties in relation to the modern bread processes in the United Kingdom, with special reference to alpha-amylase and starch damage. Cereal Chem. 41:98-111.

HATCHER, D. W., and KRUGER, J. E. 1993. Distribution of polyphenol oxidase in flour millstreams of Canadian common wheat classes milled to three extraction rates. Cereal Chem. 70:51-55.

ICC. 1980. Standard 137, Mechanical determination of the wet gluten content of wheat flour (Glutomatic). International Association for Cereal Chemistry: Vienna.

KRUGER, J. E., MORGAN, B., MATSUO, R. R., and PRESTON, K. R. 1992. A comparison of methods for the prediction of Cantonese noodle color. Can. J. Plant Sci. 72:1021-1029.

KRUGER, J. E. 1976. Changes in polyphenol oxidases of wheat during kernel growth and maturation. Cereal Chem. 53:201-213.

KRUGER, J. E., and HATCHER, D. W. 1993. The relationship between α-amylase and polyphenol oxidase activities in wheat. Pages 455-462 in: Pre-Harvest Sprouting in Cereals 1992. M. K. Walker-Simmons and J. L. Ried, eds. Am. Assoc. Cereal Chem.: St. Paul, MN.

LAMKIN, W. M., MILLER, B. S., NELSON, W. S., TRAYLOR, D. D., and LEE, M. S. 1981. Polyphenol oxidase activities of hard red winter, soft red winter, hard red spring, white common, club, and durum wheat cultivars. Cereal Chem. 58:27-31.

MAHONEY, R. R., and RAMSAY, M. 1992. A rapid tyrosinase test for detecting contamination of durum wheat. J. Cereal Sci. 15:267-270.

MARSH, D. R., and GALLIARD, T. 1986. Measurement of polyphenol oxidase activity in wheat-milling fractions. J. Cereal Sci. 4:241-248.

MISKELLY, D. M. 1984. Flour components affecting paste and noodle color. J. Sci. Food Agric. 35:463-471.

MISKELLY, D. M., and MOSS, H. J. 1985. Flour quality requirements for Chinese noodle manufacture. J. Cereal Sci. 3:379-387.

OH, N. H., SEIB, P. A., DEYOE, C. W., and WARD, A. B. 1983. Noodles. I. Measuring the textural characteristics of cooked noodles. Cereal Chem. 60:433-438.

SHELKE, K., DICK, J. W., HOLM, Y. F., and LOO, K. S. 1990. Chinese wet noodle formulation: A response surface methodology study. Cereal Chem. 67:338-341.

WILLIAMS, P. C. 1973. The use of titanium oxide as a catalyst for large-scale Kjeldahl determination of total nitrogen content of cereal grains. J. Sci. Food Agric. 24:343-348.

WILLIAMS, P. C., and SOBERING, D. 1986. Attempts at standardization of hardness testing of wheat. II. The near-infrared reflectance method. Cereal Foods World 31:417-420.

[Received June 28, 1993. Accepted January 27, 1994.]