

A Practical Spectrophotometric Approach for the Determination of Lipoxygenase Activity of Durum Wheat

V. Gökmen,¹ A. Serpen,¹ A. Atli,² and H. Köksel^{1,3}

ABSTRACT

Cereal Chem. 84(3):290–293

In this study, a practical spectrophotometric approach was used to determine the hydroperoxidation activity of durum wheat lipoxygenase (LOX). As stated in the related literature, the buffered linoleic acid solution used as the reaction medium is not optically clear enough at neutral and lower pH values due to its limited solubility. In our study, the optical clarity was obtained by the formation of sodium-salt of unreacted linoleic acid just before absorbance measurement. The durum wheat LOX was characterized in terms of pH and temperature optima as well as kinetic parameters. The maximum linoleic acid hydroperoxidation activ-

ities were determined at pH 5.0 and 6.5 and at 40°C. This result can be considered as evidence for the presence of at least two LOX isoforms with respective optima at pH 5.0 and 6.5 in the crude durum wheat extract. The Michaelis constant (K_m) and maximum hydroperoxidation activity rate (V_{max}) of durum wheat LOX for linoleic acid were estimated to be 0.131 ± 0.019 mM and 42.37 ± 3.32 units/mg of protein/min, respectively. The method seems to be useful for the determination of LOX activity in durum wheat and its milling fractions.

Lipoxygenase (LOX, linoleate: oxygen oxidoreductase, EC 1.13.11.12), also known as lipoxidase, is an iron-containing dioxygenase. The enzyme catalyzes regio- and stereospecific oxygenation of polyunsaturated fatty acids and their esters and glycerides containing the *cis*, *cis*-1,4-pentadiene double-bond system originating in a hydroperoxide that possesses a chain with a *cis*, *trans* conjugated double-bond (Jaren-Galan and Minguéz-Mosquera 1999). It is widely distributed in plants, including a great variety of fruits and vegetables (Marcus et al 1988; Minguéz-Mosquera et al 1990), cereal grains (McDonald 1979; Hsieh and McDonald 1984; Trono et al 1999), mammals, fish, and microorganisms (Iny et al 1993; Bisakowski et al 1997).

From the food quality point of view, the potential effects of the products formed during enzymatic reactions are much more important than the reaction itself. The action of LOX on its substrate generates highly reactive compounds that are initiators of a cascade reaction in which components playing no part in enzymatic reaction may be affected secondarily, resulting in indirect losses of nutritive value, alterations of organoleptic properties, and color. During postharvest and technological treatments, foodstuffs lose their structural integrity, and substances that had been compartmentalized come into contact with each other. In such a situation, components that are stable in their natural environment, such as carotenoids, are transformed and degraded by the oxidizing environment (Jaren-Galan and Minguéz-Mosquera 1999). For example, loss of color observed during pasta processing is due to the LOX-linoleic acid system, which is responsible for carotenoid (mainly lutein) oxidation (Trono et al 1999). Although other enzymes such as peroxidases and polyphenoloxidases can contribute to semolina bleaching, a major role appears to be played by LOX (Taha and Sagi 1987).

Several different approaches have been used to measure LOX activity. These include determination of O₂ consumption during linoleic acid oxidation using an oxygen electrode or Warburg apparatus, colorimetric reactions of the hydroperoxide product with iodine or thiocyanate, spectrophotometric measurement of conjugated diene group in the hydroperoxide product, and determination of the bleaching of pigments in co-oxidation reactions (Galliard 1983).

Approved Method 22-40 (AACC International 2000) for determination of lipoxygenase activity of semolina and flour requires a Warburg apparatus and is based on manometric determination of O₂ uptake. The method is not commonly used due to various drawbacks. Appropriate controls are necessary to eliminate contributions to O₂ uptake from reactions other than LOX such as autooxidation, α -oxidation of fatty acids, and oxidation of nonlipid materials in crude extracts (Galliard 1983). Furthermore, compensation for changes in temperature or pressure is necessary using a thermobarometer. All of these factors make Approved Method 22-40 impractical. In recent years, use of Approved Method 22-40 has not been encountered in the related literature.

Most studies have used the quantitative spectrophotometric method that is based on the increase of absorbance at 234 nm due to formation of 9- and 13-hydroperoxides by LOX to assay enzyme activity in a wide variety of food and biological matrices (Surrey 1964; Al-Obaidy and Siddiqi 1981; Williams et al 1986; Halpin and Lee 1987; Lee et al 1988; Adams 1989; Sheu and Chen 1991; Bisakowski et al 1998; Busto et al 1999; Jaren-Galan and Minguéz-Mosquera 1999). However, linoleic acid, the substrate of LOX, is poorly soluble in water. Therefore, the buffered linoleic acid solution used as the reaction medium is not clear enough optically at neutral or lower pH values due to its limited solubility, preventing a precise, accurate, and reproducible estimation of LOX activity. The clarity of the reaction medium also depends on temperature, pH, etc. Researchers using spectrophotometric method have also reported the solubility problem of linoleic acid. Pastore et al (2000) reported that it was not possible to reach the required linoleate concentration to obtain maximal reaction rate because the turbidity of the assay mixture did not allow accurate determination of the reaction rate when linoleate concentration exceeded 700 μ M.

Therefore, the spectrophotometric method was modified to report the properties of LOX enzymes more accurately by preventing the turbidity of reaction medium before absorbance measurement. The modified spectrophotometric method was used to determine some properties of durum wheat LOX such as pH and temperature optima, Michaelis constant (K_m). LOX activities in different milling fractions of durum wheats were also investigated.

MATERIALS AND METHODS

Reagents

Linoleic acid (99% pure) and bovine serum albumin were purchased from Sigma (St. Louis, MO). Tween 20, potassium dihydrogen phosphate, di-sodium hydrogen phosphate, sodium hydroxide, glacial acetic acid, and sodium acetate were analytical grade

¹ Food Engineering Dept, Hacettepe University, 06800 Beytepe, Ankara, Turkey.

² Food Engineering Dept, Harran University, Sanliurfa, Turkey.

³ Corresponding author. Phone: +90 312 297 71 07. Fax: +90 312 299 21 23. E-mail: koksel@hacettepe.edu.tr

purchased from Merck (Darmstadt, Germany). Deionized distilled water was used in all experiments.

Preparation of Enzyme Extract

The durum wheat sample (cv. Kiziltan) used in this study was from the experimental research farm of Field Crops Improvement Center, Ankara, Turkey, harvested in 2002. The wheat sample was cleaned on a Carter Dockage tester and ground on a cyclone mill fitted with a 0.5-mm sieve (Udy) to obtain whole meal.

Enzyme was extracted by mixing 10 g of durum wheat whole meal with 50 mL of phosphate buffer (pH 6.5) at 4°C in a homogenizer (Virtis model 23) for 30 min. The mixture was centrifuged (Sigma model 3K18, Germany) at 25,000 × *g* for 15 min at 4°C. The supernatant containing LOX was used as the crude enzyme extract. The protein determinations were made using the dye-binding method of Bradford (1976). A standard curve was constructed using bovine serum albumin in the concentration range of 50–1,000 µg/mL in which a linear response was observed.

The LOX activities of different milling fractions (fine bran, course bran, semolina, and whole meal) were also measured. For this purpose, three advanced durum wheat lines (ANK 017, ANK 018, and ANK 021) and three durum wheat cultivars (Kiziltan, Kunduru, and Cesit 1252) obtained from the experimental research farm of Field Crops Improvement Center were used. The grain samples (1 kg) were cleaned on a Carter dockage tester, tempered overnight to a water content of 16.5%, and milled on a Buhler pneumatic laboratory mill (model MLU 202) to obtain semolina. Semolina samples were purified using a laboratory-type purifier (Namad, Italy). The enzyme extracts of the durum wheat milling fractions were prepared as described above.

Spectrophotometric Assay of LOX Activity

Linoleic acid solution (29.9 mL) was transferred into a 100-mL flask placed in a temperature-controlled water bath set at 30°C. The linoleic acid solution was aerated by a gentle stream of air for 2 min and the reaction was started by adding 0.1 mL of enzyme extract into the flask. Aliquots of 1 mL from the reaction medium were transferred into glass tubes containing 4 mL of 0.1N NaOH solutions at time intervals of 0.5, 1.0, 1.5, 2.0, and 2.5 min. The use of 0.1N NaOH stopped the enzymatic reaction and at the same time ensured the optical clarity by formation of the Na-salt of unreacted linoleic acid before absorbance reading. Formation of hydroperoxides was monitored spectrophotometrically as the increase of absorbance at 234 nm due to the presence of a conjugated hydroperoxydiene moiety. The blank solution was the mixture of 1 mL of substrate and 4 mL of 0.1N NaOH. One unit of LOX activity was defined as an increase in absorbance at 234 nm/min/mg of protein under assay conditions. A double beam spectrophotometer (Shimadzu model 2101PC, Japan) and 1-cm path length quartz cuvette were used. For each data point, LOX activity was determined in duplicate extracts of the durum wheat whole meal with parallel spectrophotometric measurements, and the mean values were reported. The precision of the method was tested by measuring the activity of LOX in a single wheat sample for 10×. The coefficient of variation was <5% for the spectrophotometric assay of LOX activity.

pH Optimum for LOX Activity

To determine the pH optimum, activity of crude durum wheat LOX was determined spectrophotometrically at pH 4.0–10.0 using the linoleic acid at a concentration of 0.25 mM. The buffer systems were prepared by mixing appropriate amounts of *M*/15 KH₂PO₄ and Na₂HPO₄ for pH 5.0–8.0, 1N CH₃COOH, and 1N CH₃COONa for pH 4.0, and *M*/15 Na₂HPO₄ and 1N NaOH for pH 9.0–10.0. 29.9 mL of the substrate solution buffered to pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, and 10.0 were placed in a temperature-controlled water bath that was set to 30°C. Following the aeration by a gentle stream of air for 2 min, adding

0.1 mL of enzyme extract to the flask started the reaction. For each data point, LOX activity was determined in duplicate extracts of the durum wheat whole meal with parallel spectrophotometric measurements, and the mean values were reported.

Temperature Optimum for LOX Activity and Stability

To determine temperature optimum, the activity of durum wheat LOX was determined spectrophotometrically in the temperature range of 3–60°C using the linoleic acid at a concentration of 0.25 mM prepared in *M*/15 sodium phosphate buffer, pH 6.5. Substrate solution (29.9 mL) was placed in temperature-controlled water baths set to 3, 10, 20, 30, 40, 50, and 60°C. Following the aeration by a gentle stream of air for 2 min, adding 0.1 mL of enzyme extract to the flask started the reaction. All measurements were replicated. For each data point, LOX activity was determined in duplicate extracts of the durum wheat whole meal with parallel spectrophotometric measurements, and the mean values were reported.

Kinetic Study of Durum Wheat LOX

Linoleic acid solutions at concentrations of 0.010, 0.025, 0.050, 0.100, 0.250, 0.500, and 1.0 mM were prepared by mixing certain amounts of linoleic acid with 157.2 µL Tween 20, 10 mL of water, and 1 mL of 1N NaOH, then diluting them to a 200 mL final volume with *M*/15 sodium phosphate buffer at pH 6.0. These solutions were used to determine the Michaelis constant (*K_m*) and the maximum activity rate (*V_{max}*) in the enzyme extract from durum wheat. The double-reciprocal method of Lineweaver and Burk (1934) and linear regression by least squares were used to determine *K_m* and *V_{max}* in the enzyme extract. For each data point, LOX activity was determined in duplicate extracts of the durum wheat whole meal with parallel spectrophotometric measurements and the mean values were reported.

RESULTS AND DISCUSSION

In spectrophotometric studies of the LOX-catalyzed reaction at values lower than pH 8.0, the low solubility of fatty acids is a common problem (Suurmeijer et al 1998; Parraud et al 1999). The detergents (Tween 20 or Tween 80) added to the substrate or to the buffer may help to clear the visual turbidity to a certain extent, but this does not completely overcome light-scattering problems. When the LOX-catalyzed reaction was started in the cuvette containing 2.5 mL of linoleic acid solution by adding 0.1 mL of enzyme extract, the initial absorbance reading at 234 nm against the water blank was >1.5 and rapidly exceeded 2.0, which indicates a light transmittance ratio of <1%. Gökmen et al (2002) previously reported that the absorbance readings in the cuvette continue to increase even if no enzyme extract is added, depending on loss of solubility of linoleic acid, which leads to an estimation of a pseudo LOX activity at certain conditions. This point is not only very important for accurate kinetic characterization of isoforms of LOX from different origins, but it is also important for accurate estimation of the effects of various technological treatments on LOX activity during food processing.

In this study, the LOX-catalyzed reaction was performed in a flask (100 mL) placed into a temperature-controlled water bath set at 40°C. The absorbance readings were performed after 1 mL of reaction medium was transferred into glass tubes containing 4 mL of 0.1N NaOH. The use of 0.1N NaOH just before absorbance readings allowed us to form Na-salt of unreacted linoleic acid, thus to obtain an optically clear solution for an accurate measurement of hydroperoxides formed due to LOX-catalyzed reaction. The typical curve of absorbance at 234 nm versus time for durum wheat LOX is shown in Fig. 1. The LOX activity was calculated from the slope of the initial linear (*r*² = 0.997) part of this curve. A reaction time of 2.5 min was enough to determine durum wheat LOX activity without any initial induction period.

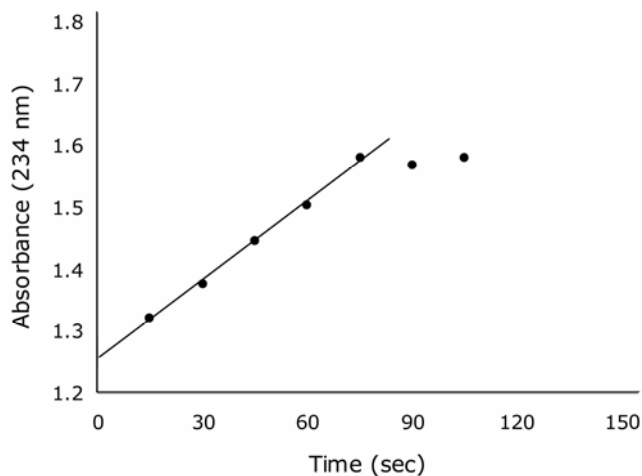


Fig. 1. Typical data for spectrophotometric measurement of LOX activity.

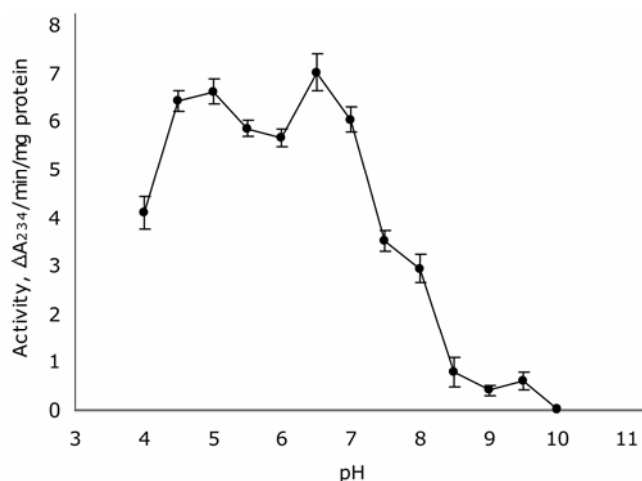


Fig. 2. Effect of pH on hydroperoxidation activity of crude durum wheat LOX. Data points are given as mean \pm standard deviation.

The pH profile of LOX from durum wheat is given in Fig. 2, which clearly shows that relatively higher linoleic acid hydroperoxidation activities were measured at pH 5.0 and 6.5. Because the enzyme extract was used for the characterization of LOX from durum wheat, this pH profile with two pH optima can be considered as an evidence for the presence of at least two isoforms of LOX in this sample. Very little linoleic acid hydroperoxidation activity was observed at pH 8.5 or above. Pastore et al (2000) measured the maximum activity of durum wheat semolina LOX at pH of 5.3 in the crude extract.

The study of durum wheat LOX extract at different temperatures revealed that enzyme activity gradually increased with temperature until it reached 40°C. Relatively high activity was also measured at 50°C, but enzyme activity started to decrease sharply after that (Fig. 3). These results compared well with the optimum temperature reported for wheat germ, which was 45°C and sweet corn germ, which was 50°C (Shiiba et al 1991).

The linoleic acid hydroperoxidation rate followed Michaelis-Menten kinetics equation ($r^2 = 0.994$) (Fig. 4). The Lineweaver-Burk plot of hydroperoxidation activity rates (V) versus linoleic acid concentrations (S) ranging from 0.01 to 1.0 mM was constructed to determine K_m and V_{max} for durum wheat LOX. V_{max} was calculated to be 42.37 ± 3.32 units/mg of protein/min from the intercept of the Lineweaver-Burk plot. K_m value for linoleic acid for crude LOX extract was 0.131 ± 0.019 mM by extrapolating the Lineweaver-Burk plot to the hypothetical point where

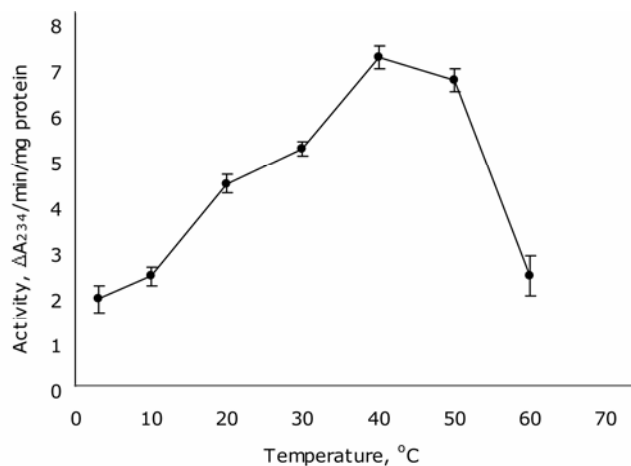


Fig. 3. Effect of temperature on hydroperoxidation activity of crude durum wheat LOX. Data points are given as mean \pm standard deviation.

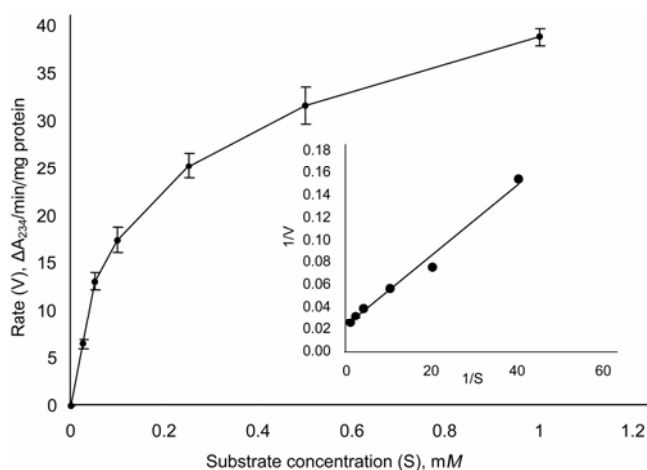


Fig. 4. Plot of substrate (linoleic acid) concentration vs. durum wheat LOX hydroperoxidation activity rate. Data points are given as mean \pm standard deviation.

$1/V = 0$. Pastore et al (2000) reported the K_m for linoleate to be 60 ± 0.67 μM for LOX from durum wheat semolina extract. However, they have also indicated that it was not possible to reach the required linoleate concentration to obtain maximal reaction rate because of the turbidity problem.

The LOX activities in different milling products of six durum wheat samples (3 advanced lines and 3 cultivars) were also measured (Table I). The LOX activities of the fine bran and coarse bran samples varied widely and were in the range of 3.98–13.67 and 4.89–17.22 $\Delta A_{234}/\text{min}/\text{mg}$ of protein, respectively. The LOX activities of the semolina samples were much lower as compared with the respective bran samples and were in the range of 0.60–3.34 $\Delta A_{234}/\text{min}/\text{mg}$ of protein. Rani et al (2001) also reported that the lipoxygenase was highly concentrated in different bran fractions and least active in semolina. The method seems to be suitable for detecting the differences of durum wheat genotypes and their different milling products in terms of LOX activities.

CONCLUSIONS

A practical spectrophotometric method was used to characterize LOX from durum wheat in terms of pH and temperature optimum and kinetic parameters. The elimination of the turbidity of linoleic acid solution enabled the optical measurement of LOX activity more accurately in durum wheat samples.

TABLE I
LOX Activity in Different Milling Fractions of Different Durum Wheat Genotypes^a

| Genotype | LOX Activity ($\Delta A_{234}/\text{min/mg of protein}$) | | | |
|----------|--|--------------|-------------|-------------|
| | Fine Bran | Coarse Bran | Semolina | Whole Meal |
| ANK 017 | 3.98 ± 0.06 | 4.89 ± 0.07 | 1.26 ± 0.02 | 1.29 ± 0.03 |
| ANK 018 | 6.83 ± 0.11 | 7.38 ± 0.13 | 1.59 ± 0.01 | 2.25 ± 0.02 |
| ANK 021 | 5.19 ± 0.09 | 6.94 ± 0.15 | 1.28 ± 0.05 | 1.87 ± 0.04 |
| C-1252 | 7.47 ± 0.13 | 8.48 ± 0.21 | 0.60 ± 0.01 | 1.31 ± 0.01 |
| Kiziltan | 6.16 ± 0.17 | 9.51 ± 0.19 | 0.95 ± 0.04 | 2.93 ± 0.03 |
| Kundurur | 13.67 ± 0.21 | 17.22 ± 0.27 | 3.34 ± 0.09 | 4.34 ± 0.07 |

^a LOX activity values are given as mean ± standard deviation.

Further studies are required for the exact characterization of appropriately isolated and purified LOX isoforms from different durum wheat cultivars. This method offers an accurate and precise measurement of LOX activity in wheat. The method also proved to be suitable for measuring LOX activities in different durum wheat genotypes and their milling fractions.

LITERATURE CITED

- AACC International. 2000. Approved Methods of the American Association of Cereal Chemists, 10th Ed. Approved Methods 22-40, 26-41. The Association: St. Paul, MN.
- Adams, J. B. 1989. Inhibition of green bean lipoxygenase by cyanide. *Food Chem.* 31:243-250.
- Al-Obaidy, H. M., and Siddiqi, A. M. 1981. Properties of broad bean lipoxygenase. *J. Food Sci.* 46:622-626.
- Bisakowski, B., Kermasha, S., and Spinnler, E. 1998. Characterization of purified lipoxygenase extracts from *Fusarium proliferatum*. *J. Agric. Food Chem.* 46:2382-2388.
- Bisakowski, B., Perraud, X., and Kermasha, S. 1997. Characterization of hydroperoxides and carbonyl compounds obtained by lipoxygenase extracts of selected microorganisms. *Biosci. Biotechnol. Biochem.* 61:1262-1269.
- Bradford, M. N. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
- Busto, M. D., Owusu-Apenten, R. K., Robinson, D. S., Wu, Z., Casey, R., and Hughes, R. K. 1999. Kinetics of thermal inactivation of pea seed lipoxygenases and the effect of additives on their thermostability. *Food Chem.* 65:323-329.
- Galliard, T. 1983. Enzymic degradation of cereal lipids. In: *Lipids in Cereal Technology*. P. J. Barnes, ed. Academic Press: London.
- Gökmen, V., Bahceci, K. S., and Acar, J. 2002. Characterization of crude lipoxygenase extract from green pea using a modified spectrophotometric method. *Eur. Food Res. Technol.* 215:42-45.
- Halpin, B. E., and Lee, C. Y. 1987. Effect of blanching on enzyme activity and quality changes in green peas. *J. Food Sci.* 52:1002-1005.
- Hsieh, C. C., and McDonald, C. E. 1984. Isolation of lipoxygenase isoenzymes from flour of durum wheat endosperm. *Cereal Chem.* 61:392-398.
- Iny, D., Pinsky, A., Cojocoru, M., and Grossman, S. 1993. Lipoxygenase of *Thermoactinomyces vulgaris*, purification and characterization of reaction products. *Int. J. Biochem.* 25:1313-1323.
- Jaren-Galan, M., and Minguez-Mosquera, M. I. 1999. Effect of pepper lipoxygenase activity and its linked reactions on pigments of the pepper fruit. *J. Agric. Food Chem.* 47:4532-4536.
- Lee, C. Y., Smith, N. L., and Hawbecker, D. E. 1988. Enzyme activity and quality of frozen green beans as affected by blanching and storage. *J. Food Qual.* 11:279-287.
- Lineweaver, H., and Burk, D. 1934. The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* 56:658-666.
- Marcus, L., Prusky, D., and Jakoby, B. 1988. Purification and characterization of avocado lipoxygenase. *Phytochemistry* 27:323-327.
- McDonald, C. E. 1979. Lipoxygenase and lutein bleaching activity of durum wheat semolina. *Cereal Chem.* 56:84-89.
- Minguez-Mosquera, M. I., Gandul-Rojas, B., Garrido-Fernandez, J., and Gallardo-Guerrero, L. 1990. Pigments present in virgin olive oil. *J. Am. Oil Chem. Soc.* 67:192-196.
- Perraud, X., Kermasha, S., and Bisakowski, B. 1999. Characterization of a lipoxygenase extract from *Geotrichum candidum*. *Proc. Biochem.* 34:819-827.
- Pastore, D., Trono, D., Padalino, L., Simone, S., Valenti, D., Di Fanzo, N., and Passarella, S. 2000. Inhibition by α -tocopherol and L-ascorbate of linoleate hydroperoxidation and β -carotene bleaching activities in durum wheat semolina. *J. Cereal Sci.* 31:41-54.
- Rani, K. U., Prasada-Rao, U. J. S., Leelavathi, K., and Haridas-Rao, P. 2001. Distribution of enzymes in wheat flour mill streams. *J. Cereal Sci.* 34:233-242.
- Sheu, S. C., and Chen, A. O. 1991. Lipoxygenase as blanching index for frozen vegetable soybeans. *J. Food Sci.* 56:448-451.
- Shiiba, K., Negishi, Y., Okada, K., and Nagao, S. 1991. Purification and characterization of lipoxygenase isozymes from wheat germ. *Cereal Chem.* 68:115-122.
- Surrey, P. K. 1964. Spectrophotometric method for determination of lipoxygenase activity. *Plant Physiol.* 39:65-70.
- Suurmeijer, C. N. S. P., Perez-Gilabert, M., van der Hijden H. T. W. M., Veldink, G. A., and Wliegenthart, J. F. G. 1998. Purification, product characterization and kinetic properties of soluble tomato lipoxygenase. *Plant Physiol. Biochem.* 36:657-663.
- Taha, S. A., and Sagi, F. 1987. Relationships between chemical composition of durum wheat semolina and macaroni quality. II. Ash, carotenoid pigments and oxidative enzymes. *Cereal Res. Commun.* 15:123-129.
- Trono, D., Pastore, D., and Di Fanzo, N. 1999. Carotenoid dependent inhibition of durum wheat lipoxygenase. *J. Cereal Sci.* 29:99-102.
- Williams, D. C., Lim, M. H., Chen, A. O., Pangborn, R. M., and Whitaker, J. R. 1986. Blanching of vegetables for freezing—Which indicator enzyme to choose. *Food Technol.* 40:130-140.

[Received September 25, 2006. Accepted January 10, 2007.]