A Simple Method to Measure Lipase Activity in Wheat and Wheat Bran as an Estimation of Storage Quality

Devin J. Rose and Oscar A. Pike*

Department of Nutrition, Dietetics and Food Science, Brigham Young University, Provo, Utah 84602

ABSTRACT: The purpose of this research was to develop a simple method for measuring lipase activity as an indicator of wheat and wheat bran storage quality. This simplified method does not require the separation or purification of lipase. Optimal conditions for lipase activity measurements were determined by varying the substrate (olive oil) and water concentrations, temperature, and incubation time. Following incubation, FFA were quantified spectrophotometrically using a copper soap assay, and lipase activity was expressed as units/gram (U/g), where 1 U was defined as the microequivalents of oleic acid liberated per hour. The method was tested on one commercial and four pure wheat cultivars. The lipase activity was also correlated with the development of FFA during actual storage of heat-treated commercial bran. Lipase activity in wheat bran ranged from 2.17 to 9.42 U/g, and in whole kernel wheat from 1.05 to 3.54 U/g. Optimal olive oil and water concentrations were 0.4 to 0.8 mL and 0.15 to 0.20 mL per g of defatted sample, respectively. Optimal incubation temperature was 40°C, and incubation times of up to 8 h were linear. Lipase activity was highly correlated with the buildup of FFA in stored wheat bran (R^2 = 0.97).

Paper no. J11251 in *JAOCS 83*, 415–419 (May 2006).

KEY WORDS: Lipase, lipolysis, wheat, wheat bran, wheat germ.

The outer portions of a wheat kernel, namely, the bran and the germ, contain lipase. Although lipase activity (LA) in dormant seeds is generally quite low compared with germinating seeds, the hydrolysis of storage lipids is the most rapid chemical change that occurs in dormant wheat during storage (1). Lipid hydrolysis in whole grain products may lead to undesirable baking (2), sensory (3), and nutritional (4) properties. Thus, many manufacturers implement a heating step, which partially inactivates the lipase, during preparation of these products for commercial use. The heat treatment used to inactivate lipase may cause undesirable browning (5) and destruction of antioxidants (6,7); therefore, it is important to find the shortest heating time that will sufficiently inactivate the lipase.

Numerous reviews on the present knowledge of plant lipases have been published (8–11). Some common techniques used for the quantification of lipolytic activity in cereal grains are: radioassay (12), fluorimetry (13), titrimetry (14), and colorimetry (15).

*To whom correspondence should be addressed at Department of Nutrition, Dietetics and Food Science, Brigham Young University, S-221 ESC, Provo, UT 84602. E-mail: oscar_pike@byu.edu

Lipase assays present several challenges (9), some of which are common to assays involving water-insoluble substrates. For example, in a general lipase assay, the enzyme is incubated with an emulsion created by blending or sonicating TAG with an emulsifier such as gum acacia or Triton X-100. These emulsions are unstable and create emulsified droplets of unequal size, resulting in large relative errors (9). As a result, numerous water-soluble, artificial substrates are often used to create more uniform results, including: 2,3-dimercaptopropan-1-ol tributyrate (16), *N*-methyllindoxyl-myristate (17), Tween 20 (18), esters of 4-methylumbelliferone (13), and esters of *p*-nitrophenol (19). Unfortunately, results obtained with these substrates are not acceptable for measuring true LA, particularly in cereal grains, which often contain nonspecific esterases that are inactive on TAG but exhibit activity on artificial substrates that is several orders of magnitude higher than LA in the same sample (9). Thus, artificial substrates should not be used unless the enzyme has been well characterized and the activity on TAG and the artificial substrate has been documented (10). Such is not the case with wheat lipases.

Some unique problems arise when assaying for LA in cereal grains. For example, lipases in cereal grains are often membrane bound and resist solubilization even after extensive washing with buffered solutions. Excess water has also been shown to inhibit LA (20); thus, typical lipase assays using emulsions (19) may not be useful because they contain too much water.

The purpose of this research was to develop a simple method for measuring LA as an indicator of wheat and wheat bran quality that does not require separation or purification of lipase.

EXPERIMENTAL PROCEDURES

Samples. Commercial hard red winter wheat (CW) and bran (CB) were obtained from Lehi Roller Mills (Lehi, UT). Pure wheat cultivars were obtained from Horizon Milling (Minnetonka, MN). Crude bran from these samples was obtained by grinding tempered wheat (15% moisture) in a laboratory mill (Quadramat Junior; C.W. Brabender, Hackensack, NJ).

Lipase assay. CB was ground in a cyclone mill (Model 3010-030; UDY Corp., Fort Collins, CO), and then partially defatted with 3 vol of hexane (1:10 wt/vol) for 30 min on an orbital shaker (Model 980001; VWR Scientific, West Chester, PA) at 140 rpm. Residual hexane was allowed to evaporate at room temperature (about 10 min), and 1 g of the ground, defatted CB was weighed into each of two test tubes: one blank (A_i)

and one sample (A_f). Olive oil and distilled water (optimized levels, explained below) were added to both tubes and mixed. The lipids from the blank (A_i) were immediately extracted using a stepwise procedure. Five milliliters of hexane were added, the tube was vortexed and then centrifuged on a benchtop centrifuge at $1000 \times g$ for 3 min (Model 0131; Clay Adams, Parsippany, NJ). The hexane was decanted into a 100-mL round-bottomed flask, and the extraction was repeated twice. The hexane extracts were pooled, evaporation was performed on a rotary vacuum evaporator (Model RE 121; Brinkmann Instruments, Westbury, CT) at 40°C, and the residue was redissolved in 4 mL of isooctane. The other test tube was capped and incubated for 4 h at 40°C. After incubation, lipids were extracted as described for the blank, and FFA were quantified according to the method of Kwon and Rhee (21); 1 mL of 5% (wt/vol) cupric acetate, which had been adjusted to pH 6.1 with pyridine, was added and shaken vigorously for 1 min. The tubes were centrifuged at $1000 \times g$ for 1 min, and the absorbance was read in a spectrophotometer at 715 nm (Spectronic 1001 Plus; Miltion Roy, Ivyland, PA). The sample absorbance was compared with the absorbance of oleic acid standard solutions prepared in isooctane (1–10 mM). LA was expressed as units/gram (U/g), where 1 U was defined as the micromoles of FA liberated per hour according to Equation 1:

$$\mathrm{LA} = 1000\frac{(4+v)(A_f - A_i)}{\varepsilon t l s} \quad [1]$$

where LA = lipase activity (U/g), 1000 = conversion factor from mol/L to μequiv/mL, 4 = volume of isooctane used to redissolve lipids (mL), v = volume of olive oil added (mL), A_f = absorbance of sample after incubation at 715 nm, A_i = absorbance of blank at 715 nm, ε = molar absorptivity of oleic acid at 715 nm ($M^{-1}\cdot cm^{-1}$), t = incubation time (h), l = path length (1 cm for a standard cuvette), and s = sample weight (g).

Olive oil was chosen as the substrate because it allows for quantification of true LA (unlike many artificial substrates) (10), it is widely used and inexpensive (22), and it contains about 70% oleic acid, which was the FA used to create the standard curve. Prior to use, the olive oil was tested and found to be devoid of FFA. Further purification using aluminum oxide (23) did not affect LA.

Optimization of conditions. During method development, conditions were optimized to yield the highest LA. CB was used to optimize conditions for the assay. Olive oil and water were varied between zero and 1 mL and zero and 0.5 mL, respectively. Incubation temperature was varied between 25 and 55°C, and incubation time ranged from 4 to 24 h. Extraction of lipids after incubation was also performed with the addition of HCl to liberate FA soaps that may have formed during incubation (22).

To determine whether emulsified substrate would give a higher response, the assay was performed as described previously, except olive oil was emulsified in 50 mM Tris-Cl buffer (pH 8.0) containing 10% (v/v) Triton X-100. (Preliminary tests with gum arabic and Triton X-100 indicated the latter surfactant gave the highest and most repeatable results, and was easiest to prepare. Tween 20 could not be used because it contains an ester linkage.) Five milliliters each of olive oil and Triton X-100 were weighed into a 125-mL Erlenmeyer flask, and 15 mL of chloroform was added. The flask was swirled to dissolve the lipid and detergent, and then the chloroform was removed with a stream of nitrogen. The resulting mixture was thick and turbid. With rapid magnetic stirring, 40 mL of 50 mM Tris-Cl buffer (pH 8.0) was slowly added (the first 10 mL was added dropwise, and the remainder was slowly poured in). The emulsion was continuously stirred until use and then shaken during the assay. The emulsion was prepared fresh daily. After incubation, the emulsion was broken with 5 mL of 1 M Dole reagent (isopropanol/heptane/H_2SO_4, 40:10:1 by vol) (24), 3 mL of heptane, and 2 mL of water. The tubes were capped, shaken vigorously (approximately 1 cycle every second) for 1 min, and centrifuged at $1000 \times g$ for 3 min. The top layer was transferred into a round-bottomed flask, and the extraction was repeated twice. The heptane extracts were pooled and evaporation was performed on a rotary vacuum evaporator at 40°C, and the FFA were quantified using the method of Kwon and Rhee (21).

Once the optimal conditions were determined in the commercial samples, four pure wheat cultivars were tested for applicability of the method to other varieties: Golden 86 (hard white spring), Trego (hard white winter), and Karl 92 and Jagger (hard red winter).

Correlation of LA with FFA During Storage. To test how well LA predicted the appearance of FFA during actual storage of wheat bran, duplicate 100 g portions of CB were heated at 175°C in a forced draft oven for various times up to 25 min to obtain bran with a range of LA. After cooling, a representative sample was removed for LA quantification, and the remainder was placed in a paper bag that was subsequently closed, then stored at 25°C and 60% relative humidity for up to 6 wk. Samples were removed every 2 wk for FFA quantification. The rate of FFA development in stored samples was determined by plotting FFA content of the bran against time and calculating a regression line.

FFA were measured by grinding the stored CB in a cyclone mill. Hexane (15 mL) was added to 1.5 g of ground bran in a 50-mL Erlenmeyer flask. The flasks were placed on an orbital shaker at 140 rpm for 30 min, and the hexane was filtered through filter paper (Whatman no. 1) into a 250-mL round-bottomed flask. The extraction was repeated twice, the hexane extracts were pooled, and evaporation was performed on a rotary vacuum evaporator at 40°C. FFA were quantified using the method of Kwon and Rhee (21), as above.

Data analysis. Data were analyzed using Statistical Analysis System software (Version 9.1; SAS Institute, Cary, NC) with a mixed model analysis of variance (PROC MIXED). Fisher's LSD (least significant difference) was used to determine significant differences among means. Correlation was assessed using regression analysis (PROC REG). Significant differences were defined as $P < 0.05$.

RESULTS AND DISCUSSION

Sample preparation. The first step in sample preparation involved partial lipid extraction. Attempts to circumvent this step gave undesirable results. Without lipid extraction, LA was too low to measure (data not shown). It appeared that the lipid extraction step had an "activating" effect on LA. We are uncertain of the reason for this phenomenon; however, the activation of LA through prior lipid extraction has been demonstrated by other researchers (14,15,25,26). Defatting also removed existing FFA that otherwise interfered with the measurement of FFA liberated during the reaction and caused high blank readings. By using the sequential lipid extraction procedure just described, approximately 50% of the total lipid was removed when compared with a Soxhlet extraction; however, this was sufficient to activate the lipase and remove existing FFA. Though it required only 1.5 h compared with 18–24 h for a Soxhlet extraction, it may be possible to use even shorter sequential extraction times (15).

Optimization of LA. Figure 1 shows the LA of CB as a function of olive oil concentration. As illustrated, LA peaked between 0.4 and 0.8 mL of oil per g of defatted sample. Using olive oil concentrations outside this range gave significantly lower responses. At low concentrations, the reduced LA may have been due to the lack of substrate for the enzyme. At higher concentrations LA may have been limited by the availability of water for the enzyme. However, it is probable that high amounts of TAG in the extract may have either decreased the ability of the FFA to form soaps with copper (decreasing absorbance), or simply decreased the molar absorptivity of the cupric-FA soaps (also decreasing absorbance). This would lead to lower apparent FA levels in those samples containing higher levels of TAG and explain the decrease observed in Figure 1.

Figure 2 shows the LA of CB as a function of water content. Optimal water concentration ranged from 0.15 to 0.20 mL per g of defatted sample. Using water concentrations outside this range resulted in significantly lower responses. The inhibitory effect of excess water has been demonstrated previously (10,26). Using saturated salt solutions, Drapron and Sclafani (26) found that the optimal water activity for wheat bran lipase is 0.85. In the present study, water activity was measured at the optimal oil and water concentrations and found to be 0.87. Because the reaction medium was not a buffer solution, the effects of ions (such as Ca^{2+}) and pH were not investigated.

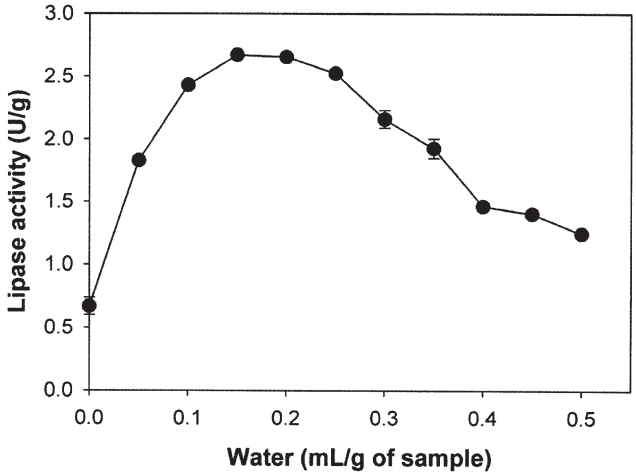

FIG. 2. Lipase activity of commercial bran with varying water concentrations. Samples were incubated with 0.6 mL of olive oil at 40°C for 4 h. Error bars represent SD. Some error bars are too small to see. For abbreviation see Figure 1.

Once the wheat bran had been mixed with olive oil and water, it was capped and incubated at a specific temperature for a specified length of time. The LA of CB as a function of incubation temperature is shown in Figure 3. The optimal temperature for incubation ranged from 40 to 55°C. Interestingly, the

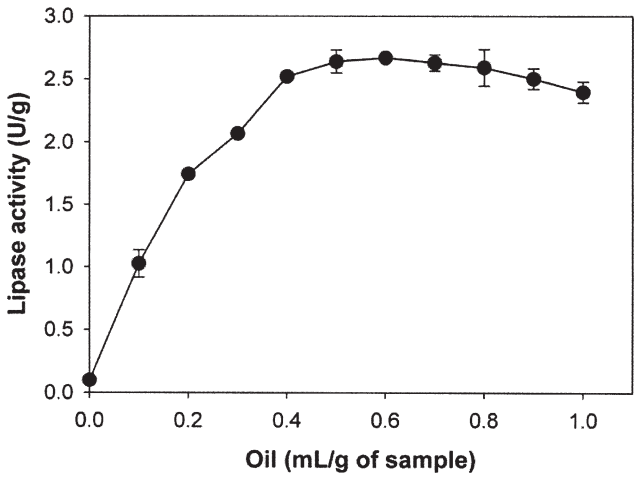

FIG. 1. Lipase activity of commercial bran with varying olive oil concentrations. Samples were incubated with 0.15 mL of water at 40°C for 4 h. Error bars represent SD. Some error bars are too small to see. U = μequiv oleic acid liberated/h.

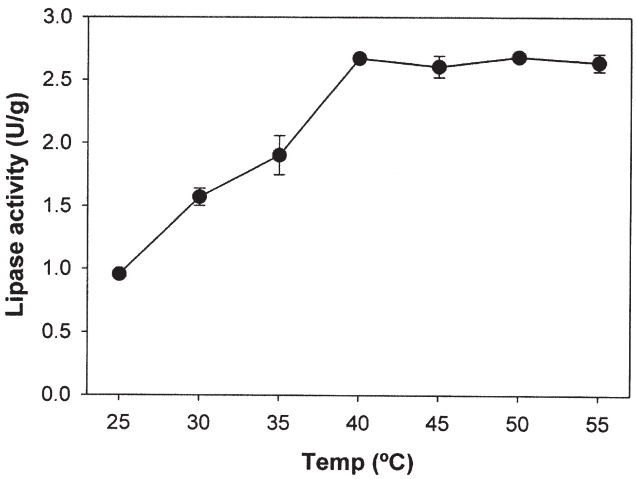

FIG. 3. Lipase activity of commercial bran incubated at different temperatures. Samples were incubated with 0.15 mL of water and 0.6 mL of olive oil at the designated temperature for 4 h. Error bars represent SD. Some error bars are too small to see. For abbreviation see Figure 1.

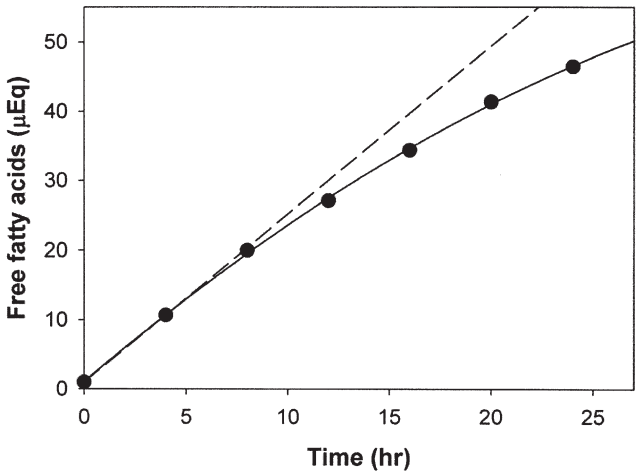

FIG. 4. Appearance of FFA in commercial bran incubated for up to 24 h. Samples were incubated with 0.15 mL of water and 0.6 mL of olive oil at 40°C for the specified time. SD was too small to plot as error bars.

enzyme activity did not decrease above 40°C, as would be expected of many enzymatic assays because of denaturation of the enzyme. This may be a result of the low water content in the assay mixture, which may impede protein denaturation despite the higher temperature (27). Figure 4 shows the appearance of FFA in CB incubated up to 24 h. As shown, the reaction becomes curvilinear with excessive incubation times. Because the calculation assumes linearity of the reaction during the incubation time, using long incubation times would result in significant error. This is particularly interesting since many existing methods use incubation times ranging from 16 to 72 h (14,15,25,26). Incubation times longer than 8 h gave significantly lower responses than expected.

The maximal LA of four wheat cultivars is shown in Table 1. In determining optimal conditions for LA, olive oil and water concentrations were varied as described above for CB. Whereas the optimal ranges of olive oil and water concentration differed slightly for each cultivar (data not shown), the ranges overlapped with those reported above using CB (0.4 to 0.8 mL olive oil and 0.15 to 0.20 mL per g of defatted sample). Using olive oil and water concentrations within these ranges will likely result in maximal LA for a variety of wheat cultivars. However, for a specific cultivar it may be necessary to verify optimal conditions.

TABLE 1
Lipase Activity of Crude Wheat Bran Measured on Four Different Cultivars[a]

Sample	Lipase activity (U/g)	RSD
Karl 92		
Whole kernel	3.54	0.42%
Bran	9.42	1.55%
Jagger		
Whole kernel	3.24	0.71%
Bran	8.88	0.74%
Trego		
Whole kernel	2.58	0.31%
Bran	6.19	1.87%
Golden 86		
Whole kernel	0.87	3.01%
Bran	2.21	3.49%

[a]Values represent means of duplicate measurements made on different days. U = µequiv oleic acid liberated/h. RSD = relative SD ([SD/mean] × 100%).

TABLE 2
Lipase Activity of Commercial Bran Using the Different Method Variations[a]

Method	Lipase activity (U/g)	RSD
Optimized conditions	2.67[a]	0.29%
Addition of 100 µL of 6 N HCl after incubation	1.78[b]	0.56%
Addition of 1.0 µL of 1 N HCl after incubation	2.73[a]	5.62%
Emulsion	1.10[c]	30.1%

[a]Values represent means of duplicate measurements made on different days. For abbreviations see Table 1.

Because wheat contains a significant amount of Mg^{2+} (and some Ca^{2+}), it was hypothesized that FA soaps of these metals might form during incubation and interfere with extraction and quantification (22). Therefore, the extraction was performed with the addition of HCl, since H^+ would displace Mg^{2+} (or Ca^{2+}) from FA soaps that might have formed. As shown in Table 2, addition of 6 N HCl resulted in lower LA. This high acid concentration resulted in an insoluble pellet in the test tube, presumably due to denatured proteins, which may have trapped some lipid and prevented extraction. The procedure was also tested with a lower concentration (1 N) and higher volume of HCl. This did not produce the insoluble pellet, yet the LA was still not significantly different from the sample using no acid (Table 2). Thus, it is likely that the formation of FFA-soaps is not of concern in this reaction.

To determine whether emulsified substrate would improve LA, the olive oil was emulsified in Tris-Cl buffer using Triton X-100. As shown in Table 2, using an emulsion gave a

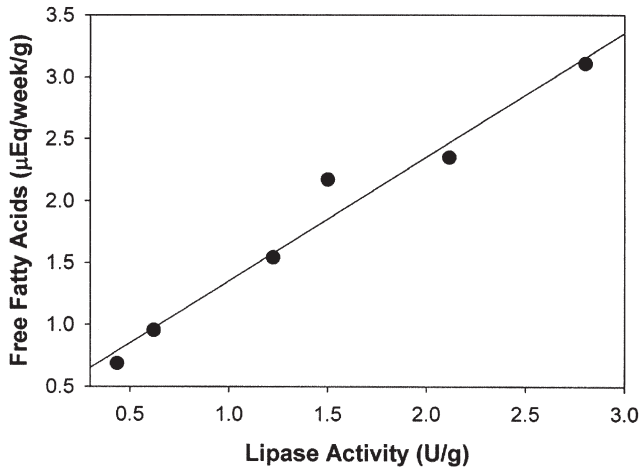

FIG. 5. Correlation of lipase activity with the rate of FFA development in wheat bran. Samples were stored at 25°C and 60% relative humidity for up to 6 wk. $R^2 = 0.96$. For abbreviation see Figure 1.

significantly lower response than the optimized conditions described above. This may be a result of the higher water content in the reaction medium when using an emulsion, as mentioned previously. Using the emulsion also resulted in a large relative SD, as expected (9).

Correlation of LA with FFA during storage. To ensure that LA measured by this method was a good predictor of the potential for FFA development in a sample during actual storage, LA was correlated with the rate of FFA development during storage (Fig. 5). LA was measured in a number of samples that had been heat treated to obtain a range of LA at baseline. These samples were then stored for a period of 6 wk, and the rate of FFA development was measured (see the Experimental Procedures section). The FFA development was linear for up to 4 wk (data not shown); therefore, data from zero, 2, and 4 wk were used to calculate a regression line for rate of FFA development over time. Figure 5 shows this rate plotted against LA measured on that sample at baseline. As expected, LA was correlated ($R^2 = 0.96$) with the rate of FFA development during actual storage of wheat bran; therefore, LA measured by this method is a good predictor of the potential for FFA development during storage.

REFERENCES

1. Pomeranz, Y., Composition and Functionality of Wheat Flour Components, in *Wheat: Chemistry and Technology,* 3rd edn., edited by Y. Pomeranz, American Association of Cereal Chemists, St. Paul, MN, 1980, Vol. 2, pp. 219–370.
2. Tait, S.P.C., and T. Galliard, Effect on Baking Quality of Changes in Lipid Composition During Wholemeal Storage, *J. Cereal Sci. 8*:125–137 (1988).
3. Galliard, T., and D.M. Gallagher, The Effects of Wheat Bran Particle Size and Storage Period on Bran Flavour and Baking Quality of Bran/Flour Blends, *J. Cereal Sci. 8*:147–154 (1988).
4. Pomeranz, Y., Biochemical, Functional, and Nutritive Changes During Storage, in *Storage of Cereal Grains and Their Products*, 4th edn,, edited by D.B. Saucer, American Association of Cereal Chemists, St. Paul, MN, 1992, pp. 55–141.
5. Vetrimani, R., and P. Haridas Rao, Studies on Stabilization of Wheat Bran, *J. Food Sci. Technol. Mysore 27*:332–335 (1990).
6. Lehtinen, P., K. Kiiliäinen, I. Lehtomäki, and S. Laakso, Effect of Heat Treatment on Lipid Stability in Processed Oats, *J. Cereal Sci. 37*:215–221 (2003).
7. Champagne, E.T., and R.J. Hron Sr., Stabilizing Brown Rice to Lipolytic Hydrolysis by Ethanol Vapors, *Cereal Chem. 69*:152–156 (1992).
8. Mukherjee, K.D., and M.J. Hills, Lipases from Plants, in *Lipases: Their Structure, Biochemistry, and Application*, edited by P. Woolley and S.B. Petersen, University Press, Cambridge, 1994, pp. 49–75.
9. Huang, A.H.C., Lipases, in *Lipid Metabolism in Plants*, edited by T.S. Moore Jr., CRC Press, London, 1993, pp. 473–503.
10. Galliard, T., Enzymatic Degradation of Cereal Lipids, in *Lipids in Cereal Technology*, edited by P.J. Barnes, Academic Press, New York, 1983, pp. 111–147.
11. Brockerhoff, H., and R.G. Jensen, *Lipolytic Enzymes*, Academic Press, New York, 1974, pp. 129–139.
12. Matlashewski, J.G., A.A. Urquhart, M. R. Sahasrabudhe, and I. Altosaar, Lipase Activity in Oat Flour Suspensions and Soluble Extracts, *Cereal Chem. 59*:418–422 (1982).
13. Heltved, F., Spectrofluorometric Assays for Hydrolytic Activity in Germinating Wheat, *J. Cereal Sci. 2*:179–185 (1984).
14. El Amrani, F., O. Fayol, R. Drapron, J. Potus, and J. Nicolas, Simplified Method for the Determination of Lipolytic Activity in Low Moisture Media, *Sci. Aliment 23*:209–221 (2003).
15. O'Connor, J., H.J. Perry, and J.L. Harwood, A Comparison of Lipase Activity in Various Cereal Grains, *J. Cereal Sci. 16*:153–163 (1992).
16. Fadiloğlu, S., and Z. Söylemez, Preliminary Kinetic Studies on the Esteratic and Lipolytic Components of a Commercial Wheat Germ Lipase*, J. Agric. Food Chem. 44*:3015–3017 (1996).
17. Lin, Y.H., and A.H.C Huang, Lipase in Lipid Bodies of Cotyledons of Rape and Mustard Seedlings, *Arch. Biochem. Biophys. 225*:360–369 (1983).
18. Goffman, F.D., and C. Bergman, Relationship Between Hydrolytic Rancidity, Oil Concentration, and Esterase Activity in Rice Bran, *Cereal Chem. 80*:689–692 (2003).
19. Pinsirodom, P., and K.L. Parkin, Lipase Assays, in *Current Protocols in Food Analytical Chemistry*, John Wiley & Sons, New York, 2001, pp. C3.1.1–13.
20. Stauffer, C.E., Ester Hydrolases, in *Enzymes and Their Role in Cereal Technology*, edited by J.E. Kruger, D. Lineback, and C.E. Stauffer, American Association of Cereal Chemists, St. Paul, MN, 1987, pp. 265–280.
21. Kwon, D.Y., and J.S. Rhee, A Simple and Rapid Colorimetric Method for Determination of Free Fatty Acids for Lipase Assay, *J. Am. Oil Chem. Soc. 63*:89–92 (1986).
22. Jensen, R.G., Detection of Lipase (acylglycerol hydrolase) Activity from Various Sources, *Lipids 18*:650–657 (1983).
23. Jensen, R.G., T.A. Marks, J. Sampugna, J.G. Quinn, and D.L. Carpente, Purification of Triglycerides with an Alumina Column, *Lipids 1*:451–452 (1966).
24. Dole, V.P., A Relationship Between Non-esterified Fatty Acids in Plasma and the Metabolism of Glucose, *J. Clin. Invest. 35*:150–154 (1956).
25. Galliard, T., Degradation of Acyl Lipids: Hydrolytic and Oxidative Enzymes, in *The Biochemistry of Plants. Lipids: Structure and Function,* edited by P.K. Stumpf and E.E. Conn, Academic Press, New York, 1980, Vol. 4*,* pp. 85–116.
26. Drapron, R., and L. Sclafani, A Method for the Determination of Lipolytic Activity in Solid or Dough-Consistency Biological Products, *Ann. Technol. Agric. 18*:5–16 (1969).
27. Damodaran, S., Amino Acids, Peptides, and Proteins, in *Food Chemistry*, 3rd edn., edited by O.R. Fennema, Marcel Dekker, New York, 1996, pp. 321–429.

[Received October 6, 2005; accepted February 27, 2006]