Lipase Activity in Oat Flour Suspensions and Soluble Extracts[1]

G. J. MATLASHEWSKI,[2] A. A. URQUHART,[2,3] M. R. SAHASRABUDHE,[4] and I. ALTOSAAR[2]

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ABSTRACT

A radioisotope assay was developed for measuring lipase (triacyl glycerol acyl hydrolase) activity in aqueous suspensions of oat flour, with glycerol tri[1-^{14}C]oleate as substrate. The pH optimum in 0.05*M* Tris-HCl buffer containing 1% (v/v) Triton-X-100 and 0.2% (v/v) benzene was 7.5; the temperature optimum was 35–39° C. Hydrolysis (followed as release of [1-^{14}C]oleic acid) was linear for 8–10 min, with rates of 5–10 μmol of fatty acid released per minute per gram of flour. Triton-X-100 (0.5–2.0%) and benzene (0.2%) were essential for activity. Michaelis-Menton kinetics were exhibited with increasing concentrations of glycerol trioleate; the apparent Michaelis-Menton constant was 3.5m*M*. Lipase activities measured by this method and by colorimetric determination of free fatty acids released in oat flour doughs were comparable. A stable, active, soluble lipase extract was prepared from defatted oat flour by extraction in 0.05*M* Tris-HCl buffer (pH 7.5) containing 1% Triton-X-100. In a comparison of oats, wheat, barley, and rye, lipase activities in the ungerminated grains were 8.4, 0.7, 0.9, and 0.9 μmol of fatty acid released per minute per gram of flour, respectively. Rates in the grain germinated for two days were 11.9, 0.8, 0.6, and 3.2 μmol per minute per gram of flour, respectively.

Lipase (EC 3.1.1.3) is a lipolytic enzyme that catalyzes hydrolysis of triglycerides, diglycerides, and in some cases monoglycerides. In plant seeds, the free fatty acids produced during hydrolysis of the stored lipids are metabolized for energy. Several lipases have been described in plants. Castor bean contains two lipases, one with an acid pH optimum that hydrolyzes both mono- and triglycerides (Ory et al 1960) and the other with alkaline pH that hydrolyzes monoglycerides (Muto and Beevers 1974). Most plant lipases have alkaline (Huang and Moreau 1978, Sanders and Pattee 1975, Theimer and Rosnitschek 1978) or neutral pH optima (Huang and Moreau 1978, Martin and Peers 1953, Tavener and Laidman 1972). In some dry, quiescent plant seeds such as rapeseed (Theimer and Rosnitschek 1978, Wetter 1957), wheat (Tavener and Laidman 1972), corn, cotton, sunflower, cucumber, and tomato (Huang and Moreau 1978), lipase was not detectable, but activities increased dramatically on germination. In other cases, lipase was found in ungerminated seed of faba bean (Dundas et al 1978), oats (Hutchinson and Martin 1952, Martin and Peers 1953) and peanut (Sanders and Pattee 1975). Because lipase can become active when seeds are damaged during shelling or dehulling, or in untreated flour, lipolysis can result in fatty acid rancidity and soapy flavors (Dundas et al 1978, Hutchinson and Martin 1952, Widhe and Onselius 1949).

In cereals, lipase is present in rice (Shastry and Rao 1976) and in oats (Martin and Peers 1953), but not in ungerminated wheat (Tavener and Laidman 1972), corn scutellum (Huang and Moreau 1978), rye, or barley (Widhe and Onselius 1949). The lipid content of oat ranges from 4 to 11%, most of this being triglycerides with a high degree of unsaturation (Sahasrabudhe 1979, Youngs 1978). Previous studies by Martin and Peers (1953) showed that oat lipase (assayed manometrically) with pH optimum of 7.4 could be extracted from the kernels. An insoluble precipitate containing lipase activity was formed from this extract after several freeze-thaw cycles, but it was not solubilized in a wide range of solvents; hence the enzyme could not be purified further.

Commercial oat milling processes include heat treatments of about 110° C for 2 hr to inactivate the enzymes, but potential for lipolysis in untreated oat flour is high (Hutchinson and Martin 1952, Widhe and Onselius 1949). Recent interest in utilization of oat protein (Shukla 1975), lipids (Sahasrabudhe 1979), and other oat fractions (Wood et al 1978) has therefore stimulated further work on oat lipase. This article describes a method for measurement of oat lipase in flour suspensions and soluble extracts by following hydrolysis of ^{14}C-labeled triolein. Comparisons were made between this radioisotope assay and a colorimetric assay measuring fatty acid released in doughs. Lipase activity was also assayed in other cereals.

MATERIALS AND METHODS

Materials

Cereal grains were obtained from the Ottawa Research Station, Agriculture Canada. In most of the experiments on oat (*Avena sativa* L.), the cultivar Hinoat was used; Elgin, Harmon, and Sentinel were used where indicated. In one experiment, wheat (Neepawa), barley (Perth), and rye (Gazelle) were used. Grain was stored at −20° C until required. Gum arabic, olive oil, sodium dodecyl sulfate (SDS), sodium desoxycholate, sucrose, triolein, and Triton-X-100 were obtained from Sigma Chemical Co. Glycerol tri[1-^{14}C]oleate (46.1 milli-Curie/mmol) was obtained from the Radiochemical Center, Amersham, IL, and diluted with cold triolein or olive oil in benzene before use. Silica gel G was obtained from Merck. All other chemicals were reagent grade.

Radioisotope Lipase Assay

Dry, ungerminated oats were manually dehulled and ground for 30 sec in a Phillips domestic electric coffee mill. After Soxhlet lipid extraction in petroleum ether (40–60° C) for 2 hr, the defatted flour was air-dried and reground in a mortar and pestle to disperse the powder. The standard assay mixture for oat flour suspensions contained 0.05*M* Tris-HCl buffer, pH 7.5, 1% Triton-X-100, 0.2% benzene, and 10m*M* glycerol tri[1-^{14}C]oleate (0.5 micro-Curie/mmol) in a final volume of 10 ml in a 50-ml Erlenmeyer flask. The assay mixtures were agitated vigorously and then placed in a shaking water bath (37° C, 160 cycles per minute). After equilibration for 15 min, the reaction was started by adding 1-g samples of the defatted oat flour. Aliquots (1 ml) were transferred at intervals of 0 to 30 min into 15-ml glass centrifuge tubes containing 2 ml of chloroform/methanol (1:1). This mixture terminated the reaction and extracted the lipids. The tubes were then centrifugd at 3,000 × *g* for 5 min, the lipid-containing, chloroform layer transferred to small test tubes, and the chloroform evaporated to dryness under N_2. The lipid was then redissolved in small volumes of chloroform, and aliquots were spotted on silica gel G thin-layer plates (0.25 mm, prepared in the laboratory). The lipids were fractionated with petroleum ether/diethyl ether/acetic acid (85:15:1, v/v). Iodine vapor was used to locate the separated lipid components. The free fatty acid, triglyceride, and other lipid fractions were then scraped into separate scintillation vials and radioactivity determined by liquid scintillation counting in 5 ml of Aquasol (New England Nuclear, Kritchevsky and Malhotra 1970). Variations in the procedure (eg,

[1]Contribution 479 from the Food Research Institute, Agriculture Canada, Ottawa, Canada.
[2]Department of Biochemistry, University of Ottawa, Ottawa, Ontario, Canada K1N 9B4.
[3]Present address: Department of Plant Science, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2.
[4]Food Research Institute, Agriculture Canada, Central Experimental Farm, Ottawa, Ontario, Canada K1A 0C6.

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changes in the reaction mixture, temperature, and pH) are given for individual experiments. In one experiment, the solvent for Soxhlet lipid extraction, petroleum ether, was replaced by hexane, acetone, or chloroform/ methanol (2:1).

Liberated fatty acid, labeled in the carbonyl group, was determined by calculating the ratio of radioactivity recovered in the free fatty acid fraction to that in the total lipid recovered. This ratio, multiplied by the number of moles of triglyceride supplied times 3 (moles of fatty acid per mole of triglyceride), gives the moles of fatty acid hydrolyzed per gram of flour for each time interval. Rates of triglyceride hydrolysis were calculated from the linear part of the time course. One unit of activity was defined as 1 μmol of fatty acid released per minute per gram of flour.

Colorimetric Lipase Assay

Defatted oat flour (cv. Sentinel) was prepared as described from dry, ungerminated groats and 0.5-g samples mixed with 90 mg of glycerol trioleate. Buffer (0.05 M Tris-HCl, pH 7.5, containing 1% [v/v] Triton-X-100) was added to give a final moisture content of 40%. The resulting dough was mixed with a glass rod for 2 min in a test tube, and then incubated at 37° C in a water bath. After intervals of 0 to 30 min, samples were removed, 0.1 ml of 1N HCl added, and free fatty acids extracted by homogenizing the acidified dough in 10 ml of chloroform/heptane/methanol (49:49:2, v/v). The extract was filtered, the residue washed with additional aliquots of the solvent mixture, and the combined solvent filtrate made up to 50 ml. Aliquots (5-ml) were mixed with copper reagent and the resulting fatty acid copper soaps measured colorimetrically (Shipe et al 1980).

Oat flour suspensions were prepared as described for the radioisotope assay. In one series of samples, unlabeled glycerol trioleate replaced the radioisotope. Samples were incubated in a shaking water bath at 37° C. The labeled samples were treated as described for the radioisotope assay. The unlabeled samples were acidified with 1 ml of 1N HCl and the free fatty acids extracted in chloroform/heptane/methanol as described.

Triolein hydrolysis was therefore followed by radioisotope and colorimetric assays in oat flour suspensions incubated under identical conditions. These rates of hydrolysis were compared with rates obtained in doughs.

Enzyme Extraction

Five-gram portions of the Soxhlet lipid-extracted oat flour were stirred at 4° C in 10 ml of 0.05M Tris-HCl buffer, pH 7.5, under the conditions described in the Results and Discussion section. These preparations were centrifuged at 6,000 $\times g$ for 15 min and aliquots of the post-6,000 $\times g$ supernatants assayed for lipase activity. Assay conditions were those described above. The assay mixtures were adjusted to give final concentrations of 1% Triton-X-100, 0.2% benzene, and 10 mM triolein in a volume of 5 ml.

Cereal Lipases

Ungerminated samples of dehulled oats (Hinoat), wheat (Neepawa), barley (Perth), and rye (Gazelle) were ground and lipid extracted as described above. Samples of each cereal were also allowed to germinate on moist filter paper in the dark at 25° C for two days. The germinated grains were lyophilized, ground, and lipid extracted as before. Lipase activity was measured by the radioisotope assay in flour suspensions of both ungerminated and two-day germinated grains.

RESULTS AND DISCUSSION

Lipase Activity in Oat Flour

The time course for triglyceride hydrolysis was linear for 8–10 min. Initial rates of activity were 5–10 μmol per minute per gram of flour with triolein concentration of 10 mM at pH 7.5 and at 37° C. Variation between batches of flour prepared from the same source was 15–20%. Lipase activity was assayed over the pH range 5.5–9.0. The pH optimum for three oat cultivars, Hinoat, Elgin, and Harmon, was 7.5, as shown in Fig. 1. The temperature optimum was 30–39° C. These optima for pH and temperature were the same as those described by Martin and Peers (1953) for oat lipase assayed manometrically with tributyrin as substrate.

A linear relationship was obtained between initial velocity and enzyme concentration in the range 0.5–2.0 g of flour per 10 ml of reaction mixture (Fig. 2). Michaelis-Menton kinetics were demonstrated with increasing concentration of triolein (Fig. 3). The apparent Michaelis constant with triolein was 3.5 mM. This value compares with the earlier report of 6.5 mM (with tributyrin as

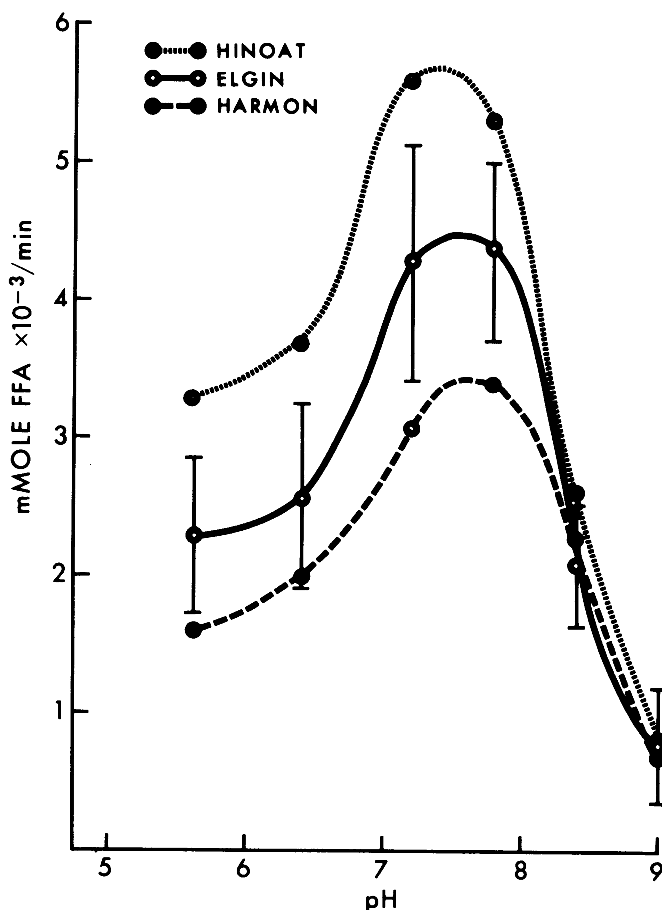

Fig. 1. Effect of reaction buffer pH on lipase activity in three oat cultivars (Hinoat, Elgin, Harmon). Standard deviations are shown for Elgin. They were the same magnitude for the other two cultivars. The points are means of three determinations.

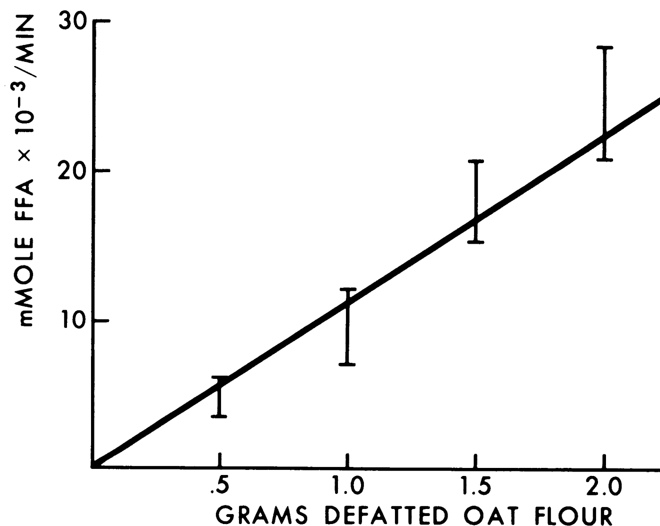

Fig. 2. Linear relationship between initial rates of lipase and weight of defatted flour added. The assays were done in triplicate.

substrate) of Martin and Peers (1953), and is similar in range to several plant lipases (Dundas et al 1978, Rosnitschek and Theimer 1980, Sanders and Pattee 1975). The limit of sensitivity of the assay, at optimum pH and temperature was estimated to be 0.2 μmol fatty acid per minute per gram of flour (approximately 55 g of free fatty acid/min/g). Olive oil could replace the unlabeled triolein in the assay.

When petroleum ether was replaced by either acetone or hexane as solvent for Soxhlet lipid extraction, no change in lipase activity of the defatted flour was observed. After chloroform/methanol extraction, however, lipase activity was completely inactivated.

Lipase activity required Triton-X-100 (Table I). Controls lacking the detergent were almost inactive. In samples containing 0.5–2.0% (v/v) Triton-X-100, lipase activity was significantly higher than the controls ($P = 0.05$); no significant difference existed between 0.5, 1.0, and 2.0% Triton-X-100. Concentrations of 3 and 5% Triton-X-100 were also tried, but separation of the lipid fractions by thin-layer chromatography was severely impaired at these higher levels. Subsequently, 1% Triton-X-100 was chosen for the assay. Low levels of benzene (0.2 and 0.3%) significantly ($P = 0.05$) increased lipase activity above the controls with no benzene (Table II). Higher levels (0.5 and 1.0%) significantly decreased the activity. Hexane at 0.2% did not have the same effect; higher levels of hexane were not tried. Lipase was also assayed in the presence of gum arabic. Concentrations of 1.0–8.0% (w/v) had no significant effect on initial rates ($P = 0.05$), but decreased the maximum extent of triglyceride hydrolyzed in 15 min (Table III).

In flour suspensions with triolein as substrate, therefore, Triton-X-100 (0.5–2.0%) and benzene (0.2%) were necessary for optimum lipase activity (Tables I and II). Lipase is believed to act on the substrate at a water-lipid interface (Desnuelle 1961). Triton-X-100 and benzene may facilitate arrangement of the long-chain fatty acids at this interface. In rapeseed, however, Triton-X-100 inhibited lipase (Rosnitschek and Theimer 1980). Several studies on plant lipases have used gum arabic or gum acacia to stabilize water-lipid emulsions (Dundas et al 1978, Muto and Beevers 1974, Theimer and Rosnitschek 1978). Berner and Hammond (1970) used gum arabic in an assay mixture for measuring oat lipase. In contrast, our study showed no marked effect of gum arabic on initial oat lipase rates (Table III). The effect of gum arabic in the absence of Triton-X-100 or benzene was not tried.

The methods used most commonly for assaying plant lipases are titrimetric (eg, Tavener and Laidman 1972, Theimer and Rosnitschek 1978), colorimetric determination of copper soaps of liberated fatty acids (Huang and Moreau 1978) and fluorimetric (Muto and Beevers 1974, Theimer and Rosnitschek 1978). Oat lipase has been assayed manometrically (Martin and Peers 1953) and titrimetrically in aqueous extracts (Berner and Hammond 1970) and in doughs (Hutchinson and Martin 1952, Martin and Peers 1953).

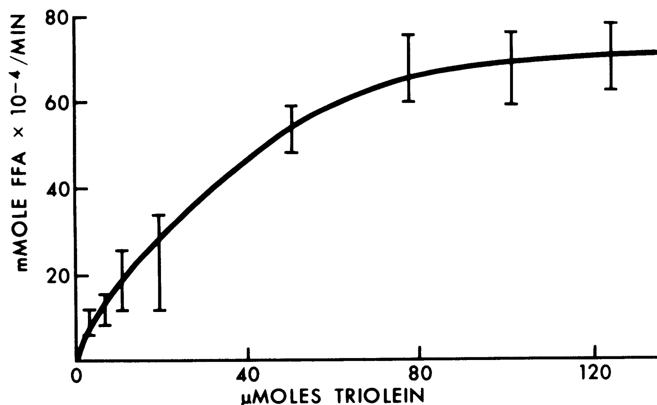

Fig. 3. Effect of substrate (triolein) concentration on lipase activity. Triolein concentration is given in μmol per 10-ml assay mixtures. The assays were done in triplicate.

TABLE I
Effect of Triton-X-100 on Lipase Activity in Oat Flour Suspensions[a]

Triton-X-100[b] (%)	Lipase Activity (μmol/min/g flour)
0	0.25 ± 0.06
0.5	5.56 ± 0.40
1.0	4.40 ± 0.69
2.0	6.19 ± 0.57

[a]Reaction mixtures contained 0.05*M* Tris-HCl buffer, pH 7.5, 0.2% benzene, and 10 m*M* triolein. Values are means of duplicate experiments.
[b]Percent Triton-X-100 (v/v) in 10 ml of reaction mixture.

TABLE II
Effect of Benzene and Hexane on Lipase Activity in Oat Flour Suspensions[a]

Solvent[b] (%	Lipase Activity (μmol/min/g flour)
Hexane	
none[c]	1.44 ± 0.82
0.2	1.17 ± 0.55
Benzene	
none[c]	1.40 ± 0.80
0.2	4.76 ± 0.97
0.3	3.30 ± 0.02
0.5	0.50 ± 0.02
1.0	0.21 ± 0.06

[a]Reaction mixtures contained 0.05*M* Tris-HCl buffer, pH 7.5, 1% Triton-X-100, and 10 m*M* triolein.
[b]Solvent as percentage (v/v) of the 10-ml reaction mixture.
[c]The substrate was added to the reaction vessel in either hexane or benzene and evaporated before addition of buffer.

TABLE III
Effect of Gum Arabic on Lipase Activity in Oat Flour Suspensions[a]

Gum Arabic[b] (%)	Lipase Activity (μmol/min/g flour)	Lipase Activity (μmol/g flour after 15 min)
0	6.05 ± 1.82	38.50 ± 8.18
1	7.02 ± 2.18	35.76 ± 6.61
2	6.89 ± 2.45	31.77 ± 7.93
4	6.24 ± 1.94	21.32 ± 4.09
6	4.26 ± 0.64	17.40 ± 1.92
8	5.05 ± 0.19	18.70 ± 0.45

[a]Reaction mixtures contained 0.05*M* Tris-HCl buffer, pH 7.5, 1% Triton-X-100, 0.2% benzene and 10 m*M* triolein. Values are means of duplicate experiments.
[b]Percent (w/v) gum arabic in 10-ml reaction mixture.

TABLE IV
Extraction of a Soluble Lipase Fraction (post-6,000 x *g* supernatant) from Oat Flour

Extraction Conditions	Extraction Time (hr)	Lipase Activity[a] (μmol/min/g flour)
Buffer alone[b]	1	0.3
Buffer		
+ 0.15*M* KCl	1	0.8
+ 3% sodium dodecyl sulfate	1	2.1
+ 3% Na desoxycholate	1	1.9
+ 1% Triton-X-100	1	1.3
+ 1% Triton-X-100	2	2.3
+ 1% Triton-X-100	5	4.9
+ 1% Triton-X-100	10	5.3
+ 3% Triton-X-100	1	9.5

[a]Lipase activity in the supernatant was assayed in 0.05*M* Tris-HCl buffer, pH 7.5, so that the final concentration in the reaction mixture was 1% Triton-X-10, 0.2% benzene, and 10 m*M* glycerol tri[1-^{14}C]oleate.
[b]0.05*M* Tris-HCl buffer, pH 7.5.

Titrimetric methods are time-consuming, and only one sample can be assayed at a time. With the rapid radioisotope procedure, several samples can be assayed simultaneously. Radioactive substrates have been used with castor bean (Borgstrom and Ory 1970) and rapeseed (Rosnitschek and Theimer 1980). Moreover, the substrate (triolein) used in the radioisotope assay contains long-chain (18:1) fatty acids. It is thus more similar to the natural oat lipids in which the major fatty acids are oleic (30–40%), linoleic, and palmitic (the three being 90% of the total). Martin and Peers (1953) used tributyrin as substrate for assaying lipase activity in aqueous extracts. This short-chain fatty acid ester does not occur naturally in oats. Berner and Hammond (1970) demonstrated lipase in oat extracts with cocoa butter and lard containing about 35 and 45% oleic acid, respectively, as substrates.

Frey and Hammond (1975) reported a 20-fold variation in lipase activity among different oat cultivars. They used a fatty acid cobalt soap assay. Only three cultivars were tested in the present study. At pH 7.8 Hinoat had significantly higher lipase activity than Elgin ($P = 0.05$). No significant difference occurred at other pH, or between Elgin and Harmon or Hinoat and Harmon.

Comparison of Radioisotope and Colorimetric Assays

The comparison of radioisotope and colorimetric methods in oat flour suspensions showed that initial rates were almost identical, 1.8 ± 0.2 μmol per minute per gram of flour (radioisotope) and 1.7 ± 0.5 μmol per minute per gram of flour (colorimetric). This is equivalent to 50.9 and 50.8 mg of fatty acid released per minute per 100 grams of flour, respectively. The two methods were therefore not significantly different ($P = 0.01$).

Lipase Activity in Doughs

Lipase activity was, however, significantly higher ($P = 0.05$) in doughs than in flour suspensions. Rates in dough were $1,900 \pm 100$ mg of fatty acid released per 100 grams of flour per 15 min, and in suspensions they were $1,360 \pm 102$ mg per 100 grams of flour per 15 min. Martin and Peers (1953) also reported higher rates of lipolysis in doughs than in extracts, although they had used different substrates in the two systems. With the same substrate, our results still show higher lipase activity in doughs.

Extraction of Lipase

Various methods were used to extract a soluble, stable lipase from oat flour. Very little lipase was extracted into the post-6,000 $\times g$ supernatant with 0.5 *M* Tris-HCl buffer, pH 7.5 alone. Table IV shows that addition of 0.15*M* KCl did not significantly increase solubilization of the enzyme. SDS, Na desoxycholate, and Triton-X-100 all improved recovery of soluble lipase. The best extraction of lipase was with 3% Triton-X-100 for 1 hr or with 1% extraction for 5–10 hr (Table IV). These extracts were stable for one week at 0–4°C and for four weeks at −20°C.

Previous attempts to purify oat lipase (Martin and Peers 1953) had used different substrates in the initial oat flour dough, and in the aqueous extract. Moreover, they were unable to maintain lipase activity in subsequent purification steps. No detergents or emulsion stabilizers were used. Our results showed that several detergents increase extractability of an active enzyme. Berner and Hammond (1970) also extracted lipase from oat flour in aqueous extracts containing gum arabic.

Using the same substrate throughout extraction and purification of a lipase is essential. Martin and Peers (1953) successfully used olive oil as substrate in oat flour doughs of 40% moisture content or less. They could not demonstrate activity with olive oil in aqueous extracts from pericarp washings but did show lipase activity in these extracts with tributyrin. Our results show that triolein can be used as substrate for oat lipase in doughs (40% moisture), aqueous flour suspensions (1 g of flour per 10 ml), and in soluble lipase extracts (6,000 $\times g$ supernatant).

TABLE V
Lipase Activity in Germinated and Ungerminated Cereals

Cereal	Lipase Activity[a] (μmol FFA[c]/min/g flour) Ungerminated	Germinated[b]
Oat (Hinoat)	8.43 ± 1.01	11.90 ± 1.05
Wheat (Neepawa)	0.65 ± 0.04	0.80 ± 0.07
Barley (Perth)	1.09 ± 0.16	0.60 ± 0.19
Rye (Gazelle)	0.93 ± 0.05	3.20 ± 0.08

[a] Lipase activity was determined from hydrolysis of glycerol tri[1-^{14}C]oleate in 0.05*M* Tris-HCl, pH 7.5, 1% Triton-X-100, 0.2% benzene, 10 m*M* triolein, at 37°C.
[b] Soaked overnight in water; germinated on damp filter paper at 25°C for two days.
[c] FFA = free fatty acid.

Cereal Lipases

In flour suspensions from ungerminated cereal grains, wheat, barley, and rye had little lipase activity compared with the high levels measured in oats (Table V). Widhe and Onselius (1949) reported similar results. They also found no increase in lipase activity in oats or rye after four days of germination. In comparison, our results showed significant increases ($P = 0.05$) in both oats and rye after two days of germination (Table V). We found very little activity in wheat or barley either before or after germination. Tavener and Laidman (1972), on the other hand, demonstrated an increase in lipase activity in wheat at two days of germination, with a maximum at six days of germination. Varietal differences may account for these discrepancies.

CONCLUSIONS

Lipase activity (triglyceride hydrolysis) can be assayed in oat flour suspensions and in soluble extracts by measuring the rate of hydrolysis of glycerol tri[1-^{14}C]oleate. Triton-X-100 (0.5–2.0%) and benzene (0.2%) were essential for activity. This radioisotope assay gave identical rates with a colorimetric assay in oat flour suspensions. Glycerol trioleate could be used as substrate for oat lipase in doughs (40% moisture content), flour suspensions, and in soluble extracts. The assay procedures described gave pH and temperature optima identical to those described for oat lipase assayed by different methods. The assay can also be used with other cereals. A stable, soluble lipase extract from oat flour can be prepared when detergents are included in the extraction buffer. Of the detergents tried, Triton-X-100 (1–3%) gave the best results.

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