Studies on Acyl Hydrolases in *Avena sativa* L.

by

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to my mom and dad,

because they've always been there.

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List of Abbreviations

AACC	American Association of Cereal Chemists
CHM	chloroform:heptane:methanol (49:49:2)
DDC	sodium diethyldithiocarbamate
DFP	di-isopropylfluorophosphate
FDB	fluorescein dibutyrate
FDL	fluorescein dilaurate
FFA	free fatty acid
FFA_{15}	rate of FFA release calculated after 15 min. incubation
FFA_{30}	rate of FFA release calculated after 30 min. incubation
MU	4-methylumbelliferone
MUB	4-methylumbelliferyl butyrate
MUH	4-methylumbelliferyl heptanoate
MUO	4-methylumbelliferyl octanoate
MUP	4-methylumbelliferyl palmitate
MUS	4-methylumbelliferyl stearate
PPL	porcine pancreatic lipase
RFI	relative flourescence intensity
SD	standard deviation
Tris	Tris(hydroxymethyl)aminomethane
cv	cultivar

ABSTRACT

A simple, rapid assay using the fluorogenic substrate 4-methylumbelliferyl heptanoate (MUH) to measure hydrolytic activity was adapted for use with oats, and compared under varying conditions with a conventional but lengthier lipase assay in which the free fatty acids (FFA) released from endogenous triglycerides by lipolytic activity were determined. Experiments using methylumbelliferyl esters with acyl chains of varying length, and results obtained using defatted flour suggested that the two assays were measuring different hydrolytic activities. This was confirmed by the use of di-isopropyl-fluorophosphate, which indicated that the MUH assay was measuring primarily esterase activity. However, the ease and rapidity of the MUH assay prompted further experimentation. High correlations were demonstrated between the two assays during moist heat inactivation (r=.85). Significant correlation (r=.83) between the two assays was also demonstrated in a screening of 21 domestic cultivars of mature oats. A 4-fold variation in lipase activity, and a 2-fold variation in esterase activity were observed.

The results presented indicate that esterase activity, as measured by cleavage of MUH, could be a useful indicator of lipase activity in specific situations.

1. INTRODUCTION

1.1 General Introduction

Acyl hydrolases are a group of enzymes which includes lipases and esterases. These enzymes catalyze cleavage of ester bonds, releasing an acyl chain, or, more specifically, a free fatty acid, from the substrate.

Lipases play an important role in food biochemistry. The impact of these enzymes is felt in agriculture, and particularly in the food and beverage industries. For example, in brewing, the monoglycerides released by lipase activity during barley malting tend to improve the head retention capacity of beer (Narzi and Sekin 1974). The production of monoglycerides by lipase in breadmaking improves the resistance of bread to staling, and microbial and milk lipases aid in the development of cheese flavour (Brockerhoff and Jensen 1974). The breakdown of stored oil into free fatty acids in rice bran that begins immediately after milling renders the oil unusable except for soap manufacture (Saunders and Heltved 1985). Lipase is also an important factor in the keeping quality of oat products, where residual enzyme activity has been implicated in undesirable colour changes in oat cakes (Martin 1956), and can lead to soapy and bitter off-flavours due to the released free fatty acids (Hutchinson and Martin 1952, Moran 1952, Kazi and Cahill 1969, Welch 1977).

Polyunsaturated fatty acids released by lipolytic activity in a sample can be further acted upon by lipoxygenases and converted to

hydroperoxides (Galliard 1983) which, depending on the quantity present, and the food material, can produce desirable or undesirable flavours.For example, in tea, traces of hexanal and hexenal impart a desirable flavour, but these flavours in raw soybeans are considered unpleasantly "green" and "beany" (Gardner 1980). Lipid hydroperoxides can also lead to loss of nutritive values, such as destruction of some vitamins and protein (Gardner 1980). The most common substrates for lipoxygenases in foods are the free fatty acids linoleic acid and linolenic acid (Galliard 1983).

Lipases are widespread in nature, hydrolyzing triglycerides to yield glycerol and free fatty acids (FFA). For example, they are found as digestive enzymes in animals, where they are required for the utilization of dietary fat. Lipases are also required for mobilization of fat reserves in plants and animals. In germinating seeds, lipases are essential to break down storage triglycerides, providing energy for the young plant. Lipophilic microorganisms secrete extracellular lipases, allowing them to derive nourishment from their environment.

A true lipase, (triacylglycerol acylhydrolase,EC 3.1.1.3), by definition hydrolyses triglycerides at an oil-water interface, and will not hydrolyse lipids in aqueous solution (Desnuelle 1951, Brockerhoff and Jensen 1974, Galliard 1980, 1983, Brockman 1984). An esterase catalyzes cleavage of ester bonds of substrates in aqueous solution. A more functional approach requires that an enzyme must be capable of hydrolyzing esters of long-chain fatty acids such as oleic acid to be considered a lipase as opposed to an esterase.

For a typical esterase, hydrolysis rates increase with increasing substrate concentration up to a saturation level, at which point

continued addition of substrate does not produce an increase in hydrolysis rate (Galliard 1983). Lipases, on the other hand, have little activity at concentrations below that at which oil droplets form, and activity increases as the interfacial area of the oil increases.

One of the first lipolytic enzymes to be recognised was pancreatic lipase. The requirement, in dogs and rabbits, of "pancreatic juice" for the digestion and absorption of dietary fat was noted by Claude Bernard in 1849 (Bernard 1849). A more detailed description of pancreatic lipase was published seven years later (Bernard 1856). The first plant lipase was observed in oil-bearing seeds when Muntz, in 1871, noted that the disappearance of oil in germinating castor beans coincided with the appearance of fatty acid in the seed (cited in Green 1890). In 1890, Green, working with extracts of germinated castor beans, demonstrated that there was a "ferment" in the seeds which developed fatty acid from the oil (Green 1890).

Since those first early observations, a large body of work has been published on both plant and animal lipases. As a detailed examination of this literature is beyond the scope of this thesis, for comprehensive reviews on lipolytic enzymes the reader is referred to the following: Brockerhoff and Jensen (1974), Galliard (1980,1983), and Börgstrom and Brockman (1984).

1.2 Literature Review : Oat Lipase

In 1920, Berry noted that the "free acid" content in the oil of freshly ground oats was quite low, but that as the time elapsing between grinding and time of extraction in diethyl ether increased, the acidity

increased also (Berry 1920). This was the first indication of hydrolytic activity in oats, as earlier work by Stellwaag in 1874, and Konig in 1890 merely reported that oat oil contained a high proportion of fatty acids (cited in Berry 1920).

In 1951, Hutchinson et al.(1951) reported that under normal storage conditions, sound, undamaged grain underwent very little increase in free fatty acid content, but if the grain was cracked or crushed, or milled into meal, lipases were activated, and within two or three days the production of free fatty acids was quite significant. In their experiments on the location and inactivation of lipase in oats, Hutchinson et al. were among the first to note that the temperature required to inactivate lipase was inversely proportional to the moisture content of the dehulled oat kernels, or groats. Inactivation was also achieved by soaking the groats in 1.0 N HCl. Hutchinson et al.(1951) noted that removal of the outer pericarp of groats reduced their lipase activity by 98%, with the pericarp scrapings having a very high activity.

The methods used to measure lipase activity by these workers were tedious and time consuming. Neutral fat and water were added to defatted oat meal, and the free fatty acid formed per gram of meal at 37°C was measured by titration. For comparison of the keeping qualities of several different samples of oatmeal, an incubation period of 3 days at 12% moisture was required, while suitability for biscuit and oatcake manufacture could be assessed after 3 hours at 36% moisture.

This assay method was later refined to incubations of 2 hours or less, and in some cases, with the use of natural rather than defatted meal (Hutchinson and Martin 1952). However, the method still involved

time consuming steps for the extraction and determination of the free fatty acids released. The incubated material was extracted with light petroleum, which was then evaporated, and the oil was titrated in boiling alcohol-benzene against alcoholic sodium hydroxide using phenolphthalein as an indicator.

Templeton and Carpenter (1953) used the methods of Hutchinson and Martin to examine lipase activity in a number of cereal products. Their results showed that lipase activity in oatmeal was thirty to forty times greater than that in the same amount of wheat wholemeal flour, wheat malt flour, or barley malt flour.

Martin and Peers (1953) were the first to describe oat lipase in any biochemical detail. They partially purified the lipase by removing the pericarp from groats in a wet-brushing technique. The highest lipase activity was found in an opalescent aqueous extract of the pericarps. Their only successful attempt to precipitate the active component from the aqueous extract consisted of repeated freezing and thawing. By this method a 2000-fold purification (on a dry weight basis) compared with whole groats was obtained. These workers used two assays to measure lipase activity: one which used olive oil as a substrate was essentially the same as that of Hutchinson et al.(1951), and the second was a manometric assay using tributyrin as the substrate. Although the two substrates were not interchangeable in the two assays, both were still considered by Martin and Peers to measure the same enzyme.

Using the manometric assay, some of the properties of the semi-purified lipase were determined: optimum activity occurred at pH 7.4, the temperature optimum was 37°C, and a Lineweaver-Burke plot yielded a

Michaelis constant of 0.0062M. Further experiments by Martin and Peers using tributyrin as a substrate indicated that the "purified" oat lipase would specifically split off one butyric acid residue from tributyrin (Martin and Peers 1953, Peers 1953).

Later experiments implicated lipase activity, and the resulting increase in free fatty acids, in the occurrence of undesirable colour changes in oatcakes (Martin 1956). These analyses showed a more than ten-fold increase in lipase activity in "complaint" oatmeal, which produced oatcakes of a peculiar bright orange-brown colour, rather than the usual pale brown, as well as a three-fold increase in free fatty acid content. Experiments showed that the colour change involved both a heat-labile component found in the pericarp, which could be inactivated by steaming (the enzyme), and a heat-stable component found in the endosperm (the triglyceride substrate), which required the unsteamed component from the pericarp to produce the colour change. Total inactivation ~~of~~ the enzymes in oatmeal was achieved by heating in a sealed tube for 30 minutes at 100°C. The moisture content of the samples used was not mentioned, although previous work by these authors had shown it to be important.

Thermal inactivation of oat lipase has also been studied using dry heat (Pokorny et al.1963). In these experiments, samples of oat flour were spread on filter paper and dried in an electric oven at a specified temperature for a given period of time. The assay used to determine lipase activity was extremely time consuming (requiring a week of incubation at 30°C, before extraction and titration of the released free fatty acids). Pokorny et al. found that at temperatures of 100°C and

lower, the lipase activity rose initially, and decreased again only after a prolonged heating (2 hours or more) at the same temperature. The effect of moisture on inactivation rate was studied, and at 120°C, only a wetted sample (21% moisture) was effectively inactivated, while the air-dried (7% moisture) and dessicator-dried samples actually showed a slight increase in activity after 2 hours of heating. It was concluded by these workers that hydrothermic processes are required for oat lipase inactivation.

A faster, but non-quantitative method for detecting residual lipase in oats was suggested by Kazi and Cahill (1965). In their assay, the sample was incubated for 30 minutes in the presence of phenol red, starting with a pink solution at neutrality. A colour change from pink to yellow (indicating a drop in pH) was taken to indicate the production of fatty acid, and hence the presence of residual lipase. Neutralization of the solution with NaOH (to a pink colour), and a further 30-minute incubation confirmed the activity in these samples.

The specificity of oat lipase was studied by Berner and Hammond (1970) using both natural and randomized cocoa butter and lard. In randomized fats, the triglycerides have been hydrolyzed and reformed in such a way that the distribution of fatty acids on the various positions on the molecule is random, thus eliminating any positional specificity. These workers used the partial purification and assay method of Martin and Peers (1953), which has been described above. Although chemical hydrolysis showed approximately equal amounts of palmitic, stearic, and oleic acids in cocoa butter (approximately 30% each), enzymatic hydrolysis yielded 84-95% palmitic and oleic acid after a 2-minute reaction in the presence of oat lipase. Similar results were obtained

using lard as substrate. The lard was analysed by chemical hydrolysis to contain approximately 27% palmitic acid, 15% stearic acid, 46% oleic acid, and 9% linoleic acid, with traces of myristic and palmitoleic acids. Enzyme hydrolysis of lard and randomized lard yielded 25-30% palmitic acid, 2-3% stearic acid, 39-47% oleic acid and 22-23% linoleic acid, indicating a preferential release of linoleic acid regardless of position. These workers also noted that inclusion of 1% mono- and diglyceride in the substrate did not alter the reaction, indicating that the reaction is not influenced by the accumulation of hydrolysis products. However, later work on seeds of a different plant, Nigella damascena L. indicated that the released free fatty acids were inhibitory to lipase activity (Korchagina and Rudyuk 1979). Similar observations were made by Rosnitschek and Theimer (1980) in their study of the triglyceride lipase of rapeseed (Brassica sp.) cotyledons.

An agar plate test for oat lipase was later developed by Frey and Hammond (1975). Groats were pressed into tributyrin-containing agar, and lipase activity was assessed by the diameter of the clear zones around the groats, where the lipase had diffused into the agar and solubilized and cleared the dispersion. The tributyrin test was used to screen 352 oat species and cultivars. After 24 hours, 58 samples showed "no lipase activity", 217 samples showed "a detectable zone of clearing", 65 samples showed "an obvious clear zone", and 12 samples had "a large clear zone".

A more precise method was also used by Frey and Hammond (1975) to determine lipase, by bringing the moisture content of the sample to 30% and incubating for 1 hour at 37°C in the presence of soybean oil. The released free fatty acids were detected using the method of Novak (1965)

by forming cobalt soaps of free fatty acids, and a subsequent colourimetric reaction with α-nitroso-β-naphthol. Little association was found between the results of the two tests. The colourimetric method revealed a 20-fold variation in lipase activity among the cultivars examined.

In the study by Frey and Hammond (1975), it was found that lipase activity in oat dough was strongly influenced by the amount of water included in the dough, with maximum activity at 25-50% of the weight of the groats, while at 75-100%, the activity was inhibited. Lipase could not be detected at water levels above 100% of the weight of the groats. In experiments designed to inactivate lipase, these investigators found that the most effective treatment was boiling the groats in water, while steaming was found to be less effective. The second most effective treatment was grinding in 95% ethanol. Treatment with acid to destroy lipase, as reported by Hutchinson et al.(1951) was found to be ineffective.

An assay to measure lipase activity in single grains of oats was developed by Sahasrabudhe (1982). In this assay, rather than determining enzyme activity by titration of released FFA, the released FFA were detected and quantified similar to the colourimetric assay of Frey and Hammond. After incubation of the crushed seed sample at 38°C for 30 minutes, using endogenous lipid as substrate, the released free fatty acids were extracted and detected using a modification of the copper soap method of Shipe et al.(1980). Briefly, this method involved conversion of the free fatty acids to copper soaps by shaking the solution of the extracted fatty acids with a copper-containing reagent at alkaline pH, followed by colourimetric detection of the copper soaps

with diethyldithiocarbamate (DDC). This method is quite sensitive, and microgram amounts of free fatty acid can be determined.

Sahasrabudhe measured lipase activity in individual oat grains from the "dough" stage (about 2 weeks before normal harvest) to two years post-harvest storage. The "dough" stage showed the highest lipase activity. Samples of mature grains showed 45-50% decreases in lipase activity after 2 years storage. Sahasrabudhe reported that lipase activity was found in "the outer aleurone layers" of the kernels.

Matlashewski et al.(1982) measured oat lipase in flour suspensions and aqueous buffer extracts by following the hydrolysis of ^{14}C-labelled triolein. Parallel measurements were made using a colourimetric assay adapted from that of Sahasrabudhe (1982). A 2-hour Soxhlet lipid extraction, and subsequent air-drying of samples before assay added considerably to the time required for these assays. After incubation in the presence of ^{14}C-triolein, aliquots of the reaction mixture were extracted with chloroform-methanol (1:1) and the extracts were fractionated using thin layer chromatography. After locating the separated lipid components with iodine vapour, the fractions were scraped into separate scintillation vials and radioactivity was measured. A comparison of this method with the colourimetric method using oat flour suspensions showed that the two methods were not significantly different, producing initial rates of lipase activity that were almost identical. Lipase activity was found to be significantly higher in doughs (with approximately 40% water) than in flour suspensions.

Urquhart et al. performed further experiments with oat lipase using the same ^{14}C-triolein assay, undertaking both a morphological study to

localize lipase activity in oat grains (1983), and a developmental study, to examine lipase activity during oat maturation and germination (1984). Their results indicated that the majority of the lipase in ungerminated grains is found in the bran, with little or none in the embryo (Urquhart et al. 1983). Lipase activity was also assayed in developing oats, from 2 to 44 days after flowering (post-anthesis) (Urquhart et al.1984). In the three cultivars examined, activity was detected throughout kernel development. Total activity per 100 kernels increased to a maximum at 23-30 days post-anthesis, and decreased again as the grains completed maturation (44 days post-anthesis). During germination, a 60% increase in activity on a whole kernel basis occurred in the first 12 hours following the start of imbibition. Activity in the embryo did not start to increase until more than 24 hours after the start of imbibition.

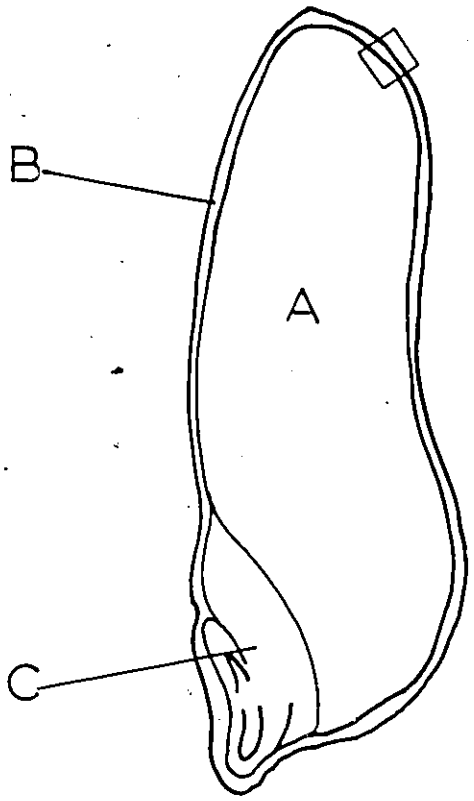
Precise localization of lipase activity remains unclear. Sahasrabudhe (1982) stated that "...localization of the lipase in the outer aleurone layers of the grain [citation of Martin and Peers (1953)] was confirmed by scraping the outer layers of individual grains using a dentist's drill...". Martin and Peers (1953) stated that "...oat lipase is located in the outer pericarp layers of the groat...". In fact, in an earlier paper by Hutchinson, Martin and Moran (1951) it was clearly stated that "...the active lipase in oats lies within the pericarp, that is, above the testa, and not in the aleurone layer...". Urquhart et al. (1983) stated that "...most of the oat lipase in ungerminated grains occurs in the outer layers (bran) of the kernels...". The milling term 'bran' includes both of the botanical tissues in question (see Figure 1), and thus does not shed any further light on the question.

Figure 1.

a) Simplified diagram of longitudinal cross section of an oat kernel, showing the starchy endosperm (A), the bran (B) and the embryo, or germ (C).

b) Enlargement of inset in (a), showing the tissues present in the bran (pericarp, testa and aleurone layer) and beginning of the starchy endosperm (the subaleurone layer).

a)



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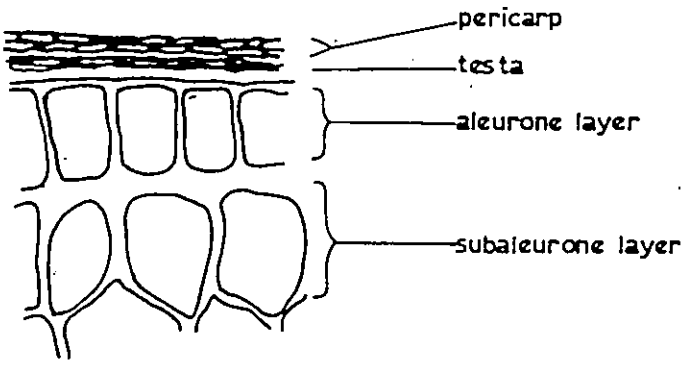


The use of fluorogenic substrates to measure lipolytic activity was first reported in the early 1960's (Yagi et al.1961, Guilbault and Kramer 1963). Throughout that decade, a number of papers were published documenting the use of a variety of non-fluorescent substrates which could be cleaved by purified or semi-purified lipase preparations to give fluorescent products (Guilbault and Kramer 1964, Jacks and Kircher 1967, Guilbault et al.1968, Guilbault and Hieserman 1969). Interest in the use of these substrates has revived in recent years, particularly with respect to the detection of hydrolytic activities of germinating grains of barley, wheat, rye and sorghum (Jensen and Heltved 1982, Jensen and Heltved 1983, Heltved 1984, Jensen et al.1984), and in rice bran (Saunders and Heltved 1985), and in wheat fractions for baking (Galliard 1986). In these studies, the enzymes were not extracted and purified, but were assayed in the seed tissues. In this thesis, the MUH assay of Saunders and Heltved (1985) using the fluorogenic substrate 4-methylumbelliferyl heptanoate (MUH) was adapted to assay activity in oat flours.

1.3 Objectives of Thesis

From a food biochemistry standpoint, lipase activity gives rise to products which cause undesirable changes in the colour and flavour of oats and oat products. This deterioration in quality, resulting in unsuitability of the product for marketing, will, in turn, be an economic disadvantage to producers of oat products. Clearly, a rapid assay which could detect lipase activity in whole or partially processed oats before their incorporation into a final product would be an asset,

allowing treatment to inactivate the enzyme before final processing. Such an assay would also be useful to oat breeders, allowing convenient screening of genetic stock, and the potential of retaining cultivars exhibiting low lipase activity, while those with unacceptibly high lipase activities could be discarded.

In the preceding literature review, several conventional assays which have been used to measure lipase activity in oats are discussed. Virtually all of these assays require an incubation period, in which the enzyme is exposed to endogenous or exogenous substrate, after which the released free fatty acids are determined by titrimetric or colorimetric means. While some of these assays are capable of considerable accuracy and sensitivity (eg. Sahasrabudhe 1982, Matlashewski et al.1983), all are time consuming to perform.

Cleavage of non-fluorescent esters to yield easily detectable fluorescent products has been used as an indicator of lipolytic activity, both in purified plant and animal enzyme preparations (Kramer and Guilbault 1963, Guilbault and Kramer 1964, Jacks and Kircher 1967, Guilbault et al.1968, Guilbault and Sadar 1969) and in cereal seed tissues (Jensen and Heltved 1982, Heltved 1984, Saunders and Heltved 1985, Galliard 1986). The most common of these fluorogenic substrates are mono-esters of 4-methylumbelliferone (MU), and di-esters of fluorescein. The general structures of these compounds, and their cleavage to fluorescent products, are illustrated in Figure 2. As this rapid type of assay had not yet been applied to oats, the MUH assay of Saunders and Heltved (1985) was adapted for use with oats.

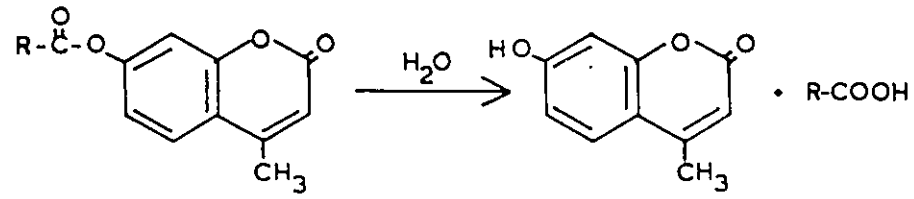
To evaluate the MUH assay, experiments were conducted in which the FFA assay of Sahasrabudhe (1982) and the adapted MUH assay were

Figure 2.

a) Schematic representation of the cleavage of a non-fluorescent 4-methylumbelliferyl ester (MUR) to release a fatty acid (R-COOH) and the highly fluorescent molecule 4-methylumbelliferone (MU). R represents the acyl chain of a fatty acid.

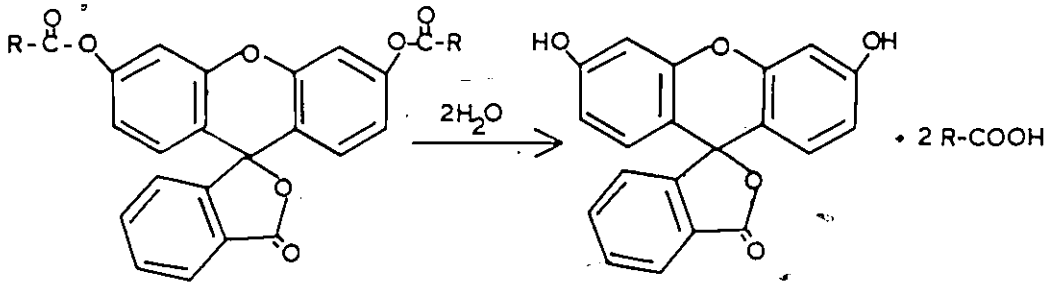
b) Schematic representation of the cleavage of a non-flourescent fluorescein di-ester (FDR) to release 2 fatty acids (R-COOH) and the fluorescent molecule fluorescein.

a)



MUR MU

b)



FDR F

performed in parallel, and the results compared. Preliminary experiments with methylumbelliferyl esters of varying chain length, and also experiments using both natural and defatted flours suggested that the two assays were not measuring the same activity. For this reason, experiments with the esterase inhibitor di-isopropylfluorophosphate(DFP) (Saunders and Stacey 1948, Means and Feeney 1971) were performed.

Although the experiments with DFP indicated that the MUH assay was, in fact, measuring esterase activity, because of its ease and rapidity, experiments were initiated to ascertain whether the MUH assay could be used as an indicator of lipase activity under some conditions. With this in mind, further experiments were performed in which the FFA and the MUH assays were performed in parallel on the same samples. Activity during germination of oat kernels was examined, as well as a moist heat inactivation process. Twenty one cultivars of oats were screened for lipolytic activity in mature, ungerminated kernels.

2. MATERIALS AND METHODS

2.1 Plant Materials:

Samples of oat (Avena sativa L.) cultivars (cvs) used in heat inactivation and germination experiments were obtained from Dr. V.D. Burrows of the Cereal Section, Ottawa Research Station, Agriculture Canada. Oat samples for cultivar screening were obtained from Dr. F.H. Webster of the Quaker Oats Company, Barrington, Illinois, grown at the University of Wisconsin in 1984).

2.2 Reagents:

Chloroform, heptane, methanol, 0.1 N HCl, the dye Nile Blue A, sodium diethyldithiocarbamate, triethanolamine and copper nitrate were obtained from the Fisher Scientific Company (Fairlawn, New Jersey). Trizma base, porcine pancreatic lipase, and methylumbelliferyl and fluorescein esters (4-methylumbelliferyl butyrate, heptanoate, octanoate, palmitate and stearate, and fluorescein dibutyrate and dilaurate) were obtained from the Sigma Chemical Company (St. Louis, Missouri). Triton X-100 was obtained from Calbiochem (San Diego, California). Di-isopropylfluorophosphate (DFP) was obtained from the Aldrich Chemical Company (Milwaukee, Wisconsin). Sodium hypochlorite, or Javex was obtained at the supermarket, distributed by Bristol-Meyers Inc. (Toronto, Ontario). The olive oil used was produced by Bertolli-Lucca (Novara, Italy).

2.3 Enzyme Assays:

Two assays were used in this study. The copper soap method of Sahasrabudhe (1982) for detecting free fatty acids (FFA assay) was used with very little modification to monitor the release of free fatty acids from endogenous triglyceride by oat lipase. Cleavage of 4-methyl-umbelliferyl heptanoate (MUH) has also been reported to indicate lipase activity (Guilbault et al.1968, Heltved 1984, Saunders and Heltved 1985). In this thesis, the MUH assay of Saunders and Heltved (1985) was adapted for use with oat flour. The FFA assay was considered the "standard" method, with which the results of the much faster MUH assay were compared. A schematic representation comparing the two assays with respect to number of manipulations, and time required is presented in Figure 3.

2.3.1 Free Fatty Acid (FFA) Method:

A modification of the copper soap method of Sahasrabudhe (1982) was used to monitor release of free fatty acids by lipases in the oat flour. Oats (2-3 g) at ambient moisture were dehulled and ground to pass through a 0.5 mm screen using a UDY Cyclone Sample Mill (UD Corporation, Boulder, Colorado), and the resulting flour was well mixed to ensure uniformity. For each test sample, 0.1 ml of 0.5M Tris-HCl buffer (pH 7.4, with 1% Triton X-100 vol/vol) was added to 50.0 mg of oat flour in a round bottomed tube and mixed well for 30 seconds with a glass rod. Tubes were then incubated in a 37°C water bath for 15 or 30 minutes. At the end of the incubation period, the reaction was stopped

Figure 3. Flow chart comparing manipulations required for the FFA and MUH assays.

dehull 2-3 g oats

grind in UDY mill

weigh 50mg/tube

For FFA Assay:

add 0.1ml buffer, and mix with glass rod for 30 seconds

incubate (15-30 mins)

inactivate (0.2 ml HCl) (mix again 30 sec)

extract FFA with CHM (3 min/sample)

make copper soaps of FFA (vortex 15 sec/sample)

remove 4 ml of CHM layer

add DDC and vortex

read absorbance at 440 nm

Time for 6 samples:
4 hours

For MUH Assay:

suspend in 20 ml of buffer by vigorous shaking for 30 sec

remove 3 ml aliqot to cuvette

add MUH
invert 3 times to mix

read increase in fluorescence at 448 nm (48 sec)

30 minutes

by addition of 0.2 ml of 0.1 N HCl, and again mixed well with a glass rod for 30 seconds. For controls, the HCl was added immediately to the oat flour-buffer mixture. To extract the released free fatty acids, 5.0 ml of chloroform:heptane:methanol (CHM 49:49:2) was added to the tube, and mixed using a Kinematica homogenizer (distributed by Sybron Brinkman, Westbury, New York) at setting 7 for 45 seconds (continuous). The mixture was filtered through Whatman No. 2^{V} filter paper into a 50 ml volumetric flask. The incubation tube was rinsed three times with CHM (20 second bursts with the Kinematica), with the rinsings also being filtered into the 50 ml volumetric flask. For the colour reaction, 5.0 ml of the above extract was placed in a conical tube with 2.0 ml of copper reagent (0.1M $Cu(NO_3)_2.3H_2O$ and 0.4M triethanolamine in saturated NaCl, pH 8.3), and vortexed vigourously for 15 seconds. The tube was then centrifuged at 2000 rpm for 10 minutes. A 4 ml sample of the clear CHM layer was aspirated off and mixed with 0.1 ml of 0.5% sodium diethyldithiocarbamate (DDC) in butanol. The absorbance of the resulting coloured solution was read in 1 cm cuvettes at 440 nm using a Beckman DU-7 Spectrophotometer. For each assay, duplicate samples were incubated, and duplicate determinations performed on each sample extract, for a total of 4 values per enzyme assay.

2.3.2 4-Methylumbelliferyl heptanoate (MUH) Assay:

For the adapted MUH assay, 20 ml of 0.2M Tris-HCl buffer (pH 8.0) was added to a tube containing 50.0 mg of oat flour and the tube was shaken vigorously. A portion (3.0 ml) of the resulting suspension was removed to a cuvette and 20.0 ul of 0.1M 4-methylumbelliferyl heptanoate (MUH) was added. The reaction was followed using a Hitachi Perkin-Elmer

Fluorescence Spectrophotometer MPF-2A with a Xenon lamp at an excitation wavelength of 397 nm and emission wavelength of 448 nm. Initial scans which were performed to determine the excitation and emission maxima are presented in Figure 4. Both excitation and emission slit widths were set at 3.5 nm. The sensitivity setting was 3. The spectrophotometer was connected to a chart recorder. The rate of reaction was calculated from the slope of the line resulting from the increase in fluorescence as a function of time, as the substrate was cleaved. Other fluorogenic substrates used were 4-methylumbelliferyl butyrate (MUB), 4-methylumbelliferyl octanoate (MUO), 4-methylumbelliferyl palmitate (MUP), fluorescein dibutyrate (FDB), and fluorescein dilaurate (FDL). These substrates were added in the same concentrations as MUH.

2.3.3 Assays Using Porcine Pancreatic Lipase:

To help clarify which of the MU esters was the most appropriate substrate for lipase, assays were performed using porcine pancreatic lipase (PPL), and the following substrates: MUB, MUH, MUO, MUP and 4-methylumbelliferyl stearate (MUS). For each determination, 3 ml of buffer (.05M Tris-HCl, pH 8.0) was placed in a 1 cm cuvette, and 10 ul of a stock solution (.01 mM) of substrate was added. This was the blank for the determination. 100 ul of a 1.0 mg/ml solution of enzyme was then added, and mixed by inversion. Enzyme activity was calculated from the increase in fluorescence with time as the substrate was cleaved.

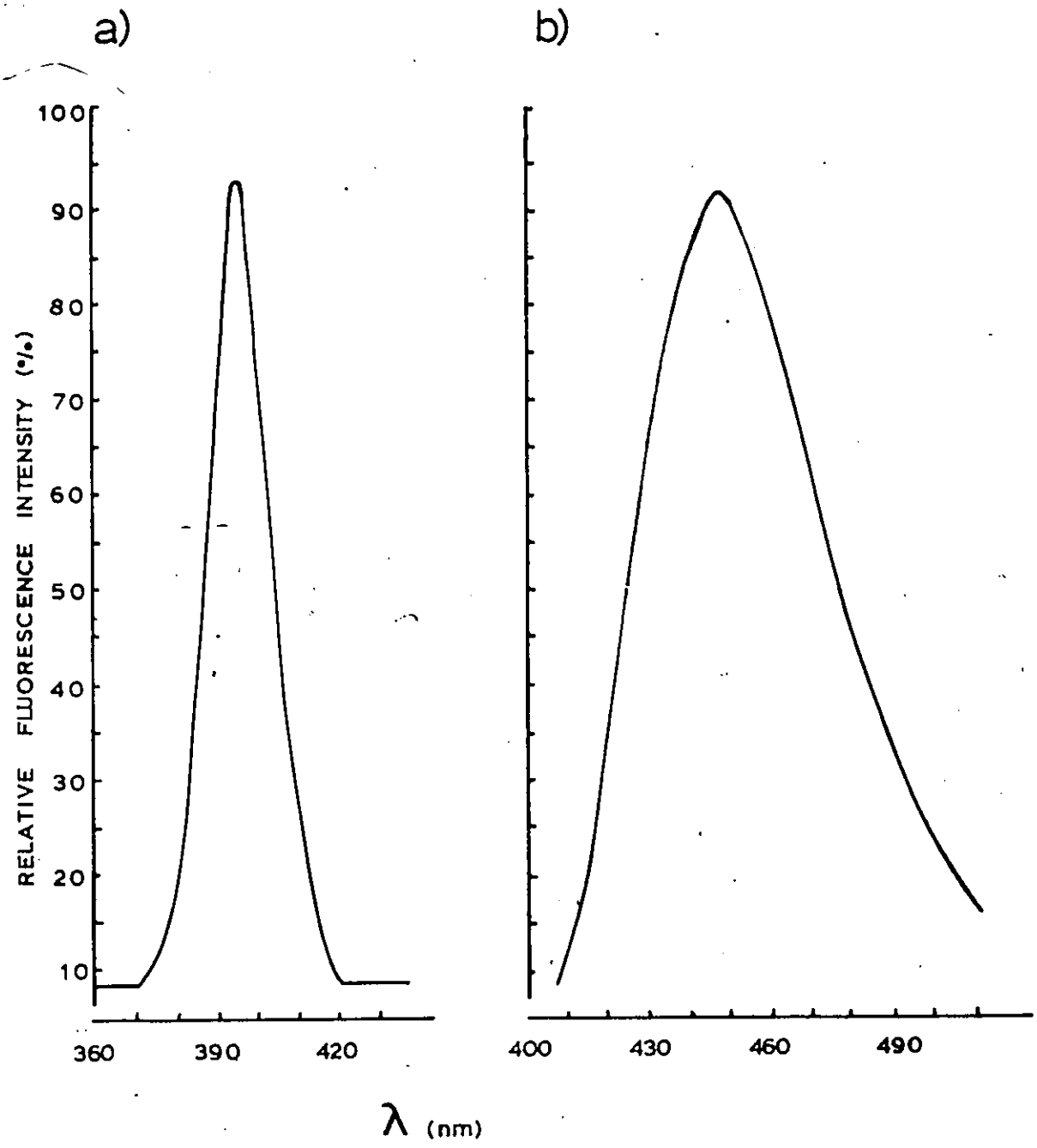
2.3.4 Preparation and Assay of Defatted Material:

To determine the maximum potential rate of lipolysis in oat flour, defatted flour was prepared, and assayed with known amounts of exogenous

Figure 4. Fluorescence spectra of 4-methylumbelliferone (MU) in 0.2M Tris buffer at pH 8.0.

a) scan of excitation wavelengths with emission wavelength set at 450 nm.

b) scan of emission wavelengths, with excitation wavelengths set at 397 nm.


a)
b)
100
90
80
70
60
50
40
30
20
10
RELATIVE FLUORESCENCE INTENSITY (%)
360
390
420
400
430
460
490
λ (nm)

substrate. Since samples of oat oil were not obtainable, olive oil, which is approximately 80% triolein (Linstromberg 1974) was used. Oats have been found to contain 28-41% oleic acid, depending on the cultivar (Youngs 1978, Sahasrabudhe 1979). It was also thought, in the MUH assay, that removal of endogenous substrate might make MUH a more attractive substrate for lipase.

Defatted material was prepared by stirring oat flour in diethyl ether for 1 hour using a magnetic stirrer, with 2 subsequent washings in diethyl ether. The resulting extracted flour was air dried, then to confirm lipid extraction, a small portion was prepared as a wet mount using 0.01% aqueous Nile Blue A and examined using a Zeiss Universal Research Microscope equipped with a III RS epi-illuminating condenser for fluorescence analysis. The condenser contained a fluorescence filter combination with a dichromatic beam splitter and exciter/barrier filters with maximum transmission at 450-490 nm/520 nm.

For assay of defatted flours with the FFA method, emulsions of olive oil and the Tris buffer (as above) were prepared using the Kinematica Homogenizer. The optimum substrate concentration was determined to be 450 mM triolein. At this concentration, the optimum sample quantity for defatted flours was found to be 50 mg flour per assay. For the MUH method, the assay conditions were the same as those outlined in section 2.3.2 except that 30 ul of substrate (0.1M) was added for these determinations.

2.3.5 Inhibitor Studies:

To further characterize the enzyme activities measured by the two assays, experiments were performed using di-isopropyl-

fluorophosphate (DFP), a known esterase inhibitor (Saunders and Stacey 1948). Both the FFA and MUH assays were performed as described, with DFP added to the assay buffer at 2 mg/100 ml.

2.4 Germination Studies:

Experiments were conducted to observe changes in lipase activity in the initial stages of plant development, ie. during germination. For germination tests, the hull-less cultivar Tibor was used, as no tedious dissection of hulls by hand was required. The oats were visually examined to ensure that no damaged seeds were used. Seeds were first surface sterilized by the following procedure: immersion in 70% ethanol (with shaking) for 2 minutes, rinsed with distilled water, then two 10-minute immersions in 1.2% sodium hypochlorite (J. Frégeau, personal communication). In the second hypochlorite immersion, the sample was placed under vacuum for 5 minutes to aid penetration into the crease area of the kernels. The seeds were rinsed with distilled water and allowed to dry in a laminar flow cabinet. The seeds were then placed crease down on 2 layers of filter paper in a petri dish (50 seeds per plate) moistened with 5 ml of distilled water. The filter paper was changed daily, and fresh distilled water added. The plates were incubated at 12°C or 20°C in the dark. At the end of the germination period, the seeds were frozen rapidly by mixing with crushed dry ice, and stored at -10°C. For freeze drying, the stored samples were homogenized in a small amount of cold (4°C) distilled water and shell-frozen in large round-bottomed tubes before freeze-drying overnight. Because of depletion of endogenous triglyceride reserves during germination, the resulting flour was assayed using olive oil

emulsified in Tris-HCl buffer (to give approximately 450 mM triolein).

2.5 Inactivation Studies:

Studies were performed to examine the behavior of lipase during heat treatment, as encountered in an industrial setting. Prior to heat inactivation, the moisture content of the groats was determined, and enough distilled water added to bring the moisture content to 20% (D. Paton, personal communication). The container of groats was then sealed, and allowed to equilibrate at 4°C overnight. Equal amounts (2.0 g) of the equilibrated groats were then measured into vials, and the vials sealed and placed in heated water baths at varying temperatures, for varying periods of time. At the end of the heat treatment, the groats were mixed immediately with crushed dry ice, and ground in the UDY Cyclone Mill. For both the FFA and the MUH methods, 60 mg of the material was used per tube due to the increased water content of the groats.

2.6 Proximate Analyses:

2.6.1 Moisture Determinations:

Moisture content of the oats was determined using the standard American Association of Cereal Chemists (AACC) method number 44-15. Briefly: a 2.0 g sample of oat flour was dried in an oven at 130°C in a moisture dish for one hour, then allowed to cool in the moisture dish with lid in a dessicator over NaOH/silica gel before weighing. The resulting dry weight was used to calculate the % moisture of the oats.

2.6.2 Crude Fat Determinations:

Crude fat was determined using an automated Soxhlet apparatus (Soxtec, Tecator AB, Höganäs, Sweden). Briefly: 1.0 g samples of oat flour were soaked in Skellysolve B for 30 minutes, then distilled for 1 hour. The solvent was then evaporated, and dried in an 85°C oven for 30 minutes, before cooling, and weighing the extracted oil.

2.6.3 Total Nitrogen Determinations:

Total nitrogen was determined using an automated Kjeldahl system (Kjeltec, Tecator AB, Höganäs, Sweden). Briefly: 0.5 g samples of oat flour were digested with concentrated H_2SO_4 for 55 minutes, which converted protein nitrogen into ammonium sulphate. After cooling, 70 ml of distilled water was added. 40 ml of 35% NaOH was then added to liberate the ammonia, which was then distilled into a solution of boric acid. The ammonia in this solution was then titrated with 0.1 N HCl to an orange endpoint using Methyl Red/Bromcresol Green as an indicator. The nitrogen value obtained was multiplied by 6.25 to give % protein of the sample.

2.7 Statistical Analyses:

Throughout this thesis, data (both in numerical and graphical form) are presented as the means of 2-4 determinations (n is indicated on each figure or table) ± the standard deviation (SD). On all figures, where bars are shown for SD, the value is either zero, or too small to extend beyond the symbol on the graph. Variation between samples was assumed to be independent of environmental influence, as all cvs were grown in a single location in the same year. These samples were assumed to be

totally random and representative of the whole population. For each experiment, the samples were thoroughly mixed, and random subsamples of about 100 oats were taken. The subsamples were ground, and 50 mg portions were used for each determination in both assays. For the germination, inactivation, and cultivar screening experiments, the data were analyzed using either Duncan's Multiple Range test, or the Student-Newman-Keul's Multiple Range test, both of which compare means within groups, and determine whether the differences between them are significant.

3. RESULTS

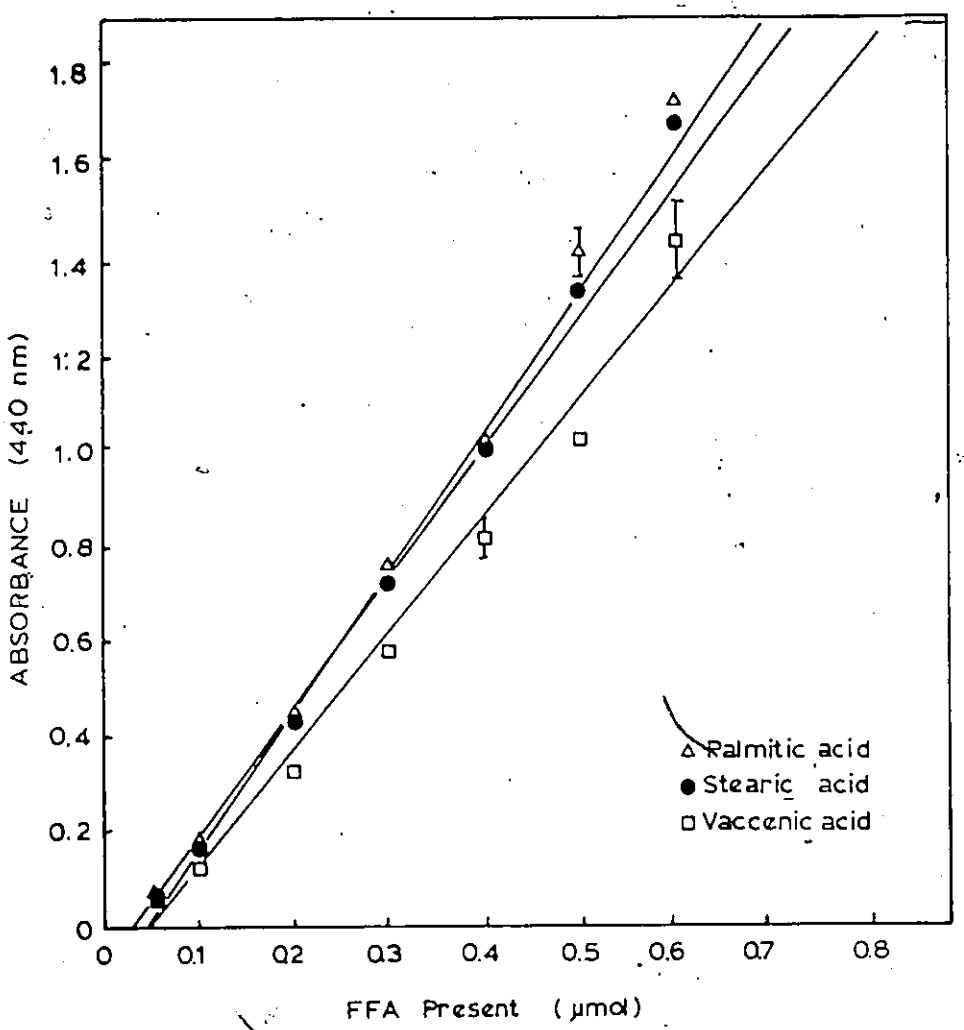
3.1 Standard Curve for FFA Assay:

To determine the amount of FFA released in the lipase assays, a standard curve was constructed using three long-chain fatty acids: palmitic (16:0), stearic (18:0), and vaccenic (18:1,trans) (Figure 5). Determinations were performed in duplicate, and the resulting lines were similar, with the calculated parameters for linear regression being very close. The line for stearic acid was arbitrarily chosen as the one from which to make further calculations. The units of lipase activity are expressed as nmoles FFA released/mg oat flour/minute.

3.2 Development of MUH Assay:

Although cleavage of MUH has been used as an indicator of lipolytic activity in a number of systems (Guilbault and Kramer 1964, Jacks and Kircher 1967, Guilbault et al.1968, Guilbault and Sadar 1969, Heltved 1984, Saunders and Heltved 1985, Galliard 1986), the method has not yet been applied to oats. A review of the literature reveals several conventional assays which have been used to measure oat lipase (see Literature Review). Without exception, these methods are time consuming, and sometimes complicated to perform. With this in mind, the simple, rapid method used by Saunders and Heltved (1985) to detect lipolytic activity in rice bran by monitoring cleavage of MUH has been developed in this thesis for measuring the same activity in oats.

Figure 5. Determination of free fatty acids (FFA) using diethyldithiocarbamate (DDC) as a colour reagent to detect copper soaps of FFA. The absorbance of the resulting coloured complex was read at 440 nm. Standard curves for 3 fatty acids: a)palmitic acid (△), slope=.029, intercept=-.14,r=.99; b) stearic acid (●), slope=.030, intercept=-.13, r=.99; c)vaccenic acid (□), slope=.025, intercept=-.14, r=.99. For each data point, n=3.


ABSORBANCE (440 nm)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
FFA Present (μmol)
Palmitic acid
Stearic acid
Vaccenic acid

3.2.1 Effect of pH on MU release:

Since pH is known to affect the fluorescence of MU (Jacks and Kircher 1967), a preliminary experiment to determine the optimum pH for enzyme activity was conducted before determining the standard curve. Enzyme activity as measured by release of MU increased with increasing pH from pH 7.0 to 8.0, and remained the same from pH 8.0 to 8.5 (Figure 6). Thus, pH 8.0 was chosen for all subsequent experiments, as beyond that, some hydrolysis of MUH in the buffer was observed, as well as a noticeable increase in turbidity of the sample. A similar pH curve was obtained when turbidity was eliminated by boiling and centrifuging the assay material (data not shown).

3.2.2 Standard Curve for MU:

A standard curve for detection of MU in a 2.5 mg/ml (arbitrary concentration) oat flour suspension was constructed at pH 8.0 by adding increasing amounts of MU to the suspension, and measuring the relative fluorescence intensity (RFI) that resulted (Figure 7). Each point is the average of three determinations. MU released by cleavage of MUH in the oat flour suspension was calculated using this standard curve. Enzyme activity was expressed as nmoles of MU released/mg oat flour/minute. For reproducibility, in further experiments with MU or MUH, at the start of each day's experiments, a sample containing 3.3 uM MU in an oat flour-buffer suspension was placed in the spectrofluorometer, and the fluorescence intensity adjusted to 20 units, as determined from the standard curve. In this way, inconsistencies in the light source, such as fluctuations in intensity, were eliminated.

Figure 6. Effect of pH on MUH assay. Rate of MU release as measured by increase in RFI units at 448 nm was recorded in the first 60 seconds after formation of the oat flour suspension (2.5 mg flour/ml of 0.2M Tris-HCl buffer) and addition of substrate (.67uM). For each point, n=3.

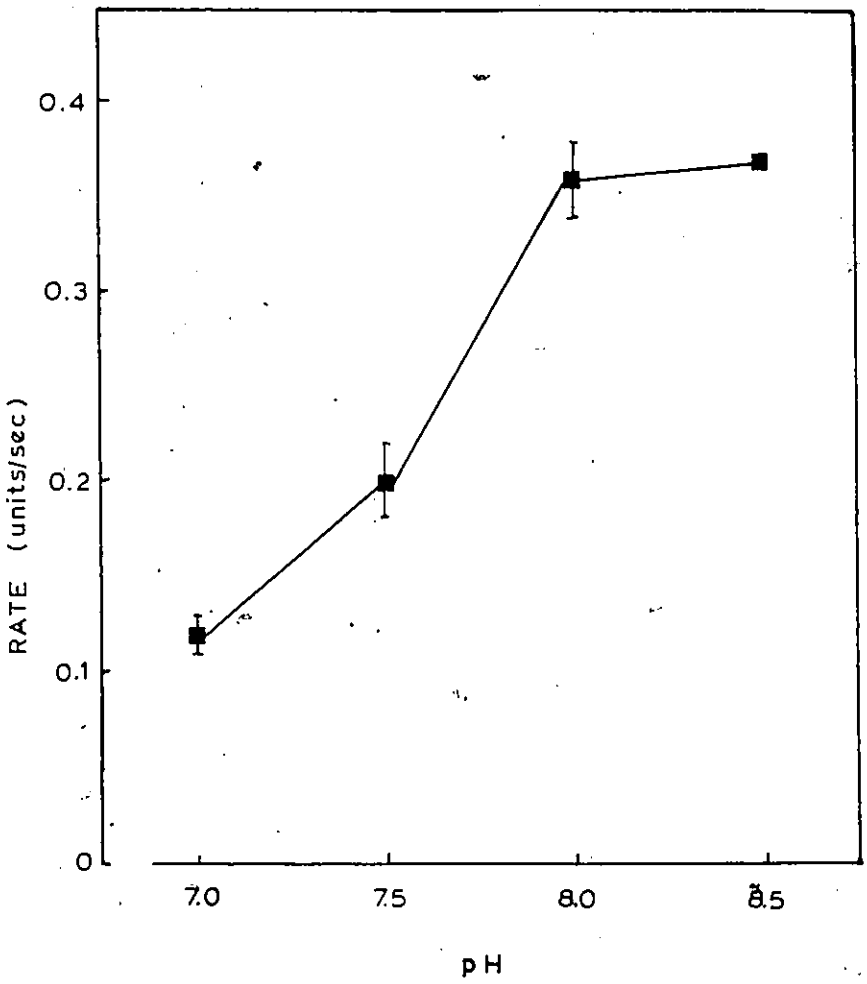
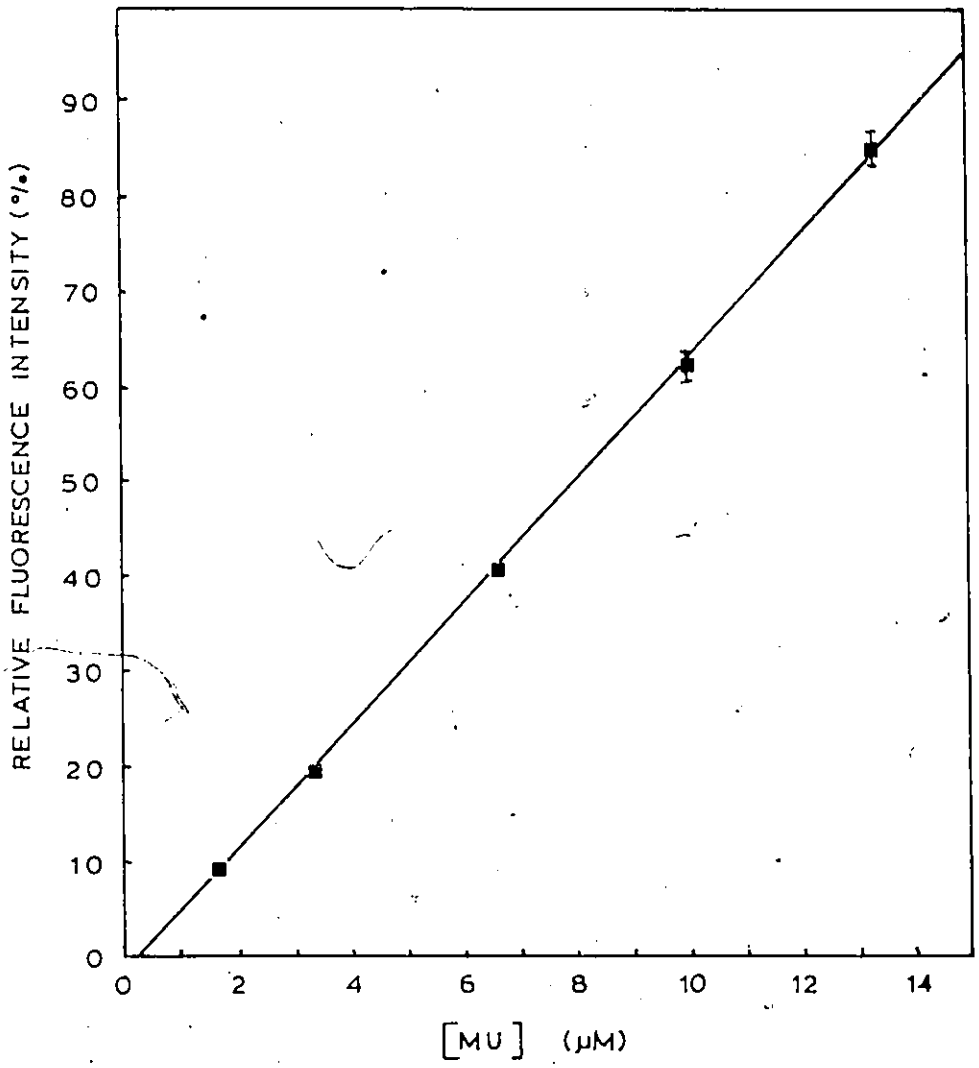

0.4
0.3
0.2
0.1
0
RATE (units/sec)
7.0
7.5
8.0
8.5
pH

Figure 7. Standard curve for detection of 4-methylumbelliferone (MU) by RFI in 0.2M Tris buffer at pH 8.0. Excitation wavelength: 397 nm. Emission wavelength: 448 nm. Fluorescence intensity was measured in an oat flour suspension (2.5 mg flour/ml buffer), as for the enzyme assay. The blank value was obtained using the oat flour suspension with no added fluorochrome. For each point, n=3.


RELATIVE FLUORESCENCE INTENSITY (%)
0
10
20
30
40
50
60
70
80
90
0
2
4
6
8
10
12
14
[MU] (μM)

3.2.3 Effect of Oat Flour Concentration on MUH Assay:

The effect of oat flour concentration on enzyme activity is shown in Figure 8. The increase in rate of enzyme activity with increasing oat flour concentration was linear (r=0.98) from 1.75 to 3.0 mg/ml oat flour. At concentrations greater than 3.0 mg/ml, the turbidity of the suspension greatly increased the probability of inaccuracies due to light scattering and fluorescence quenching. A flour concentration of 2.5 mg/ml was arbitrarily chosen from the middle of this range as a reasonable compromise between activity and turbidity.

3.2.4 Effect of Substrate Concentration on MUH Assay:

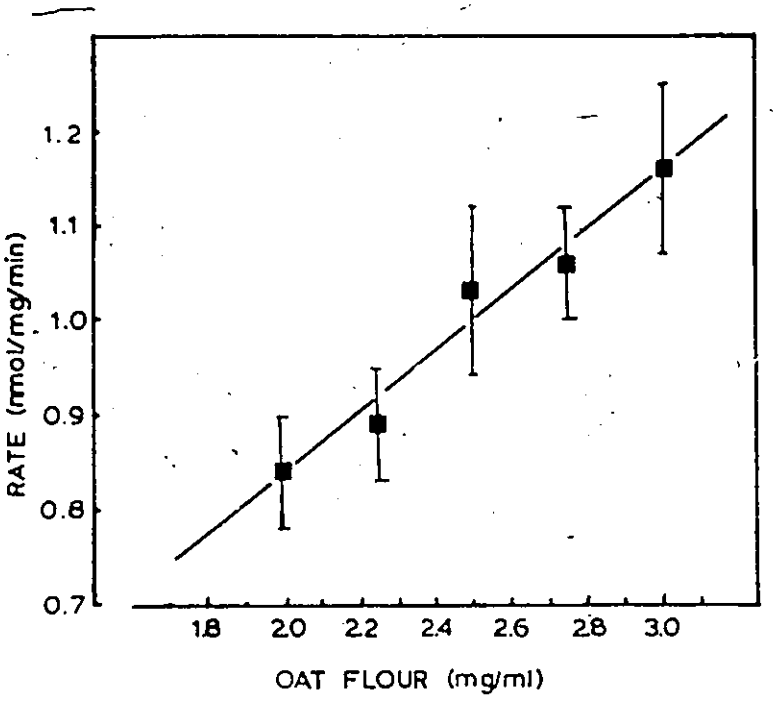
The effect of substrate concentration on enzyme activity is shown in Figure 9. The rate of MUH cleavage increased with increasing substrate concentration to a maximum at 0.67 uM, after which further increases in substrate concentration did not produce increased rates of hydrolysis. Thus, a concentration of 0.67 uM MUH was used in further experiments using natural oat flour.

3.2.5 Effect of Triton X-100 on MUH Assay:

It was thought that the detergent Triton X-100 might enhance rates of hydrolysis by solubilising the enzyme, and making it more accessible to the MUH. The effects of Triton X-100, and preincubation of oat flour in buffer, are shown in Figure 10. Both concentrations of Triton used (0.5% and 1.0%) resulted in a decreased rate of MUH cleavage by the suspension. Preincubation of the oat flour suspension also decreased rates of cleavage, both in the presence and absence of Triton X-100. The rate of MUH cleavage is at a maximum immediately after formation of

Figure 8. Effect of oat flour concentration on MUH assay. Rate was calculated by measuring the slope of the increasing fluorescence intensity produced after the addition of MUH (to 0.67 uM) to the oat flour suspension (in Tris-HCl, 0.2M, pH 8.0). For each point, n=3.

Figure 9. Effect of substrate (MUH) concentration on rate of MU release by oat flour suspensions (2.5 mg/ml in Tris-HCl buffer, 0.2M, pH 8.0). For each point, n=3.


RATE (nmol/mg/min)
1.2
1.1
1.0
0.9
0.8
0.7
1.8
2.0
2.2
2.4
2.6
2.8
3.0
OAT FLOUR (mg/ml)

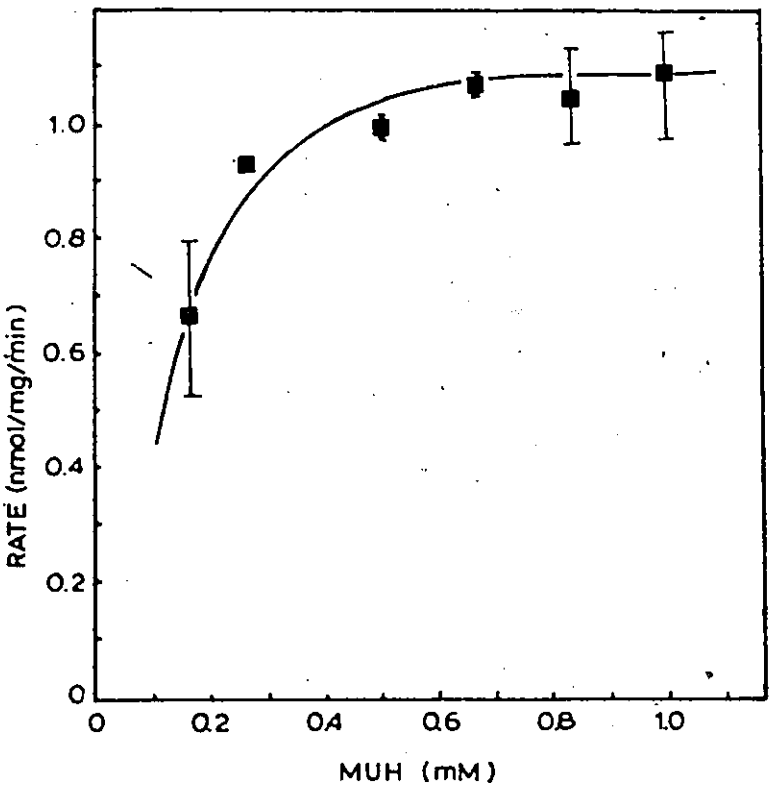
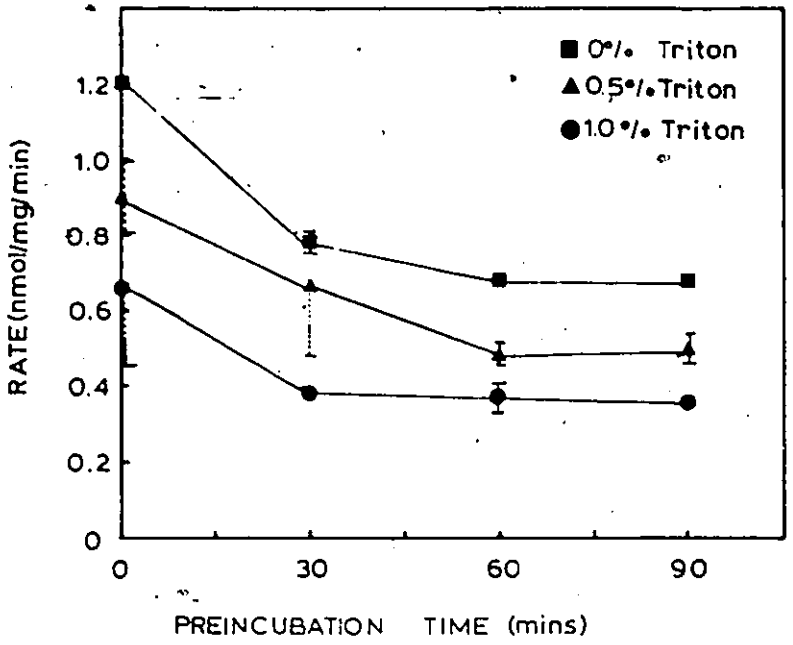
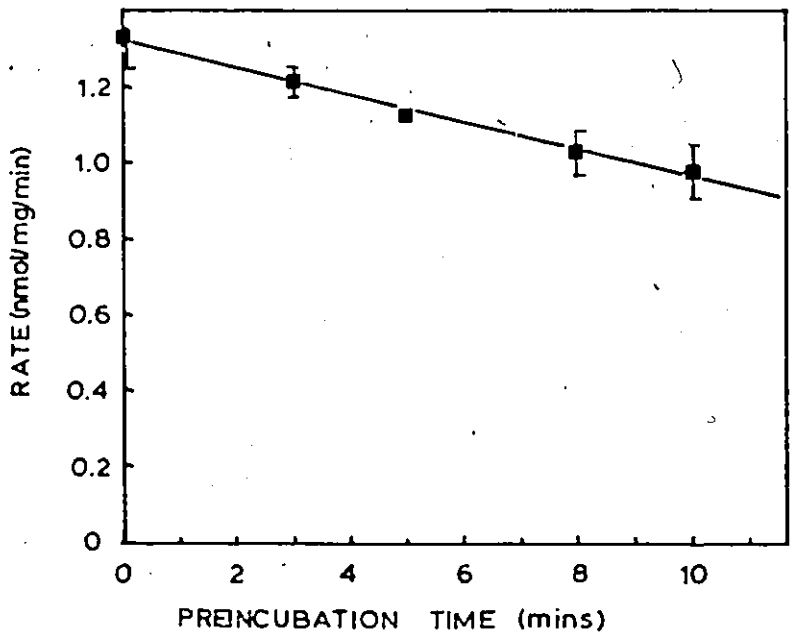

RATE (nmol/mg/min)
1.0
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1.0
MUH (mM)

Figure 10. Effect of Triton X-100, and preincubation on MU release by oat flour suspensions, using 2.5 mg/ml oat flour, and 0.67 uM MUH. 0% Triton added (■), 0.5% Triton added (▲), and 1.0% Triton added (●). For each level of Triton (0,0.5% and 1.0%), flour was suspended in buffer (Tris-HCl, 0.2M, pH 8.0) and preincubated at 23°C varying lengths of time before addition of MUH. For each point, n=3.

Figure 11. Effect of short term preincubation on MU release in the absence of Triton X-100. Oat flour was suspended in buffer (2.5mg/ml in 0.2M Tris-HCl, pH 8.0) and preincubated at 23°C for varying lengths of time before addition of MUH (.67uM). For each point, n=3.


■ 0% Triton
▲ 0.5% Triton
● 1.0% Triton
RATE (nmol/mg/min)
1.2
1.0
0.8
0.6
0.4
0.2
0
0
30
60
90
PREINCUBATION TIME (mins)


RATE (nmol/mg/min)
1.2
1.0
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
PREINCUBATION TIME (mins)

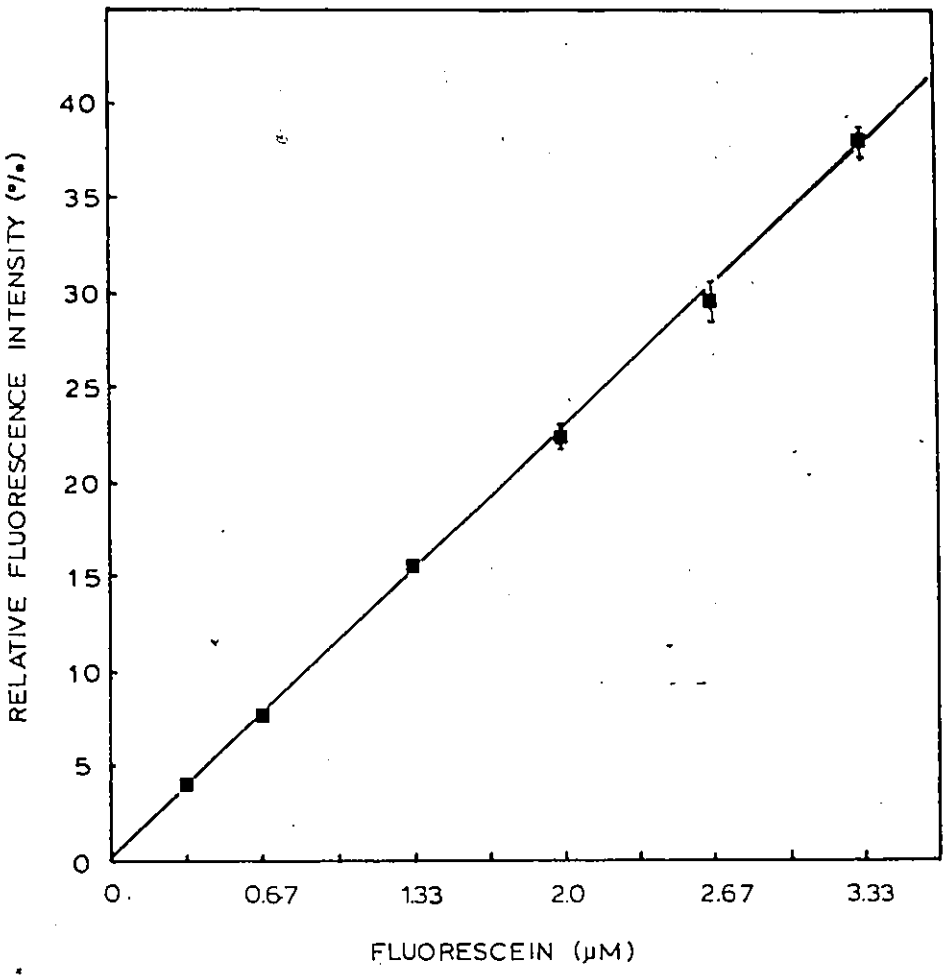
the oat flour suspension. After 30 minutes preincubation, hydrolysis rates had decreased by approximately 25%, with and without Triton. Further preincubation of the suspension did not lead to any further significant decrease in enzyme activity. A second experiment, shown in Figure 11, shows the 25% reduction in activity in the absence of Triton occurred in the first 10 minutes of preincubation. The decrease in activity appears to begin immediately after formation of the suspension.

3.2.6 Experiments using Other Fluorogenic Substrates:

Experiments were conducted to examine the suitability of other methylumbelliferyl esters, as well as fluorescein esters, as substrates for 'lipolysis'. Prior to the experiments using fluorescein esters, scans were performed to determine the optimum excitation and emission wavelengths for detection of fluorescein (data not shown). A major excitation peak was observed at 490 nm. This was, however, too close to the emission maximum at 510 nm, forming a scatter peak found as a shoulder on the emission peak. To avoid interference by this scatter peak, a secondary excitation maximum, at 323 nm was used with emission at 510 nm. For quantitation of assays using fluorescein esters, a standard curve for fluorescein was constructed by adding increasing amounts of fluorescein to an oat flour suspension, and measuring the RFI (Figure 12). Enzyme activity was expressed as nmol fluorescein released/mg oat flour/minute. Since it was assumed that all of the fluorimetric substrates were being acted on by the same enzyme(s), the substrate and flour concentrations were used as determined for the MUH assay.

The hydrolysis of several fluorometric substrates of varying chain

Figure 12. Standard curve for fluorescein. Excitation wavelength: 323 nm. Emission wavelength: 513 nm. Fluorescence intensity was measured in oat flour suspension (2.5 mg oat flour/ml of 0.2M Tris-HCl buffer, pH 8.0) as for the enzyme assay. For each point, n=3.


RELATIVE FLUORESCENCE INTENSITY (%)
0
5
10
15
20
25
30
35
40
0
0.67
1.33
2.0
2.67
3.33
FLUORESCEIN (μM)

length by oat flour suspensions is shown in Table I. MUH was considered to be the 'reference' substrate for this experiment. For the methylumbelliferyl esters, it can be seen that as the length of the fatty acyl chain increased, the rate of hydrolysis decreased. Only methylumbelliferyl butyrate (MUB) was hydrolyzed at a rate rivalling, and in fact exceeding, that of MUH. As palmitic acid is one of the major fatty acids found in oat triglyceride (Youngs 1978, Sahasrabudhe 1979), it would not have been unreasonable to expect that the methylumbelliferyl palmitate (MUP) ester would be a good substrate for hydrolysis. Lin et al.(1986) have found in a survey of several seed types that, in general, the lipase from a given species has the highest activity on triglycerides of the same fatty acid composition as their own storage triglycerides. Of the fluorescein esters examined, only the fluorescein dibutyrate (FDB) showed any activity. No hydrolysis of fluorescein dilaurate (FDL) was observed.

Further assays were performed to assess which would be the best substrate for hydrolysis. A comparison of the rates of activity achieved using MUH, MUB, and FDB in several oat cultivars is presented in Table II. MUB yielded consistently higher enzyme activities than MUH or FDB, but less variation among oat cultivars. The rates achieved with FDB were very low, and also showed little variability.

3.2.7 Activity of Porcine Pancreatic Lipase on MU Esters:

The results obtained using MUH and MUB as substrates for lipolysis in oat flour did not indicate which would be the better substrate of the two. To clarify this, the activity of a commercial porcine pancreatic lipase (PPL) preparation on 5 MU esters, including MUB and MUH, was

Table I

Initial comparison of different fluorimetric substrates using natural oat flour (substrate concentration 0.67 uM, cv Preston, n=3).

Substrate	Chain Length (acyl group)	Rate ±SD (nmol/mg/min)
MUB	C_4	1.05± .05
MUH	C_7	0.82± .05
MUO	C_8	0.13± .02
MUP	C_{16}	0.08± .05
FDB	C_4	0.09± .002
FDL	C_{12}	0

Table II

Comparison of the rates of activity achieved with the substrates MUH, MUB and FDB over several cultivars (substrate concentration 0.67 uM, n=3).

Cultivar	MUH*	MUB*	FDB*
Pierce	1.39±.03	1.40±.09	0.09±0
Lyon	0.81±0	1.32±.03	0.09± .002
Dumont	1.24±.03	1.59±.10	0.11±.01
Centennial	0.97±.05	1.37±.03	0.08±0
Dal	0.81±.03	1.34±.17	0.10± .002
Ogle	0.90±.03	1.34±.03	0.08± .002
Stout	1.09±.05	1.13±.17	0.08±0
Steele	1.12±.03	1.22±.07	0.09± .002
Marathon	0.99±.03	1.43±.17	
Webster	1.32±.03	1.21±.14	

* Rate of fluorochrome release (nmol/mg/min) ±SD

examined. The rates of MU release from the 5 MU esters by PPL are presented in Table III. The rate of cleavage of MUH was more than 6 times greater than the rate of cleavage of MUB. MUH also gave much higher rates of cleavage than MUO, MUP or MUS. MUH was thus the substrate of choice for lipolysis.

3.3 Experiments using Defatted Flour

3.3.1 Microscopic Detection of Lipid in Oats:

Samples of natural and defatted flour were examined with the fluorescence microscope using Nile Blue A. The dye produces bright yellow fluorescence in the fat deposits as reported by Hargin et al. (1980). In the natural flour preparation in Figure 13a, many fat deposits were evident. The absence of detectable fluorescence in the defatted flours was taken as an indication of thorough removal of triglycerides (data not shown). Figures 13b and 13c show the distribution of lipid in the intact kernel. The most intense fluorescence was seen in the embryo, or germ, indicating that the highest concentration of lipid was in this tissue. Slightly less fluorescence was seen in the bran, and only faint fluorescence was detectable in the endosperm, indicating the lowest concentration of lipid in that tissue.

The distribution of lipids in the oat kernels was in agreement with Hammond (1983), who reported that in the embryo axis, or germ, lipid constituted 10.6-12.6% of the dry weight of the tissue. In the bran, the lipid content was 6.4-9.5%, and in the endosperm, 5.2-6.8%. Although these distinctions could not be made quantitatively in the

Table III. Activity of porcine pancreatic lipase on methylumbelliferyl esters of varying chain length. Rates are given as nmoles of MU released/mg enzyme/minute ± SD (n=3).

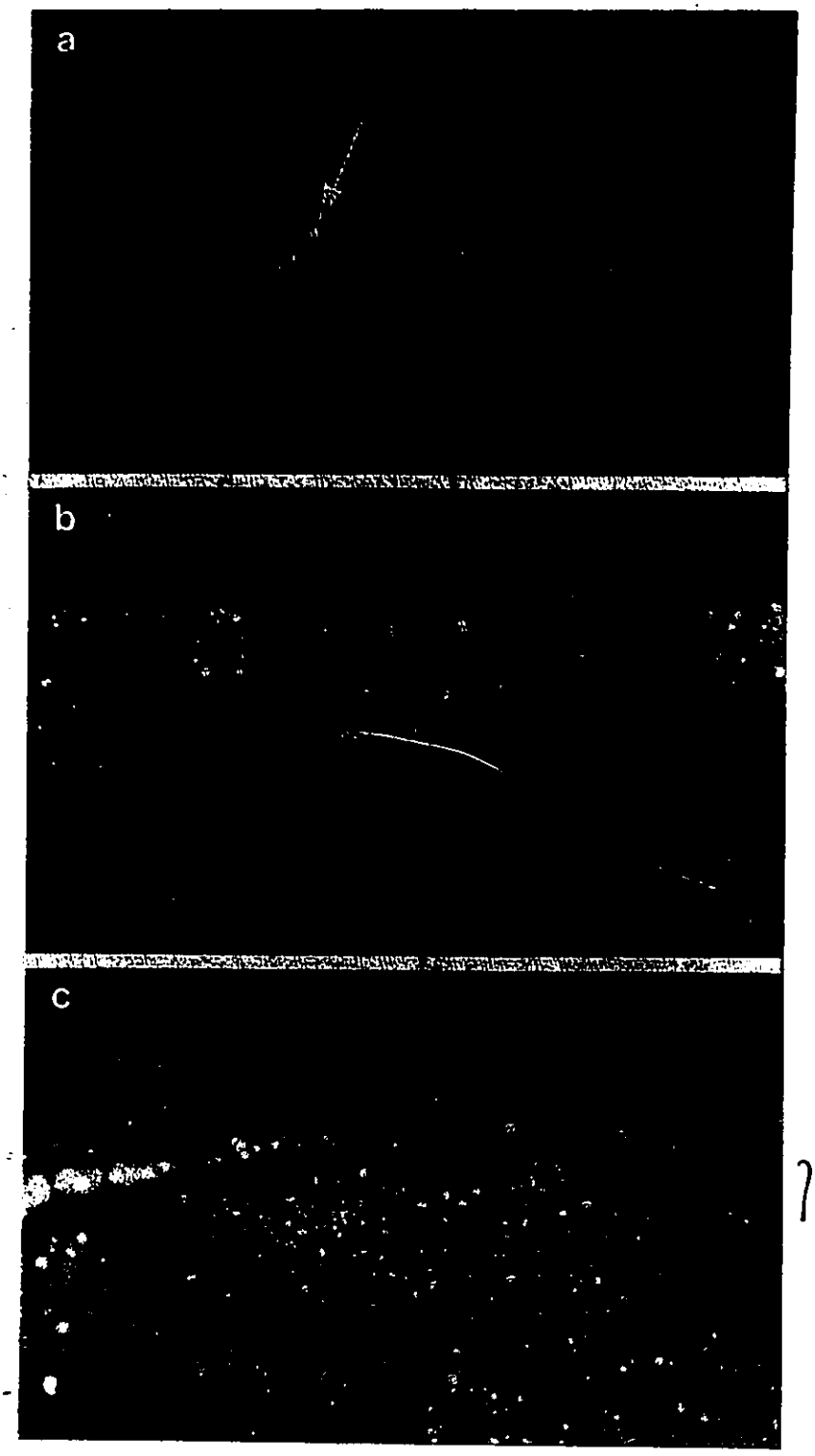
Substrate	Rate
MUB	0.98±.10
MUH	6.25±.43
MUP	1.44±.31
MUO	0.94±.09
MUS	0.37±.07

Figure 13. Fluorescence microscopic detection of lipid using the dye Nile Blue A.

a) Fluorescence micrograph of oat flour stained with Nile Blue A. Yellow fluorescence indicates presence of lipid. In some areas, discrete oil droplets can be seen (arrow).

b) Fluorescence micrograph of a plastic section of oat stained with Nile Blue A. Bright yellow fluorescence shows lipid deposits in the bran (B). Fainter yellow fluorescence shows lipid distribution in the endosperm (E).

c) Fluorescence micrograph of a plastic section of oat stained with Nile Blue A. Yellow fluorescence shows lipid deposits in the germ (G), or embryo.


a
b
c

fluorescence micrographs, the differing fluorescence intensities in the various tissues certainly reflect the distributions reported by Hammond, as determined by separation of the tissues, extraction of lipids in diethyl ether, and quantitation of the extracted lipids.

3.3.2 Parameters for FFA and MUH Assays in Defatted Flours:

For assay of FFA release in defatted flours, emulsions of olive oil and Tris buffer (as in section 2.3.1) corresponding to increasing concentrations of triolein were prepared using the Kinematica Homogenizer. As shown in Figure 14, the optimum substrate concentration was determined to be 450 mM triolein. At this concentration, the optimum sample quantity of defatted flour was found to be 50 mg of oat flour per assay, both for 15 and 30 minutes of incubation (Figure 15).

For the MUH assay of defatted flour, 2.5 mg/ml of flour was used, as in section 3.2.3. Variation in the rate of MU release with substrate concentration showed a different profile (Figure 16) than that seen in natural flour (Figure 8), with a maximum at 1.0 mM MUH. On the basis of these results, 1.0 mM MUH was used in assays of defatted flour.

3.3.3 FFA and MU Release in Defatted Flours:

Samples of six oat cultivars were defatted and assayed using added substrate for the purpose of determining maximum potential rates of lipolysis when substrate was not limiting. A summary of the activities measured using both the FFA and MUH assays is presented in Table IV, with activities in natural (or whole) flour using only endogenous substrate included for comparison.

When the defatted oat flours were assayed for FFA release with

Figure 14. Determination of optimum substrate concentration for FFA release using 50 mg defatted flour per sample. Olive oil was used as the source of triolein, with concentrations of triolein calculated on the basis of 80% triolein in olive oil. Copper soaps were detected colourimetrically using DDC as in Figure 1. Samples were incubated at 37°C in 0.5M Tris-HCl buffer (pH 7.4, with 1% Triton X-100) for 15(●) or 30(■)minutes before inactivation, and assay of FFA. For each point, n=4.

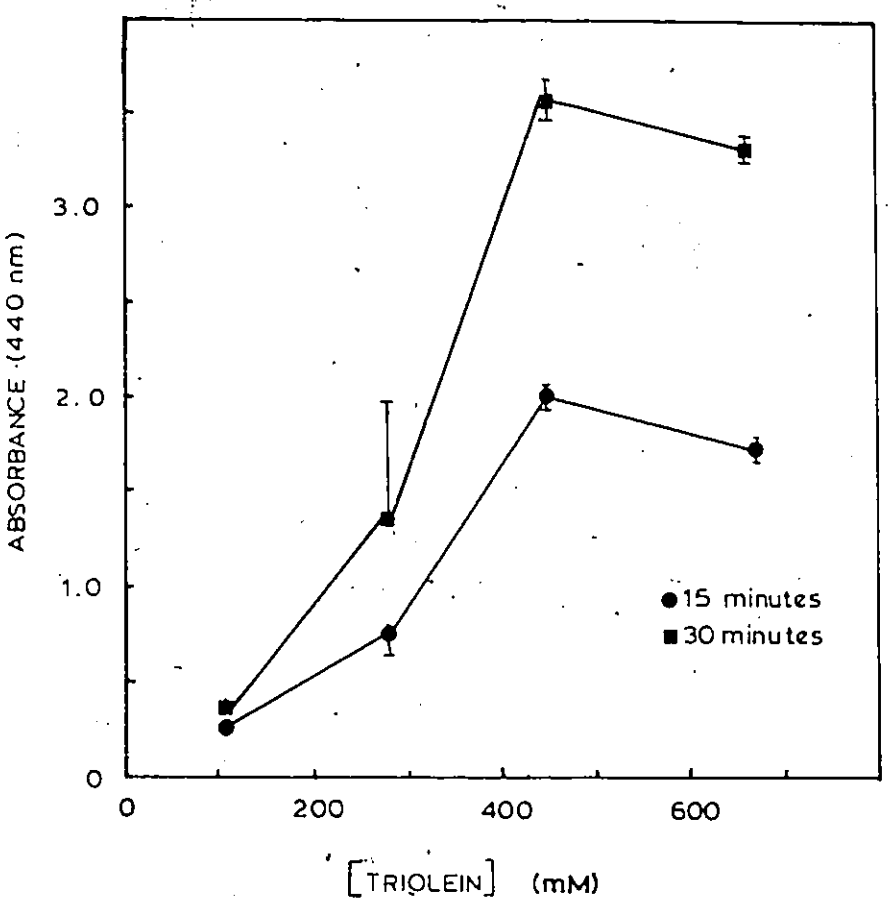

ABSORBANCE (440 nm)
3.0
2.0
1.0
0
0
200
400
600
[TRIOLEIN] (mM)
15 minutes
30 minutes

Figure 15. Determination of optimum amount of defatted flour for FFA release. Samples were incubated at 37°C, with 450 mM triolein in 0.05M Tris-HCl buffer (pH 7.4, with 1% Triton X-100) for 15(●) and 30(■) minutes before inactivation. Copper soaps of FFA were detected colourimetrically using DDC, and measuring absorbance at 440 nm. For each point, n=4.

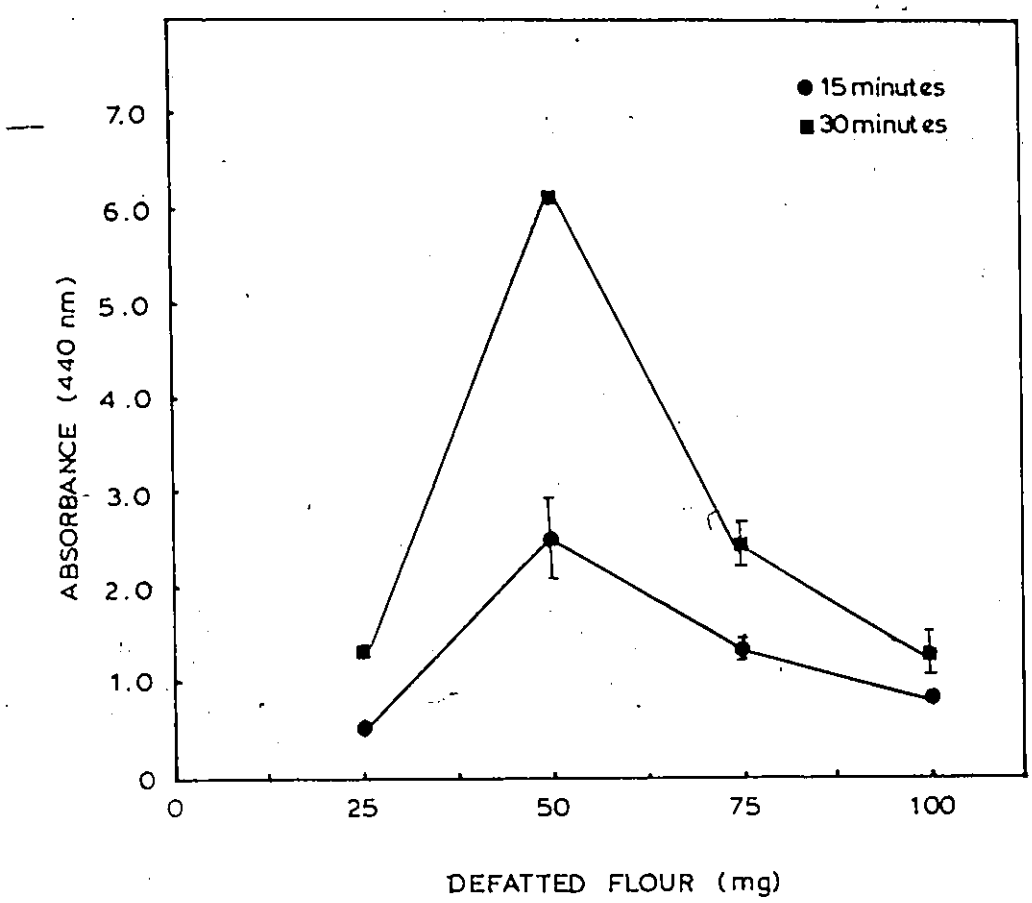

15 minutes
30 minutes
ABSORBANCE (440 nm)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
0
25
50
75
100
DEFATTED FLOUR (mg)

Figure 16. Effect of substrate concentration on MUH assay of defatted flour. 2.5 mg/ml of defatted flour in 0.2M Tris-HCl buffer, pH 8.0, were used per assay. For each point, n=3.

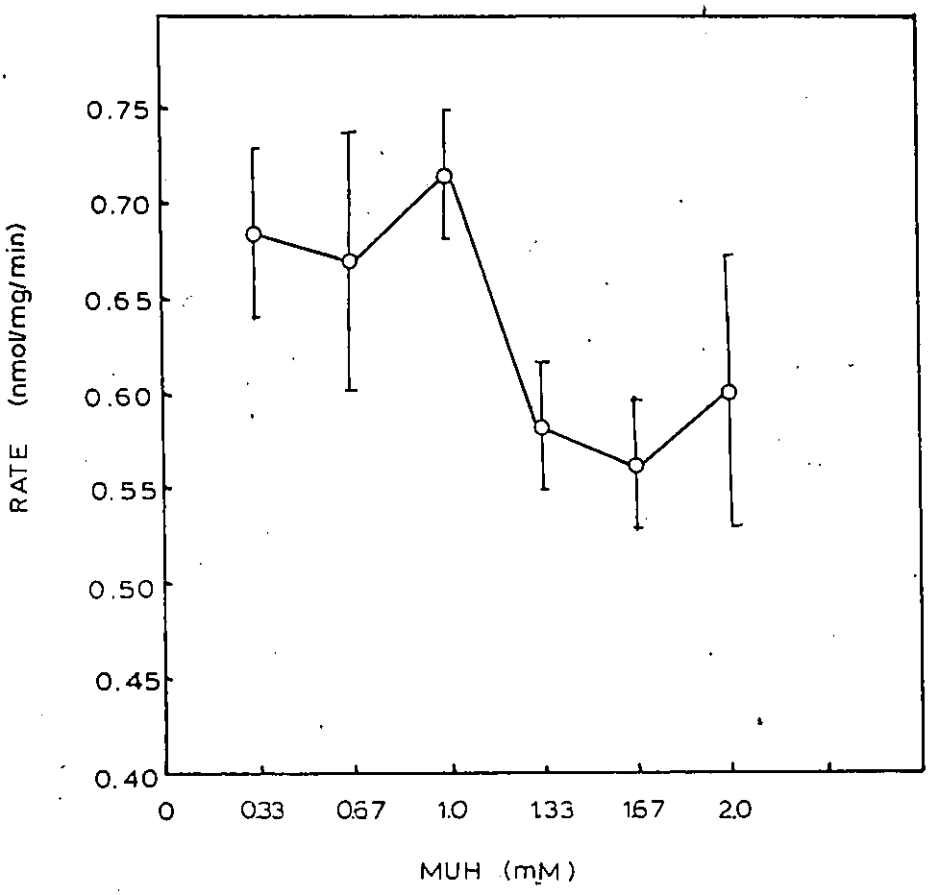

RATE (nmol/mg/min)
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0
0.33
0.67
1.0
1.33
1.67
2.0
MUH (mM)

Table IV.

Comparison of MUH (substrate concentration 1 mM, n=3) and FFA (with added substrate, 450 mM triolein, n=4) assays using defatted flours and natural flours. Values shown represent rates of release of FFA or MU (nmol/mg flour/min) ± SD.

CULTIVAR	FFA_{15}		FFA_{30}		MUH	
	defatted	natural	defatted	natural	defatted	natural
Clintland	9.55±.88	1.98±.14	10.37±.10	1.57±.02	0.98±.21	1.48±.05
Marathon	6.79±.44	1.61±.44	6.31±.96	1.40±.04	0.92±.17	1.45±.03
Froker	12.74±.02	1.78±.12	9.71±.82	1.36±.02	0.89±.21	1.65±.03
Steele	15.22±.50	2.20±.16	11.05±.32	2.15±.08	1.01±.26	1.61±.05
Webster	10.56±.66	2.40±.06	12.28±.10	2.05±.14	1.13±.19	1.76±.03
Lyon	13.95±2.1	1.52±.10	11.03±2.78	1.55±.08	0.76±.19	1.22± 0

450 mM triolein added for substrate, the increases in activity over activities measured in natural flour ranged from 4-fold (in the lowest case) to 9-fold (in the highest case). The ranking of the cultivars (from highest to lowest rates of lipolysis) also changed. When the rates of lipase activity in natural flours (using endogenous substrate) and defatted flours (using added substrate) were compared at 15 minutes of incubation, a correlation coefficient of 0.15 was obtained. A similar comparison at 30 minutes of incubation yielded a correlation coefficient of 0.66. These correlations suggested that although the maximum potential rate was considerably higher than that achieved with endogenous substrate for both incubation times, an assay of natural flour at 30 minutes incubation using only endogenous substrate would provide a more realistic prediction of the maximum potential rate.

When MUH cleavage was assayed in the defatted flour a different trend was observed. A comparison of the activities obtained with defatted oat flours with those obtained using natural flours shows a decrease in activity ranging from 35-45% in the defatted flours. The decrease in rates of MUH cleavage in defatted flours, when compared with the large increase in rates of FFA release, suggested that the two assays were, in fact, measuring 2 different enzyme activities.

3.4 Experiments using Inhibitor:

Because the experiments with defatted oat flours suggested that the FFA and MUH assays were measuring different hydrolytic activities, it was decided to conduct further studies using the esterase inhibitor di-isopropylfluorophosphate (DFP) to further characterize the two activities.

Initial experiments with DFP using cvs Lyon and Pierce showed only a slight inhibition of activity (10-15%) as measured by the FFA assay, using natural flours (Fig. 17a). In a similar determination using defatted flour, no inhibition was observed in the presence of DFP (data not shown). In the fluorometric assay, however, MUH cleavage was inhibited from 65%-80% in natural flours (Figure 17b). Similar inhibition was seen in defatted flours as well (data not shown).

Because of the significant inhibition of MUH cleavage observed in the presence of DFP, further studies were conducted to examine the effect of DFP on the MUH assay over a range of eighteen cultivars. Because of the alkali-lability of DFP (Saunders and Stacey 1948), MUH assays were performed at both pH 8.0 and pH 7.4 (Table V). In the absence of DFP, the rates of MUH cleavage were 45-50% lower at pH 7.4 than rates at pH 8.0 over the range of eighteen cultivars. At both pH's, major inhibition of MUH cleavage (50-80%) was found in the presence of DFP. These results indicate that the MUH assay is actually measuring primarily esterase activity, rather than true lipase activity. However, because of the ease and rapidity of the MUH assay, it was decided to ascertain whether the MUH assay could be used as an indicator of lipase activity under some conditions, when the more time-consuming FFA assay would be inconvenient.

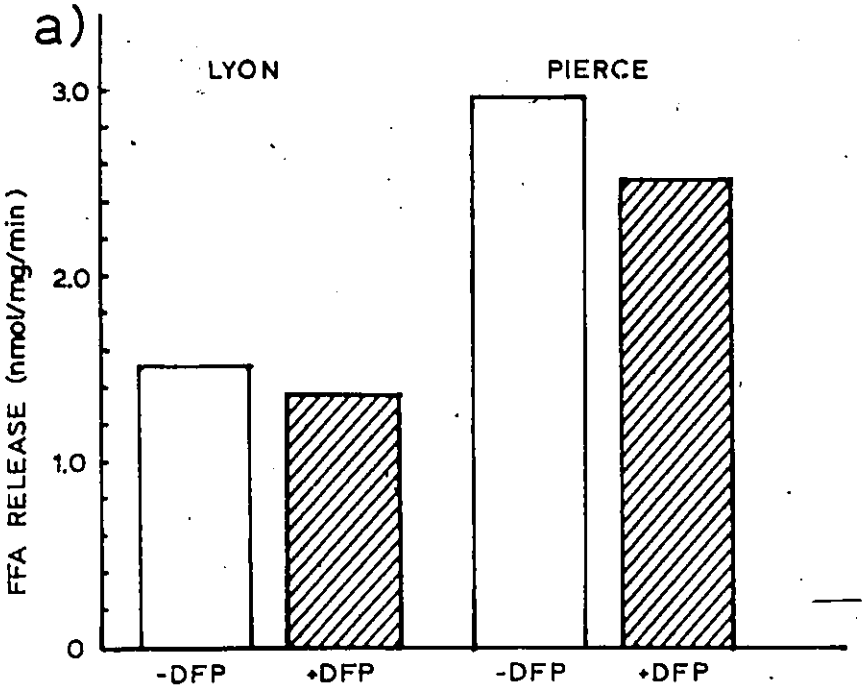
3.5 Inactivation of Oat Hydrolases:

Inactivation experiments were conducted to examine the behavior of lipase during industrial processing. In preliminary inactivation experiments, the MUH assay was used to establish approximate times of

Figure 17. Effect of the inhibitor DFP (2.0 mg/ml) on enzyme activity in oats (cvs Lyon and Pierce).

a) FFA assay. In Lyon, the activity decreased by 10%, and in Pierce, the activity dropped by 15% with addition of DFP. n=4.

b) MUH assay. In Lyon, a 65% drop in activity was observed, and in Pierce, activity decreased by 81% with addition of DFP. n=3.


a)
LYON
PIERCE
FFA RELEASE (nmol/mg/min)
3.0
2.0
1.0
0
-DFP
+DFP
-DFP
+DFP

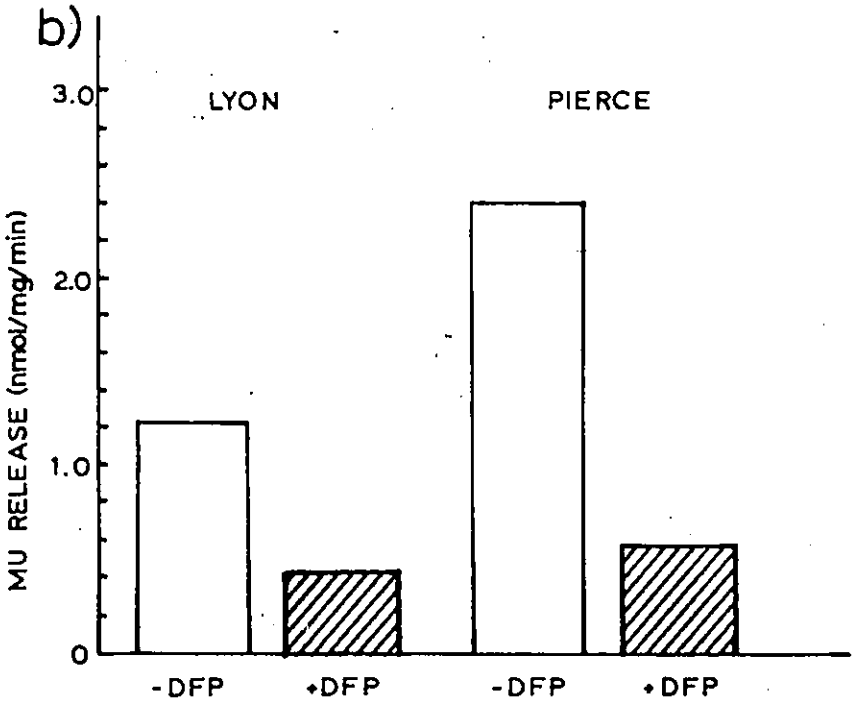

b)
LYON
PIERCE
MU RELEASE (nmol/mg/min)
3.0
2.0
1.0
0
-DFP
+DFP
-DFP
+DFP

Table V.

Effect of DFP on MUH activity at pH 8.0 and pH 7.4 over a range of 18 cultivars (MUH and MUH+DFP (2mg/ml) represent rate (nmol/mg/min) ±SD, substrate concentration 0.67 uM, n=2)

	pH 8.0			pH 7.4		
CULTIVAR	MUH	MUH+DFP	%Drop	MUH	MUH+DFP	%Drop
Dal	1.18±.03	0.38±.01	68%	0.60±.03	0.21± 0	65%
Kelly	1.91±.08	0.71±.01	63%	0.86± 0	0.36±.01	58%
Lyon	1.22± 0	0.43± 0	65%	0.75±.07	0.23±.01	70%
Ogle	1.33±.04	0.56±.01	58%	0.71± 0	0.32±.06	55%
Centennial	1.41±.06	0.29+.01	79%	0.70± 0	0.19± 0	73%
Stout	1.59±.07	0.32±.01	80%	0.67±.01	0.20±.01	70%
Steele	1.61±.04	0.55±.01	66%	0.70± 0	0.19±.03	60%
Webster	1.76±.03	0.81±.06	54%	0.87± 0	0.41±.01	52%
Pierce	1.99±.04	0.38± 0	81%	0.86±.03	0.24± 0	73%
Wright	1.44±.01	0.33±.03	77%	0.61± 0	0.24±.07	60%
Dumont	1.62±.01	0.86±.01	47%	0.77±.01	0.48± 0	38%
Clintland	1.48±.07	0.31±.03	79%	0.71± 0	0.19± 0	73%
Lang	1.87±.04	0.78±.01	58%	0.86±.06	0.42±.03	51%
Clintford	1.50±.03	0.32±.06	79%	0.73±.03	0.17± 0	77%
Froker	1.65±.03	0.31± 0	81%	0.74±.01	0.18±.01	76%
Preston	0.94±.01	0.32±.06	66%	0.69±.01	0.17± 0	76%
Marathon	1.31±.03	0.39±.01	73%	0.73±.01	0.18±.01	75%
Lodi	1.43±.04	0.41±.01	72%	0.75±.01	0.24± 0	69%

inactivation at different temperatures (Fig. 18). The initial experiment, at 97°C, using 2.5 mg/ml of flour, showed complete inactivation after 4 minutes. In subsequent inactivation experiments, flour concentration was increased to 3.0 mg/ml for both assays, due to the increased moisture content of the flour (20%). At 80°C, the rate dropped to a very low level after 5 minutes, and was essentially zero after 10 minutes. At 56°C, the drop in activity was much more gradual, with significant activity remaining after 50 minutes incubation at this temperature.

Further inactivation studies were carried out with both the FFA and the MUH assays being performed in parallel on each sample. Figure 19 shows the heat inactivation of hydrolases in Lamar oats at 95°C. The curves for inactivation of both enzyme activities were very close, with approximately 75% inactivation achieved in the first three minutes of heating. When these results were analyzed using Duncan's Multiple Range Test ($P<.05$) there was no significant difference in MU release from 3 minutes to 10 minutes. FFA release did not change significantly between 5 and 10 minutes. A comparison of the two assays during inactivation at this temperature gives a correlation coefficient of 0.86.

The inactivation of Lamar oats at 72°C is shown in Figure 20. The inactivation curves as determined using both the FFA and MUH assays were again very similar. At this lower temperature, both enzyme activities underwent an initial increase, or activation, in the first minute of heating. Activity dropped rapidly as heating continued, and analysis of the data using Duncan's Multiple Range Test ($P<.05$) showed no significant change in activity as measured by the FFA assay after 5 minutes, and no significant change in the MUH assay after 10 minutes of

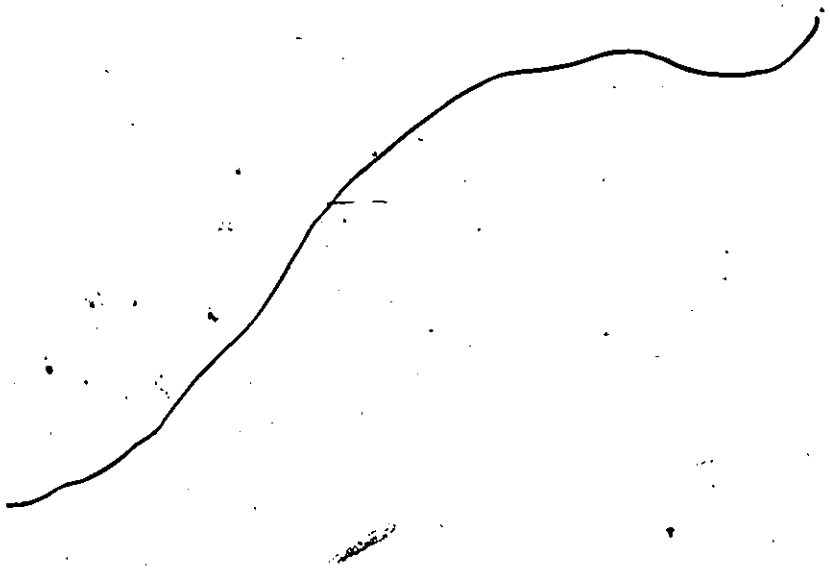


Figure 18. Effect of heat on MU release with time (cv Lamar, 20% moisture). 2.0 gram aliquots of groats were held in sealed vials for varying times at 97°C(Δ), 80°C(O), and 56°C(□), and fast frozen in crushed dry ice before grinding and assay in 0.2M Tris-HCl buffer, pH 8.0. MUH concentration was 0.67uM. At 97°C, 2.5 mg/ml oat flour was used, and at 80° and 56°C, 3.0 mg/ml oat flour was used. (note separate axis for 97°C inactivation). For each point, n=3.

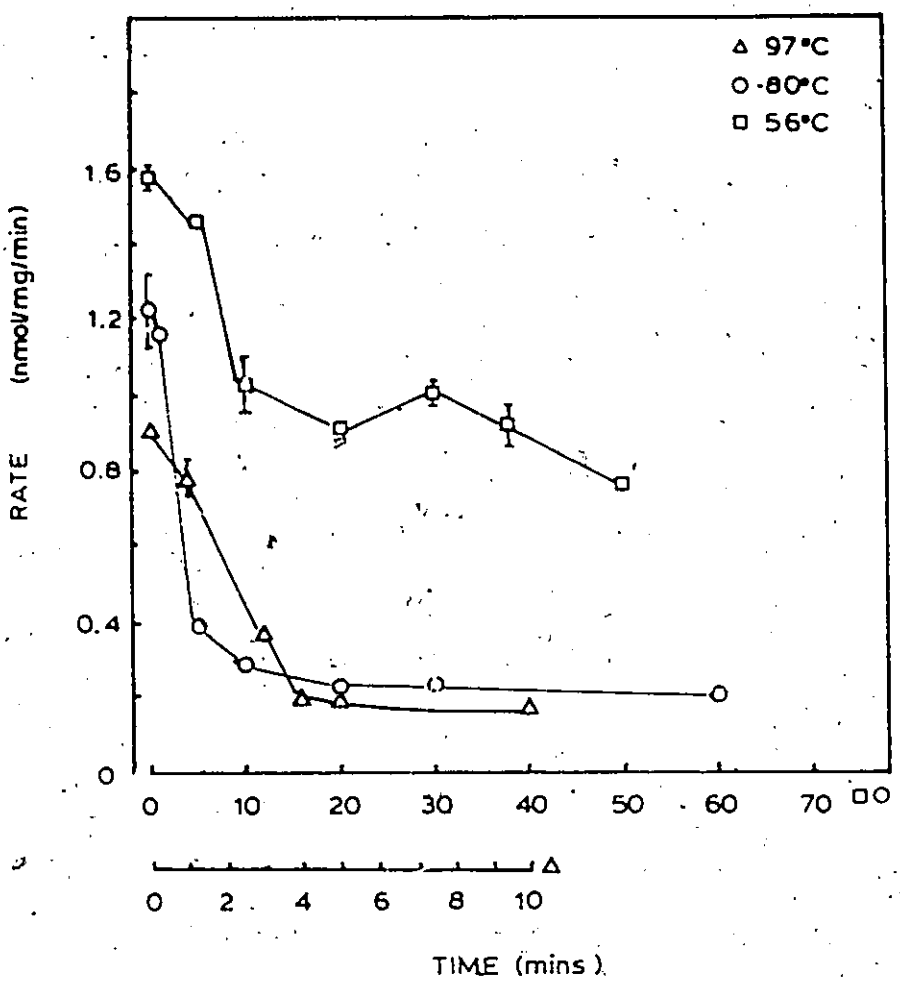
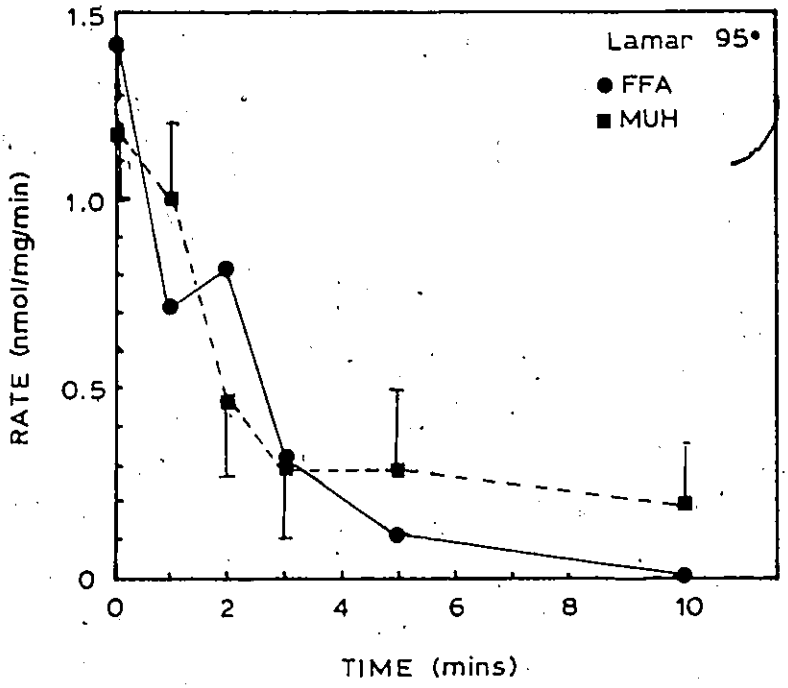
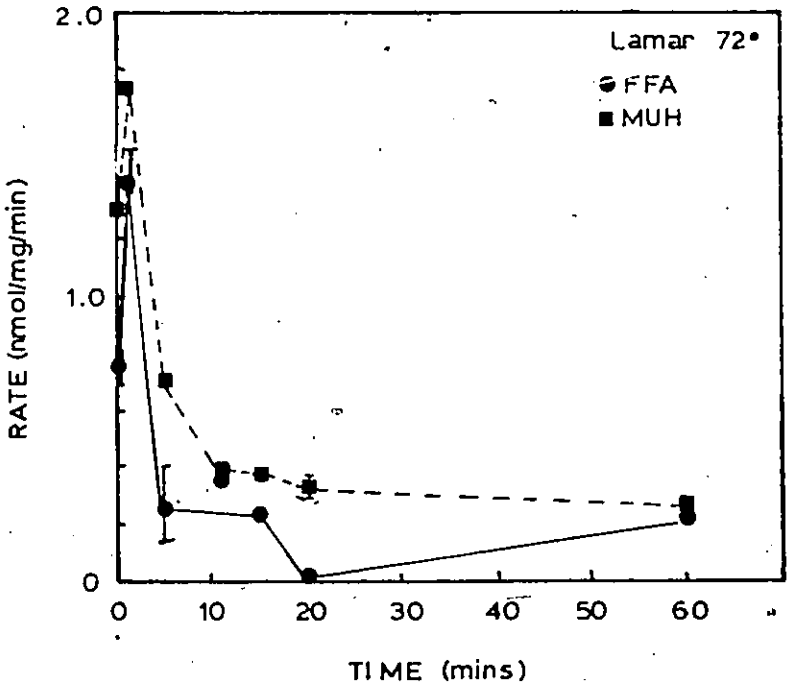

△ 97°C
○ -80°C
□ 56°C
RATE (nmol/mg/min)
1.6
1.2
0.8
0.4
0
0 10 20 30 40 50 60 70 □○
0 2 4 6 8 10 △
TIME (mins)

Figure 19. Inactivation of Lamar oats at 95°C, measured with FFA assay using 60 mg oat flour/assay (-●-)(n=4), and MUH assay using 3.0 mg/ml oat flour, and 0.67 uM MUH(--■--)(n=3).

Figure 20. Inactivation of Lamar oats at 72°C, measured with FFA assay (-●-)(n=4), and MUH assay (n=3)(--■--).
Conditions as in Figure 19.


Lamar 95°
FFA
MUH
RATE (nmol/mg/min)
1.5
1.0
0.5
0
0
2
4
6
8
10
TIME (mins)


Lamar 72°
FFA
MUH
RATE (nmol/mg/min)
2.0
1.0
0
0
10
20
30
40
50
60
TIME (mins)

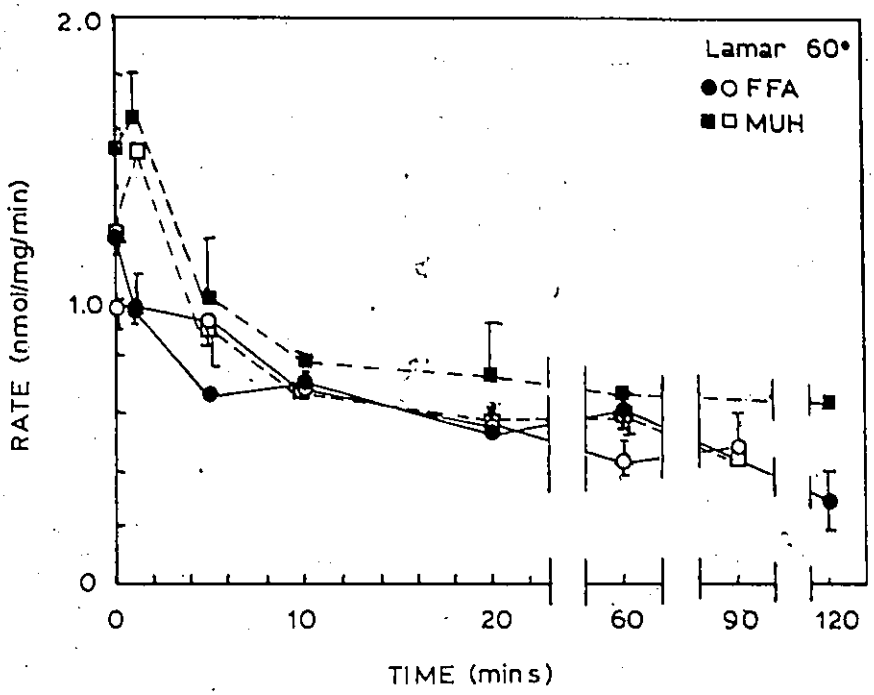
heating. Correlation between the two assays was quite significant, with a coefficient of 0.94 being obtained.

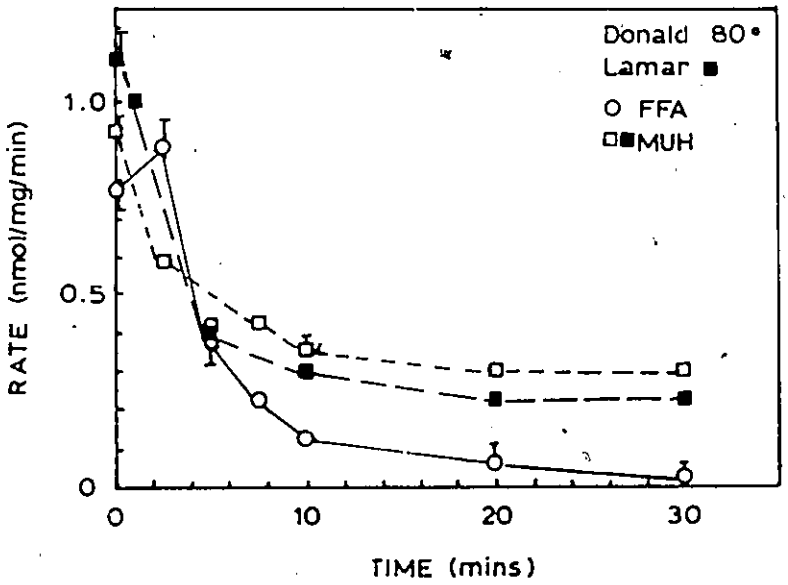
At 60°C (Figure 21), the inactivation curves of the hydrolases as measured by both the FFA and MUH assays were again very similar. Two separate inactivation experiments at 60°C were performed. In both experiments, cleavage of MUH showed a small initial rise before decreasing on continued heating. Lipase activity as measured by FFA release did not show an initial activation at 60°C. For both assays, data analysis using Duncan's Multiple Range Test ($P<.05$) showed, for the most part, no significant changes in activity after 10 minutes of heating. Activity as measured by both assays stabilized at a fairly high level, indicating that even after 2 hours of heating at 60°C, complete inactivation of the enzymes was not achieved. When the two assays were compared in both experiments, correlation coefficients of 0.87 and 0.89 were obtained.

To ensure that the close correlations obtained between the MUH and FFA assays were not restricted to one cultivar, a different cultivar, Donald, was tested for inactivation at 80°C (Figure 22). The values for the assay of MU cleavage in Lamar at the same temperature were included for comparison. At this temperature, a slight initial activation of FFA release was seen. No increase in MU release was observed in either cultivar. Analysis of the data using Duncan's Multiple Range Test ($P<.05$) showed that no significant change in MUH cleavage occurred after 5 minutes at 80°C for both Donald and Lamar. No significant change in FFA release occurred in Donald after 10 minutes. At this temperature, rates of FFA release dropped to a much lower level (essentially zero) than the rate of MU release, which stabilized at a somewhat higher level

Figure 21. Inactivation of Lamar oats at 60°C, measured with FFA assay (-●-,-○-)(n=4) and MUH assay (--■--, --□--)(n=3). Closed circles and squares refer to experiment 1, and open circles and squares refer to experiment 2. Experiments 1 and 2 refer to the same experiment repeated on different days. Conditions as in Figure 19.

Figure 22. Inactivation of Donald oats at 80°C, measured with FFA assay(-○-)(n=4), and MUH assay (n=3) (--□--). MUH assay of Lamar at the same temperature is shown for comparison (--■--)(n=3). Conditions as in Figure 19.


Lamar 60°
●○ FFA
■□ MUH
RATE (nmol/mg/min)
2.0
1.0
0
0
10
20
60
90
120
TIME (mins)


Donald 80°
Lamar ■
○ FFA
□■MUH
RATE (nmol/mg/min)
1.0
0.5
0
0
10
20
30
TIME (mins)

for both Donald and Lamar. In a comparison of the FFA and MUH assays of Donald oats at this temperature, a correlation coefficient of 0.85 was obtained.

3.6 Germination experiments:

For the germination experiments, assays were performed on each sample using both the FFA and the MUH methods. In the first germination experiment, groats were incubated at both 12°C and 20°C for 4 days. The lower temperature, 12°C, more closely approximates the temperature found under field conditions. At 20°C, germination was much more rapid, and consequently increases in hydrolysis rates were more rapid as well. Oats germinated at 12°C and 20°C for varying lengths of time are shown in Figure 23.

At 20°C, there was a consistent increase in both enzyme activities with time (data not shown). These samples were very difficult to grind and assay due to their extremely high level of hydration. The oats germinated at 12°C were freeze-dried before assay, and because of the expected depletion of triglyceride reserves during germination, assayed using the olive oil-buffer emulsion (giving 450 mM triolein) described previously. At 12°C, the increase in rate of FFA release was very slow, with only a small increase over the initial value after 97 hours (Fig 24). Analysis of the data using Duncan's Multiple Range Test ($P<.01$) indicated that the increase in FFA release did not reach significance, even after 97 hours of germination. Rates of MUH cleavage started to increase significantly after 48 hours.

The surface sterilization procedure, performed to prevent fungal growth (and hence inadvertant assay of fungal lipases), appeared to have

Figure 23 Photographs showing stages in the germination of Tibor oats with time at 12° and 20°C. Oats were germinated crease down on wet filter paper in the dark.

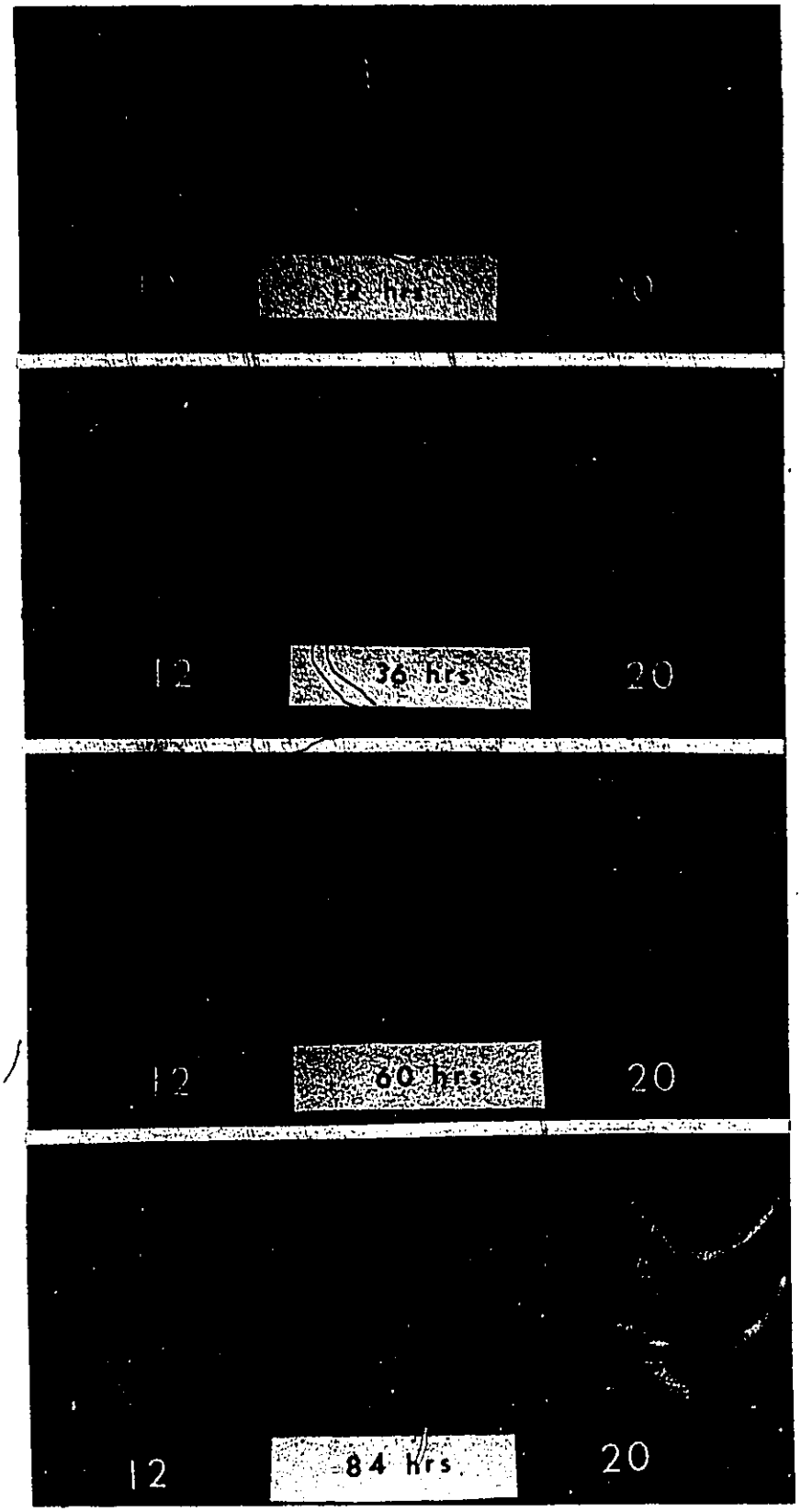
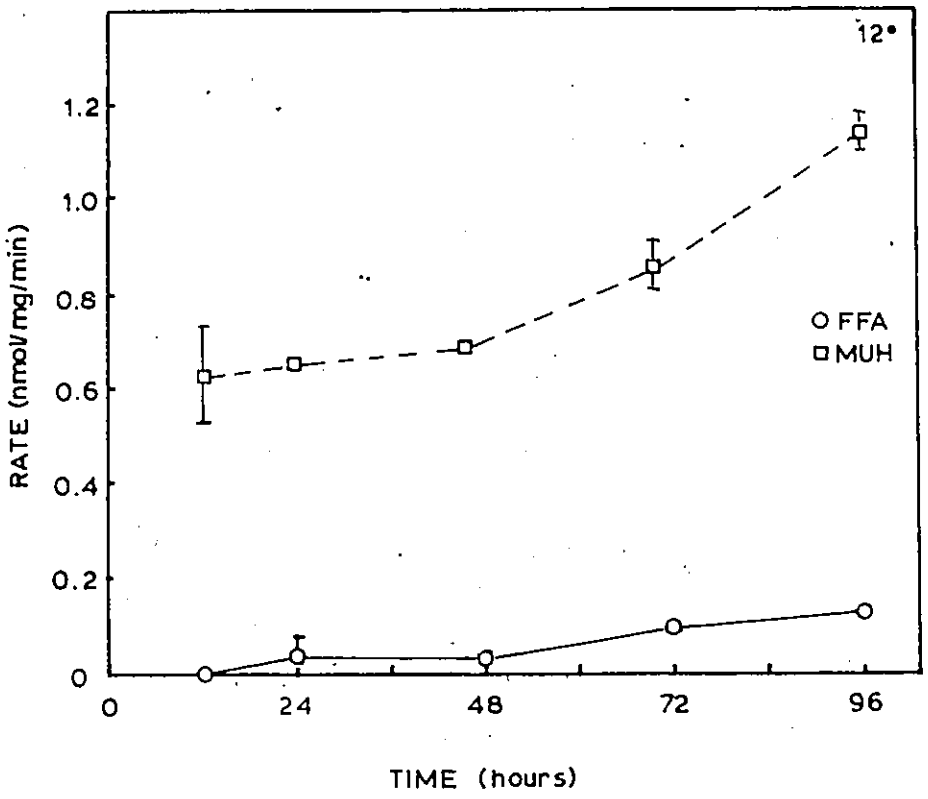

12
32 hrs
20
12
36 hrs
20
12
60 hrs
20
12
84 hrs
20

Figure 24. Germination of Tibor oats at 12°C. Oats were germinated crease down on wet filter paper in the dark. At the end of the germination time, samples were freeze-dried, and enzyme activity measured using the FFA assay (with 450 mM triolein in 0.05M Tris-HCl buffer, pH 7.4, with 1% Triton X-100, 50 mg flour/assay, n=4), and the MUH assay (0.67 uM MUH in 0.2M Tris-HCl, pH 8.0, 2.5 mg/ml oat flour, n=3).


12°
1.2
1.0
0.8
0.6
0.4
0.2
0
RATE (nmol/mg/min)
O FFA
□ MUH
0
24
48
72
96
TIME (hours)

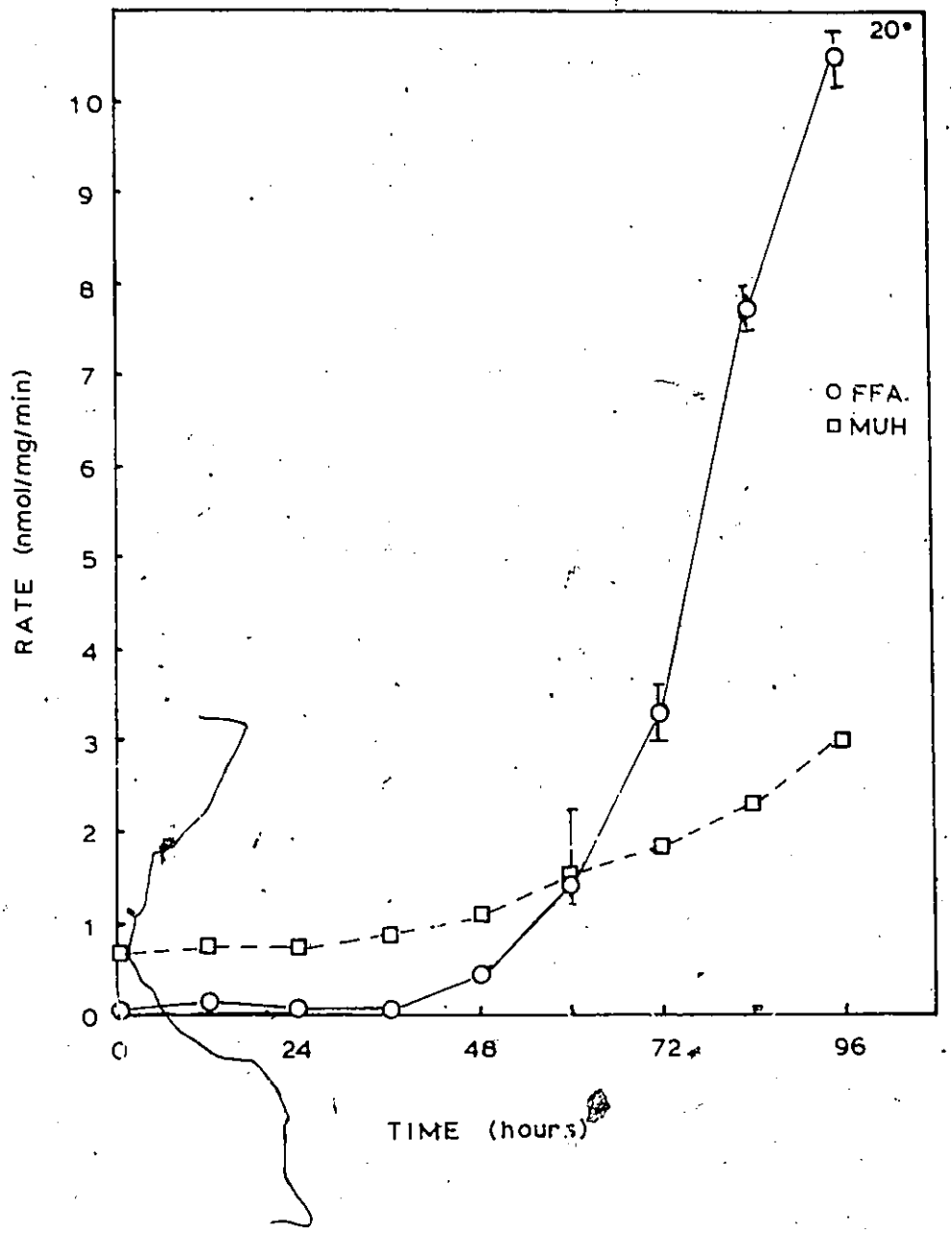
completely inactivated the lipase initially present in the oat kernels. MUH cleavage, or esterase activity, appeared relatively unaffected by the surface sterilization procedure. This suggested that esterase was located in the interior of the oat kernel, and was protected from the action of the surface sterilizing agents, while the lipase, being located in the exterior portion of the kernel (as controversially reported by Hutchinson et al. 1951, Sahasrabudhe 1982, Urquhart et al. 1983: see Literature Review) was vulnerable to the surface sterilizing agents.

Germination at 20°C was repeated, and the samples freeze-dried before assay. At 20°C (Fig.25), there was a lag period, as at 12°C, as well as a difference in time of activation between MUH cleavage and FFA release. A significant change in FFA release was detectable only after 36 hours of germination, before which lipase activity was essentially zero. MU release, which exhibited significant activity from the start of germination, increased significantly after 24 hours of germination (as analyzed by Duncan's Multiple Range Test, $P<.01$). After 48 hours, lipase activity, or FFA release, rose dramatically achieving a 100-fold increase after 96 hours of germination. The increase in MUH cleavage, or esterase activity was much more gradual, achieving only a 3-fold increase at the end of 96 hours of germination.

3.7 Cultivar Screening:

In view of the significant correlations between the FFA and the MUH assays during inactivation and germination of groats, experiments were performed to examine the possibility that the MUH assay could be used as a method for screening for lipase activity in a breeding

Figure 25. Germination of Tibor oats at 20°C.
Oats were germinated crease down on wet filter paper in the dark. Hydrolytic activity measured using FFA assay (-○-)(n=4), and MUH assay (--□--)(n=3). Conditions as in Figure 20.


20°
O FFA.
□ MUH
RATE (nmol/mg/min)
TIME (hours)
0
1
2
3
4
5
6
7
8
9
10
0
24
48
72
96

program. Twenty one cultivars were assayed using the two methods, with incubation periods of 15 and 30 minutes for the FFA assay (Table VI).

A visual inspection of Table V shows that at 15 minutes incubation, there was a 4-fold variation in lipase activity, or FFA release, between the lowest (Donald) and the highest (Pierce) cultivars. At 30 minutes incubation, the variation was slightly greater, almost 5-fold over the same range of cultivars. Variation in MUH cleavage, or esterase activity, was not as great, having only a 2-fold increase from the lowest to the highest cultivar.

Analysis of the cultivar screening data using the Student-Newman-Keuls Multiple Range Tests ($P<.05$) expands these observations. Table VII shows the differences between means for FFA release in the 21 cultivars after 15 minutes incubation. The size of the blank area under the line shows that there are a number of cultivars for which the rate of FFA release was not significantly different from each other. Table VIII shows the differences between means for FFA release after 30 minutes incubation. The smaller area below the line in Table VIII than that in Table VII indicates that, at 30 minutes incubation, more cultivars had significantly different activity than at 15 minutes incubation. For a screening program then, the 30-minute incubation would be most appropriate, as it was more sensitive to differences in rates than was the 15 minute incubation. It showed a greater range of activity, and a greater variation in activity throughout that range, than was observed for the 15-minute incubation.

Table IX shows the differences between means for MUH cleavage. Although the range from the lowest to the highest values was only 2-fold, the variability within that range was relatively high, as shown

Table VI.

Comparison of rates of activity (nmol/mg/min) for FFA_{15} and FFA_{30} (n=4) and MUH (n=3, *n=2) assays (results given as mean ± SD)

CULTIVAR	FFA_{15}	FFA_{30}	MUH
Dal	1.78±.08	1.41±.06	1.19±.03*
Kelly	2.29±.66	2.20±.14	1.91±.10
Lyon	1.52±.10	1.55±.08	1.22± 0
Ogle	1.46±.14	1.27±.06	1.33±.05
Centennial	1.72±.12	1.57±.02	1.41±.07
Stout	1.79±.08	1.88±.04	1.59±.09
Steele	2.20±.16	2.15±.08	1.61±.05
Webster	2.40±.06	2.05±.14	1.76±.03
Pierce	2.97±.12	3.14±.08	1.99±.05
Wright	2.53±.04	2.05±.10	1.44±.02
Dumont	2.42±.06	2.25±.06	1.80±.02*
Clintland	1.98±.14	1.57±.02	1.48±.09
Lang	2.10±.16	1.70±.06	1.87±.05
Clintford	2.94±.16	2.28±.30	1.50±.03
Froker	1.78±.12	1.36±.02	1.65±.03
Preston	1.94±.08	1.27±.14	0.94±.02
Marathon	1.61±.20	1.40±.04	1.45±.03
Lodi	2.06±.12	2.00±.06	1.43±.05
Lamar	1.26±.16	0.95±.04	1.37± 0*
Donald	0.67±.44	0.64±.01	0.98±.06*
Tibor	2.38±.14	1.70±.06	1.04± 0*

MUH vs FFA_{15} r=0.54, MUH vs FFA_{30} r=0.71

Table VII. Student-Newman-Keuls table of differences between means for FFA activity at 15 minutes incubation. Cultivar means move from left to right and from top to bottom in ascending order. (For actual values of means, see Table VI) * indicates significance at P<.05.

	DN	LR	OG	LN	MR	CN	FR	DL	ST	PN	CL	SE	LO	LG	KY	TB	WB	DM	WR	CF	PR
DN																					
LR	*																				
OG	*																				
LN	*																				
MR	*																				
CN	*	*																			
FR	*	*																			
DL	*	*																			
ST	*	*																			
PN	*	*	*	*																	
CL	*	*	*	*																	
SE	*	*	*	*																	
LO	*	*	*	*	*																
LG	*	*	*	*	*																
KY	*	*	*	*	*	*	*	*	*												
TB	*	*	*	*	*	*	*	*	*	*											
WB	*	*	*	*	*	*	*	*	*	*	*										
DM	*	*	*	*	*	*	*	*	*	*	*										
WR	*	*	*	*	*	*	*	*	*	*	*	*	*	*							
CF	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*		
PR	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	

Abbreviations: CF=Clintford; CL=Clintland; CN=Centennial; DL=Dal; DM=Dumont; DN=Donald; FR=Froker; KY=Kelly; LG=Lang; LN=Lyon; LO=LOdi; LR=Lamar; MR=Marathon; OG=Ogle; PN=Preston; Pr=Pierce; SE=Steele; ST=Stout; TB=Tibor; WB=Webster; WR=Wright

Table VIII. Student-Newman-Keuls table of differences between means for FFA activity at 30 minutes incubation. Cultivar means move from left to right and from top to bottom in ascending order. (For actual values of means, see Table VI) * indicates significance at P<.05.

	DN	LR	OG	PN	FR	MR	DL	LN	CL	CN	LG	TB	ST	LO	WB	WR	SE	KY	DM	CF	PR
DN																					
LR	*																				
OG	*	*																			
PN	*	*																			
FR	*	*																			
MR	*	*																			
DL	*	*																			
LN	*	*	*	*	*		*														
CL	*	*	*	*	*																
CN	*	*	*	*	*																
LG	*	*	*	*	*	*	*														
TB	*	*	*	*	*	*	*														
ST	*	*	*	*	*	*	*	*	*	*	*	*									
LO	*	*	*	*	*	*	*	*	*	*	*	*									
WB	*	*	*	*	*	*	*	*	*	*	*	*	*								
WR	*	*	*	*	*	*	*	*	*	*	*	*									
SE	*	*	*	*	*	*	*	*	*	*	*	*	*								
KY	*	*	*	*	*	*	*	*	*	*	*	*	*	*							
DM	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*					
CF	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*					
PR	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	

Abbreviations as in Table VII.

Table IX. Student-Newman-Keuls table of differences between means for MUH activity. Cultivar means move from left to right and from top to bottom in ascending order. (For actual values of means, see Table VI) * indicates significance at P<.05.

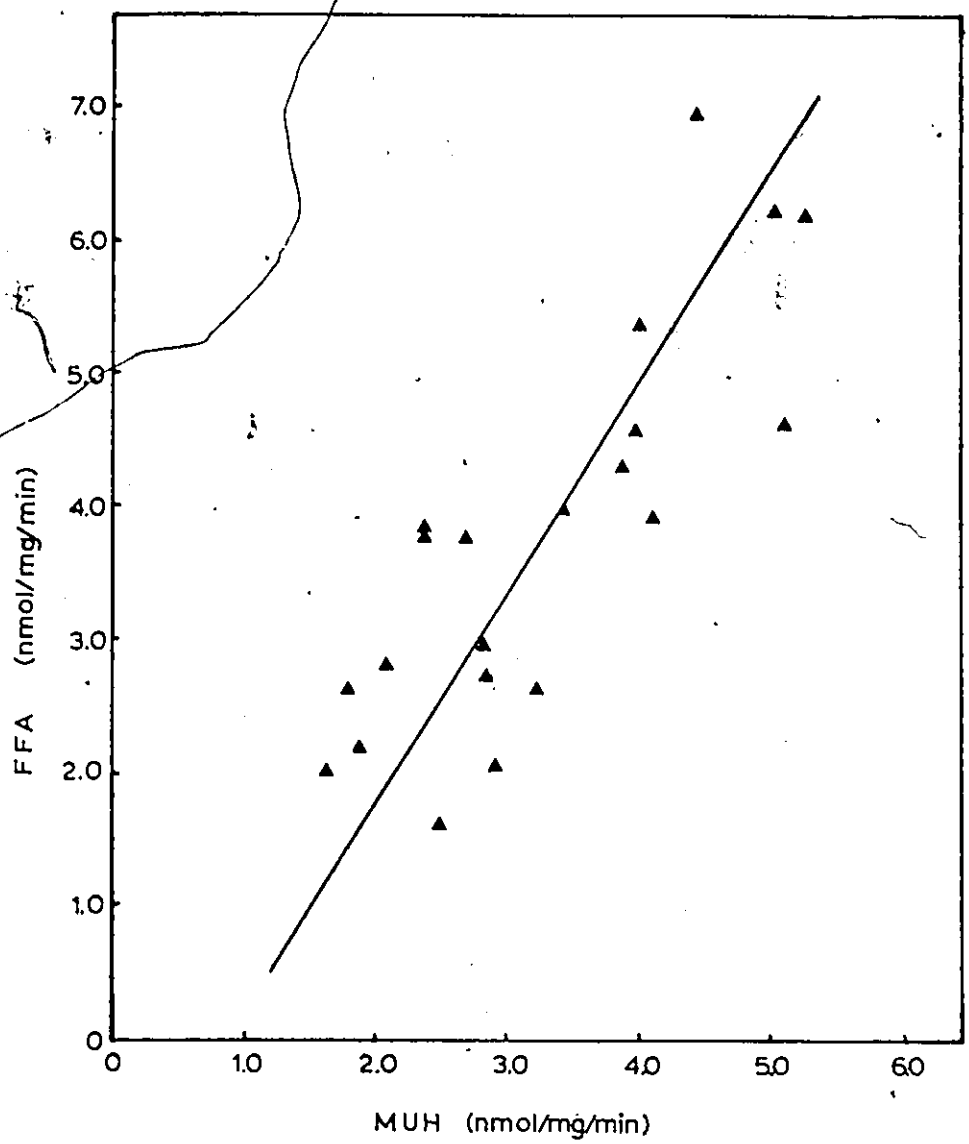
	PN	DN	TB	DL	LN	OG	LR	CN	LO	WR	MR	CL	CF	ST	SE	FR	WB	DM	LG	KY	PR
PN																					
DN																					
TB	*																				
DL	*	*	*																		
LN	*	*	*	*																	
OG	*	*	*	*	*																
LR	*	*	*	*	*																
CN	*	*	*	*	*																
LO	*	*	*	*	*																
WR	*	*	*	*	*	*															
MR	*	*	*	*	*	*															
CL	*	*	*	*	*	*															
CF	*	*	*	*	*	*															
ST	*	*	*	*	*	*	*		*	*	*		*								
SE	*	*	*	*	*	*	*		*	*			*								
FR	*	*	*	*	*	*	*		*	*			*								
WB	*	*	*	*	*	*	*	*	*	*	*	*	*	*							
DM	*	*	*	*	*	*	*	*	*	*	*	*	*	*							
LG	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*		*				
KY	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*		*	*			
PR	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	

Abbreviations as in Table VII.

by the relatively small blank area beneath the line.

When the FFA assays were compared with the MUH assay in the 21 cultivars, a correlation coefficient of 0.54 was found for the FFA incubation at 15 minutes, and 0.71 for the FFA incubation at 30 minutes. When these rates were mathematically adjusted to equivalent fat and protein contents using the values obtained from the Soxtec and Kjeltec analyses (See Appendix I), the correlation coefficent increased to 0.84. A plot of MUH versus FFA at 30 minutes incubation (adjusted values for both) is presented in Figure 26, with the calculated regression line.

Figure 26. Plot of hydrolysis rates for MUH vs FFA(30 mins) assays for 21 cultivars adjusted to 18% protein, 8% fat, dry weight basis, with calculated regression line(r=.84). For actual values, see Appendix I, Table II. Sample sizes for each cultivar are given in Table VI.


7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
FFA (nmol/mg/min)
0
1.0
2.0
3.0
4.0
5.0
6.0
MUH (nmol/mg/min)

4. DISCUSSION

Cereal grains exhibit a wide range of lipase activities. High activity has been reported in oats and rice compared with wheat and maize, while millet and sorghum appear to have intermediate levels of activity (Galliard 1983). In oats, this high lipase activity has been implicated in the production of undesirable colour changes (Martin 1956) and flavours (Hutchinson and Martin 1952, Moran 1952, Kazi and Cahill 1969, Welch 1977) in oat products which are then considered unpalatable by the consumer. Virtually all of the traditional assays for measuring lipase activity (such as measurements of free fatty acid release) are time-consuming to perform, and are impractical for routine use in an industrial setting. Rapid detection of residual lipase activity in oats before processing is complete would therefore be an economic advantage in the oat industry. A rapid method of screening for high and low lipase activities would also be useful to oat breeders, enabling them to make more informed choices in breeding programs, which often include several thousand cultivars, and to assess the heritability of lipase.

Fluorometric assays which monitor cleavage of MUH have been consistently and frequently reported as measuring lipase activity in agricultural products (Jacks and Kircher 1967, Guilbault et al. 1968, Heltved 1984, Saunders and Heltved 1985). In order to evaluate the validity of this premise in a food processing and/or genetic screening context, for this thesis, the MUH assay of Saunders and Heltved (1985) was adapted and tested for its potential use with oats. It was compared

under varying conditions with the more traditional lipase assay of Sahasrabudhe (1982), which measures free fatty acids released from endogenous triglycerides during lipolysis. Although results indicated that the MUH assay was, in fact, measuring primarily esterase activity rather than true lipase activity, further experiments showed that the MUH assay might provide a rapid method for screening for lipase activity, in selected situations (eg. monitoring of lipase during oat processing) where an absolute value was not required.

In any study involving cereal grains, one must be aware of the inherent variability of the sample. Oats are particularly ill-defined biochemically, and components of variation within a single cultivar can be seen on several levels. For example, on a single head of a plant, different physical and biochemical characteristics can be observed. Differential growth at different positions on the head, and within different florets at the same position, have been reported (Rawson and Evans 1970, Scott and Langer 1977, Bangerth et al. 1985, Feucht and Höfner 1985). Because of this differential growth, the primary kernels are invariably larger than the secondary and tertiary kernels, and the ratios of primary, secondary and tertiary kernels vary considerably among cultivars. The size differences in these kernels are accompanied by differences in surface-to-volume ratios. For an enzyme like lipase, which is found primarily in the bran, such considerations become important, and will contribute to the variations observed. Smaller kernels will have a greater proportion of bran per unit volume, and thus higher lipase activity would be measured in these kernels, or in a cv with a high proportion of these kernels, than in a cv with a greater proportion of large kernels. Ratios of primary, secondary and tertiary

kernels were not available for the cultivars used in this thesis. Differences have also been reported in phytohormone concentrations (Michael and Seiler-Kelbitsch 1977, Bangerth et al. 1985), assimilation of ^{14}C from flag leaves (Rawson and Evans 1970) and protein content (V.D. Burrows, personal communication).

On another level, at any given location, differential shading of plants leads to differences in fertility of florets (and hence, differences in yield), rates of dry matter accumulation, and in levels of water-soluble carbohydrates (Fischer and Stockman 1980). Between locations, differences in soil composition, drainage, or fertilizer application also lead to variations in several kernel characteristics. On still another level, climatic factors such as temperature and moisture also affect the assimilation of storage materials in grains. Low temperatures increase the lipid concentration of oats (Youngs 1978), and drought stress leads to higher beta-glucan content in barley (Aastrup 1979, Coles 1979, Bourne and Wheeler 1984).

Consequently, in view of these several levels and sources of variation, samples were used which were grown in the same year, at the same location, to facilitate observation of genetic differences, and minimize environmental effects.

Once harvested, biochemical changes in grains continue during storage. For example, lipase levels will decline in oats over as short a period as one or two months, even at freezer temperatures (Sahasrabudhe 1982). Because facilities to store seeds at liquid nitrogen temperatures to prevent this decline were not available, the rates of activity reported for the same cultivars in different experiments throughout the thesis vary, depending on the time elapsed

between experiments.

Mature, harvested grains also show differences in kernel hardness (de Francisco et al. 1982, Sampson et al.1983, Yamazaki and Donelson 1983). In oats, particle sizes can affect the hydrolytic activities of both the assays used in this thesis, with smaller particles (produced by finer grinding of the sample) exhibiting higher rates of hydrolysis (S. Miller, unpublished results). Because hardness is not routinely tested in oats (indeed, there are no accepted methods for measuring hardness in oats), it was not possible to assess this possible source of variation.

Ultimately, the variation in kernel size among populations, differential effects of grinding (leading to varying particle size distributions), and continual degeneration of activity during storage combine to provide highly heterogeneous samples. In spite of these difficulties, lipase activity has been measured in food products (Hutchinson and Martin 1952, Moran 1952, Kazi and Cahill 1969) and for genetic screening purposes (Frey and Hammond 1975), using the traditional, time-consuming methods. For these purposes, the results presented in this thesis suggest that the MUH assay could be employed as a crude indicator of lipase activity, in a fraction of the time required for the more conventional assays, despite the fact that it is apparent that the MUH procedure is indicative of esterase activity, and not that of true lipase.

In the preliminary studies to determine optimum conditions for MUH cleavage, it was thought that the addition of a detergent to the sample buffer would help to solubilize the enzyme, enabling easier access to the substrate. As Triton X-100 was already an ingredient in the FFA assay of Sahasrabudhe (1982), the 'reference' assay for this thesis,

this detergent was the logical choice.

The addition of Triton X-100 to the assay buffer in the MUH method caused increasing inhibition of MUH cleavage as the amount of Triton was increased (Figure 10). Similar inhibition of the lipase of rapeseed cotyledons by Triton X-100, as well as by EDTA, digitonin, Tween 85, and SDS has been reported by previous workers (Rosnitschek and Theimer 1980). Adams and Brawley (1981) reported a heat resistant lipase of Pseudomonas spp. to be sensitive to the emulsifiers Tween 20, Tween 80 and Durfax 60, while others such as sodium deoxycholate, lecithin and Santone-8-10 enhanced lipase activity. In light of these results, these investigators suggested that the use of appropriate emulsifiers in the food industry could be effective in retarding lipase activity in food products. The results of Rosnitschek and Theimer (1980) were confirmed by Lin and Huang (1983), who observed inhibition of the lipase activity in lipid bodies of cotyledons of rape and mustard seedlings by EDTA, Triton X-100 and SDS, as well as desoxycholate and Na_3PO_4. Cleavage of the fluorogenic substrate N-methylindoxylmyristate by the alkaline glyoxysomal lipase of germinated castor beans was also inhibited by Triton X-100 (Maeshima and Beevers 1985). But Matlashewski et al.(1982) found that Triton X-100 enhanced lipase activity in a soluble lipase fraction from oat flour, as did SDS and sodium desoxycholate. Contrary to the observations of Adams and Brawley (1981), Abigor et al.(1985), working with the lipase of oil palm mesocarp, found lecithin to be a strong inhibitor of lipase activity. The effect of Triton on FFA release in oats was not investigated in the present work, although presumably it has a beneficial effect, as it is included in the assay buffer used by Sahasrabudhe (1982).

One of the effects of detergents and emulsifiers is to cause a decrease in the interfacial surface tension of an emulsion. Some proteins (eg. melittin, myoglobin, serum albumin and ovalbumin) have also been reported to have this effect (Gargouri et al. 1984). In the same study, these proteins were also shown to inhibit hydrolysis of tributyrin and triolein by pancreatic lipase. These results led Gargouri et al. (1984) to speculate that the inhibition is the result of desorption of lipase from its substrate due to modification of the physicochemical properties of the oil-water interface.

In some of the early studies investigating fluorometric substrates for lipolysis, MUH was indicated as the most appropriate of the methylumbelliferyl esters. Jacks and Kircher (1967) found maximal rates of catalysis with esterified acids of medium chain length (C_7, C_8, C_9). Of the enzymes they tested, wheat germ lipase, steapsin and peanut lipase all exhibited the highest rates of activity using MUH as substrate. The nonanoyl ester of 4-methylumbelliferone was the best substrate for porcine lipase, and the octanoyl ester was the best substrate for castor bean lipase. Guilbault et al.(1968) found MUH to be the best substrate for porcine lipase. These authors claim that the disagreement between their results and those of Jacks and Kircher arose because Jacks and Kircher "did not have the optimum conditions for the precise analysis of lipase".

The exploration of fluorogenic substrates in this thesis included examination of fluorescein esters, which are less expensive and more readily available than methylumbelliferyl esters, and methylumbelliferyl esters of varying chain length (Tables I and II).

Of the fluorescein esters, only FDB showed any activity at all, but this was much lower than that shown with MUH (Table I). The lower rate of fluorescein release is due mainly to the presence of two ester bonds which must be cleaved to release the fluorescent product, as opposed to just one in the methylumbelliferyl esters. However, some of the inefficiency of the assay using FDB can be attributed to the use of the excitation peak at 323 nm rather than the major excitation maximum at 490 nm. The smaller, secondary excitation maximum was chosen to avoid the interfering scatter peak found as a shoulder at 490 nm on the emission peak at 510 nm, resulting in decreased sensitivity. Previous work in other systems has also shown FDB to lack the required sensitivity (Henry and McLean 1986), and/or to yield much lower rates of hydrolysis than MUH (Jacks and Kircher 1967, Guilbault and Hieserman 1969, Heltved 1984).

Of the methylumbelliferyl esters, only 4-methylumbelliferyl butyrate (MUB) showed hydrolytic susceptibility exceeding that of MUH. However, MUH exhibited slightly greater variability between cultivars (Table II). The octanoyl ester (MUO) with an acyl chain only one carbon longer than that of MUH, was cleaved at a much lower rate (15% of MUH cleavage). MUP, the only artificial substrate tested containing a natural oat fatty acid (palmitic), was cleaved at the lowest rate of all (only 10% of MUH activity). Since Lin et al.(1986) recently demonstrated that lipases from specific plant species had higher activities on triglycerides containing the major fatty acids of the seed storage triglycerides of the same species, and since methylumbelliferyl esters are not natural substrates of lipase, it seemed unlikely that

cleavage of the methylumbelliferyl esters was accomplished by a lipase.

To help elucidate which of the MU esters would be the most appropriate substrate in further investigations of lipolysis, in the present work a commercial preparation of porcine pancreatic lipase (PPL) was used (Table III). This enzyme exhibited the highest activity when MUH was added as substrate, with MUP a distant second. MUB, MUO and MUS produced very low rates of hydrolysis with PPL in comparison with MUH.

Experiments to determine Michaelis-Menten kinetics using defatted flour were not performed for two reasons. Firstly, the enzymes had not been purified, and the possibility of interference by other enzymes could not be ruled out. For example, Guilbault et al.(1968) showed that MUH could also be cleaved at a lower rate by esterase and protease. Also, in measurement of FFA release, the activities of esterases on, for instance, the monoglycerides produced during lipolysis, could artificially elevate the calculated lipase activity. Secondly, to derive meaningful Michaelis-Menten kinetics for lipolytic enzymes, substrate concentrations must be measured in units of interfacial area per unit volume (ie. surface concentration) in the substrate emulsion (Benzanona and Desnuelle 1965, Brockerhoff and Jensen 1974, Brockman 1984), for which the laboratory was not equipped.

Studies comparing the hydrolytic activities of defatted and natural flours suggested that the MUH assay was not measuring true lipase activity (Table IV). When defatted flours were assayed for lipase activity with added triolein, the rates of FFA release increased

dramatically when compared to the rates obtained in natural flours using endogenous substraste. MUH cleavage, on the other hand, showed a less dramatic, but consistent decrease in activity in defatted flours when compared to natural flours.

The experiments with methylumbelliferyl esters of varying chain length and defatted oat flours reported in this thesis suggested that the MUH assay was not, in fact, measuring true lipase activity. Galliard (1986) reported similar discrepancies in a comparison of the MUH assay with a lipase assay using [^{14}C]triolein in studies on wheat fractions. Galliard found that the relative distribution of hydrolytic activity towards MUH between germ and bran was very different from that of [^{14}C]triglyceride lipase activity, suggesting that a different enzyme was involved in MUH hydrolysis than that responsible for triglyceride hydrolysis.

To clarify the situation, in this work both assays were performed in the presence of di-isopropylfluorophosphate (DFP). DFP is a well-known inhibitor of many esterases and proteases (Saunders and Stacey 1948, Means and Feeney 1971), but not lipase. In fact, DFP is often used to 'stabilize' lipase preparations. The enzymes which are sensitive to DFP are inactivated through its reaction with a serine hydroxyl group in the active site (Means and Feeney 1971).

Initial experiments showed very little inhibition of FFA release by DFP (Figure 17). Inhibition of MU release by the same quantity of DFP is much more pronounced, indicating that esterase activity is responsible for a large part of MUH cleavage, although the possibility of protease activity existed also (Guilbault et al.(1968). A range of

cultivars was assayed using the MUH method, with and without DFP, at pH 8.0, the optimum for MUH activity, and at pH 7.4, both to avoid the alkaline lability of DFP (Saunders and Stacey 1948), and to provide more favorable conditions for "true" lipase activity (Table V). Even in the absence of DFP, the MUH activity at pH 7.4 was 45-50% lower than at pH 8.0. This is undoubtedly due in part to the pH sensitivity of the MU which increases in fluorescence intensity with increasing pH (Jacks and Kircher 1967) as well as that of the enzymes present. Over the range of 18 cultivars, inhibition by DFP was significant at both pH's, ranging from 40-80%. The residual activity in the presence of DFP is presumably lipase, although it did not show significant correlation with lipase activity as measured by FFA release. The lack of correlation between this residual activity and that of FFA release is, in all likelihood, due to the use of a highly aqueous assay medium, which has been shown to give poor results for lipase activity in oats (Frey and Hammond 1975).

Although the data clearly indicated that the MUH, or esterase assaywas not measuring the same activity as the FFA, or lipase assay, the ease, sensitivity and rapidity of the fluorometric assay invited further studies. If a significant correlation could be achieved between the two assays, the MUH method could then be used as an indicator of lipase activity under various conditions, for example during inactivation processes, or during germination. For this reason inactivation and germination experiments were conducted, and the samples obtained assayed using both methods. A screening of 21 cultivars using mature, ungerminated groats was also performed.

The importance of moisture content of groats in the thermal inactivation of oat lipase was recognised early by some investigators. Hutchinson et al.(1951) reported that at 6% moisture, a temperature of 120°C was required to inactivate 98% of the lipase in one hour. At 12% moisture, equivalent inactivation was achieved in one hour at 83°C. When the moisture was increased to 20%, 98% inactivation was achieved by holding the groats at 64°C for one hour. These workers did not indicate the scale of their inactivation experiments (ie. kilogram quantities or gram quantities of groats?), a parameter which would in all likelihood have a bearing on the time course of inactivation at a particular temperature, as the heat would require more time to penetrate a larger sample.

Thermal inactivation of oat lipase by dry heat has also been studied. Pokorny et al.(1968) reported that at temperatures of 100°C and lower, lipase activity rose initially, and decreased only after prolonged heating. Only at temperatures higher than 120°C did the rate of inactivation become rapid.

Pokorny et al.(1968) also examined the effect of moisture on inactivation rate, using a broader range but fewer increments in moisture content than Hutchinson et al.(1951). Using a wetted sample containing 21% moisture, an air-dried sample containing 7% moisture, and a dessicator-dried sample (for which the actual moisture content is not reported, but can be assumed to be essentially 0%), Pokorny et al.(1968) found that only the wetted sample was effectively inactivated by heating for 2 hours at 120°C. The activity of the 7% sample actually increased by the end of two hours, and the dessicator dried-sample showed activity that was significantly higher still.

In the work presented in this thesis, groats were brought to 20% moisture before heat inactivation to mimic industrial conditions (D. Paton, personal communication). Preliminary experiments using the MUH assay only (Figure 18) indicated that inactivation of the enzyme in 2.0 g of oats was essentially complete in 10 minutes at 97°C. At 80°C, a small amount of activity remained after 60 minutes heating, and at 56°C, a substantial amount of activity remained after 50 minutes.

Subsequent experiments were performed using both the MUH and FFA assays to monitor inactivation at varying temperatures. At 95°C (Figure 19), lipase activity dropped by more than 90% after 5 minutes as measured by the FFA assay. The corresponding drop as measured by the MUH method was 75%. Although there was some residual esterase activity remaining at the end of 10 minutes, while lipase had been completely inactivated, the correlation between the two assays was high ($r=0.86$).

At 72°C (Figure 20), while lipase activity was essentially gone after 20 minutes, a small amount of esterase remained. Again the correlation between the two assays was very high ($r=0.94$). At 60°C (Figure 21), inactivation was less complete, requiring a full 2 hours for lipase activity to drop to a very low level. As at other temperatures, esterase activity was higher at the end of the inactivation period than lipase. Correlation between the two assays, however, was still high, with correlation coefficients of 0.87 and 0.89 for 2 separate experiments at 60°C. Barley lipases, assayed by measuring cleavage of triacetin in defatted flours, have also been shown to retain a significant amount of activity in the 50-65°C temperature range (Narzi and Sekin 1968).

Although to be consistent, most of the inactivation studies were

conducted using the cultivar Lamar, Donald was used in addition at 80°C to ensure that the patterns of lipase and esterase inactivation were not peculiar to one cultivar (Figure 22). Activity dropped to a low level after 10 minutes at 80°C in both cultivars. The esterase activity stabilized at a higher rate than lipase, as was seen throughout the inactivation study. With Donald, as with Lamar at other temperatures, the two assays showed a high degree of correlation (r=0.85). Not surprisingly, there is a very high correlation between the two cultivars during inactivation. Comparing the rates of MU release for both cultivars at times 0, 5, 10, 20 and 30 minutes gives a correlation coefficient of 0.99. A high correlation between FFA and MU release is also seen in the work of Saunders and Heltved (1985) on heat stabilisation of rice bran . Comparison of "lipase" activity as measured by the MUH method (ie. probably esterase activity, as noted earlier in this thesis), and lipase activity as measured by a titrimetric determination of released FFA yielded a correlation coefficient of 0.94.

The consistently high correlation coefficients obtained between the MUH and FFA assays during inactivation at various temperatures suggested that, under industrial processing conditions, the esterase assay could be used as an indicator of lipase activity in oat products. The ease and rapidity of this assay would more than compensate for its lack of absolute biochemical specificity.

As reported by Pokorny et al.(1968), in some cases there was an initial rise in enzyme activity during heating, before inactivation occurred (see Figs. 19-21). This was neither as dramatic, nor as consistent as reported by Pokorny et al., possibly because inactivation

in the present work was accomplished using moist heat rather than the dry heat used in their studies. The "activation" with heating can be seen in both the FFA (Figs 19,21) and MUH (Figs 19,20) assays.

Oat lipase is not only important in food biochemistry, it has physiological significance as well. The activity of this enzyme is essential for the utilization of storage triglycerides in the germinating oat kernel, providing energy for the young seedling that cannot yet photosynthesize its own.

In a previous study of germinating oats, a 60% increase in lipase activity was reported during the first 12 hours following imbibition, with a subsequent slow decrease ~~up to~~ 96 hours (Matlashewski et al. 1984). The germination temperature in these experiments was 25°C. Drapron et al.(1969) found activity increases in wheat to take a little longer, although their reporting of results according to plumule length rather than germination time makes comparison difficult. These workers noted that lipolytic activity of wheat grains first underwent a slight decrease, then increased during germination. Both germination temperature and light were found to affect development of lipase activity. Much greater activity was seen in wheat germinated at 30°C than at 15°C, and decreased lipase activity resulted when wheat grains were germinated in the light. Tavener and Laidman (1972), measuring triglyceride levels rather than lipase activity in germinating wheat, reported decreases within 12 hours of the start of germination at 25°C. Heltved (1984) and Jensen et al.(1984) have used fluorometric methods to monitor lipolytic activity in germinating wheat. At an incubation temperature of 15°C, using fluorescein dibutyrate (FDB) as a substrate,

activity was first detected at 24 hours germination (Heltved 1984). MUH proved to be a more sensitive substrate, showing an increased activity at 12 hours germination. Jensen et al.(1984), also using FDB, reported the first noticeable increase in activity at 24 hours germination as well, in spite of a higher incubation temperature (19°C). Results obtained with barley seem to follow the same pattern. Narzi and Sekin (1968) reported that lipase activity increased slightly up to the first day of germination (ie. during the soaking process), and then increased steadily up to the seventh day. Both temperature and moisture content were found to be important: lipase activity increased with increasing moisture content (40-48%) and increasing temperature (from 12-18°C).

In the experiments reported here, lipolytic activity was found to be similarly dependent on germination temperature (Figures 24 and 25). Activity increased much more rapidly at 20°C than at 12°C. This difference was predictable simply from examination of the germinating seeds (Figure 23). After approximately 48 hours at 20°C, the seedlings had reached a stage which required 96 hours to achieve at 12°C.

The initial lipase activity was zero in the germination experiments at both temperatures. This was in direct contrast with the lipolytic activities measured in mature, ungerminated kernels which had not been subjected to surface sterilization. In all probability the absence of activity in the groats at the start of the germination experiments was due to the destruction of existing lipase by treatment with 70% ethanol during surface sterilization of the groats. Frey and Hammond (1975) reported that ethanol was second only to boiling of groats in water in efficacy of lipase inactivation. Esterase activity appeared to be much less sensitive to alcohol treatment.

The initial increase in hydrolytic activity durng germination was faster for esterase than for lipase at 12°C (Figure 24) and at 20°C (Figure 25), and remained so even after 96 hours at 12°C. At 20°C, however, the lipase activity had caught up with the esterase activity by 60 hours of germination, after which it rapidly outstripped the more gradual increase in esterase activity.

The final study conducted for this thesis was an examination of liplolytic activity in mature, ungerminated groats over a range of 21 cultivars. Frey and Hammond (1975) found a 20-fold variation over a range of more than 400 cultivars and collections using a colourimetric assay similar to the FFA assay. This wide variation is due not only to their large number of samples assayed, but also to the inclusion of several different species of Avena (A.brevis, A.ludoviciana, A.pilosa, A.strigosa, A.wiestei, A.barbata, A.fatua, and A.sterilis) in addition to A.sativa. Even in the domestic cultivars used in the present study, significant variation was found between cultivars, as measured by both FFA (4- to 5-fold variation) and MUH (2-fold variation) activities. A much broader variation would be expected between species, than between cultivars within a single species. Pokorny et al.(1968) also noted varietal differences within A.sativa. These workers used only a few cultivars, and hence did not observe the same magnitude of variation as in the present study. Narzi and Sekin (1974) reported varietal differences in lipase in barley, again on a small sample size, and hence on a small scale.

When the results of the two assays were compared (Table VI), there was a positive correlation between esterase activity and lipase activity at 15 minutes of incubation (r=0.59). When the lipase assay was

incubated for 30 minutes, the correlation improved considerably (r=0.71), although it was still not as high as the correlations achieved during inactivation and germination. This is because the lipase exhibited a broader range of activity (4- to 5-fold) than esterase (2-fold) in the 21 cultivars. The variability within the range is also somewhat higher for lipase activity at 30 minutes incubation than for the esterase activity (Student-Newman-Keuls Multiple Range Test, P .05), (Tables VIII, IX).

The rates of hydrolysis reported in Table VI represent enzyme activity on a dry weight basis for each cultivar. When these rates were mathematically adjusted to bring all the cultivars to equivalent fat and protein contents as well (see Appendix I, Table II), the correlation coefficient increased to 0.84 (Figure 26), although due to the variation in endogenous lipid and protein contents, the ranking of the cultivars shifted slightly. These results indicate that the esterase assay could be a useful tool in a breeding program, where a reasonable estimate of activity could allow the breeder to eliminate the high cultivars, and select those with lower activity.

5. CONCLUSION

The results presented in this thesis show that the FFA method and the MUH method are measuring two separate hydrolytic activities in oats. The FFA assay measures lipase activity, while the MUH assay measures esterase activity. However, under some conditions, a high correlation has been demonstrated between the two enzyme activities. In view of these high correlations, it is suggested that the MUH assay

could be a rapid indicator of lipolytic activity in oats, in circumstances where an absolute measurement of lipase is not required. For example, in the oat industry, the MUH method could be used to determine if lipase in a grain or flour sample was sufficiently inactivated, before that sample was incorporated in further processes. The MUH assay would also be useful in a breeding program, allowing rapid estimation of the high and low activity cultivars for selection.

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APPENDIX I

Table I. Proximate analysis of groats of 21 cultivars, determined using the Kjeltec (for protein) and Soxtec (for fat) automated systems, and moisture contents determined using the AACC oven method.

Cultivar	% Protein	% Fat	% Moisture
Dal	16.0	5.1	6.5
Kelly	16.5	3.7	7.8
Lyon	15.4	7.1	8.9
Ogle	14.3	3.3	8.8
Centennial	14.9	3.5	7.8
Stout	16.2	2.7	8.6
Steele	15.9	3.7	7.6
Webster	16.4	4.5	8.3
Pierce	16.1	7.5	8.3
Wright	15.5	5.0	8.2
Dumont	13.2	3.9	8.7
Clintland	16.2	4.7	8.7
Lang	15.2	3.5	8.4
Clintford	15.7	3.0	8.7
Froker	15.3	4.9	8.8
Preston	14.7	4.5	8.2
Marathon	16.2	4.6	7.6
Lodi	14.2	7.8	7.5
Lamar	16.4	4.2	9.3
Donald	16.7	3.5	9.5
Tibor	17.9	3.6	9.3

APPENDIX I

Table II. Rates of hydrolysis measured using the FFA (after 30 minutes incubation) and MUH assays, reported on a dry weight basis, and adjusted to equal protein (18%) and fat (8%) contents

Cultivar	FFA_{30}	MUH
Dal	2.49	2.10
Kelly	5.20	4.52
Lyon	2.04	1.61
Ogle	3.89	4.07
Centennial	4.29	3.85
Stout	6.19	5.23
Steele	5.33	3.99
Webster	3.96	3.40
Pierce	3.75	2.38
Wright	3.79	2.66
Dumont	6.23	4.98
Clintland	2.94	2.78
Lang	4.60	5.06
Clintford	6.92	4.56
Froker	2.62	3.18
Preston	2.79	2.06
Marathon	2.72	2.81
Lodi	2.60	1.86
Lamar	2.01	2.89
Donald	1.60	2.45
Tibor	3.82	2.34