THE UNIVERSITY OF MANITOBA

PURIFICATION AND CHARACTERIZATION OF LIPASE
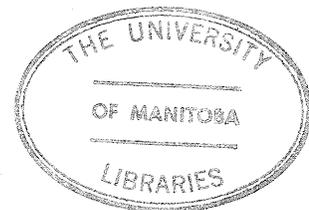
FROM VICIA FABA MINOR

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF FOOD SCIENCE

WINNIPEG, MANITOBA

January 1977

"PURIFICATION AND CHARACTERIZATION OF LIPASE

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A dissertation submitted to the Faculty of Graduate Studies of
the University of Manitoba in partial fulfillment of the requirements
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ABSTRACT

The purpose of this study was to ascertain whether a lipolytic enzyme, lipase, was present in faba bean (_Vicia faba_ L var. _minor_). Lipase is believed to be one of the enzymes responsible for the initiation of the development of rancidity and off-flavour in processed faba bean.

Faba bean lipase was partially-purified from a faba bean acetone powder by ethanol fractionation. Purification was approximately 9-fold over the crude extract. Further purification was attempted by filtration on Sephadex G - 100 gel columns with a linear NaCl gradient, or by the incorporation of sodium deoxycholate which were responsible for 18-fold and 40-fold increases in lipase activity respectively. Comparison of the gel filtration profile of faba bean lipase was made against that of commercial hog pancreatic lipase.

The assay of the enzyme was based on a potentiometric titration of released fatty acid utilizing a pH-stat method.

The electrophoretic patterns of the faba bean lipase and commercial hog pancreas lipase were compared using polyacrylamide disc gel electrophoresis. There was one active lipase band detected in the Sephadex G - 100 purified faba bean lipase, with good correlation between protein bands and lipolytic bands. The isoelectric point of the enzyme was determined to be 4.8.

The molecular weight of faba bean lipase was estimated

by sodium dodecyl sulphate-gel electrophoresis to be
$210,000 \pm 20,000$.

The course of the hydrolytic reaction was linear with respect to enzyme concentration. The enzyme exhibited greater activity towards short-chain triglyceride emulsions rather than long-chain triglyceride emulsions. The K_m for the reaction was determined to be 22.0 mM using tributyrin as the emulsified substrate.

The optimum pH of the enzyme was determined to be 8.5 while the lipase was stable over a pH range of 6.5 - 9.0 for a 10-minute period.

In solution the faba bean lipase was inactivated by exposure to $65^{\circ}C$ for 2 minutes, indicating a comparatively heat-labile enzyme. The optimum temperature was determined to be $38^{\circ}C$.

The effects of various activators on the activity of the faba bean lipase were investigated. It was shown that NaCl was necessary for the lipolytic reaction to proceed to a zero-order rate with a maximum concentration of 0.7 M. The enzyme was influenced by the bile salts, sodium deoxy-cholate and sodium taurocholate, which resulted in maximal activation at a 12.0 mM concentration. The enzyme was not sensitive to high concentrations of either calcium or magnesium chlorides.

The faba bean lipase was inhibited by a high concentration of mercuric chloride (5.0 mM), with over 60% of the original activity being inhibited at this level. Faba bean

lipase was inhibited very slightly by a high concentration
of p-chloromercuribenzoate. A 10.0 mM concentration re-
sulted in a 20% reduction in lipase activity.

ACKNOWLEDGEMENT

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INTRODUCTION

The small faba bean, or horse bean, <u>Vicia</u> <u>faba</u> L var.
<u>minor</u>, is being considered as a field crop for the Prairie
region of Canada (Presber, 1972). At present the faba is being
used to a considerable extent for livestock feed. Processed
faba beans become rancid after mechanical disruption of the
tissues, representing major storage problems and quality-
acceptability problems, to livestock, and for possible use in
human foods. The presence of increased free fatty acids during
storage of faba beans as either a concentrate or flour has been
detected (Hinchcliffe <u>et al</u>, 1974). This increase in free fatty
acid content during storage, indicates the presence of esterases
converting the stored triglycerides to free fatty acids. The
rapid development of rancidity is due to the relatively high
proportion of unsaturated fatty acids present in the lipid
fraction, especially linoleic acid. This acid is particularly
susceptible to oxidation, due to the presence of an active
lipoxygenase system (Eskin and Henderson, 1976) which is highly
specific towards unsaturated free fatty acids (Figure 1). Since
the majority of fatty acids present are predominantly in the
esterified form of either triglycerides or phospholipids, it
was decided to investigate the mechanism of release of free
fatty acid in the triglyceride fraction. Lipase (glycerol ester
hydrolase, EC 3.1.1.3) is the enzyme responsible for the
hydrolysis of acyl esters of fatty acids.

In plants, lipase investigations have been on olea-

FIGURE 1

Lipid oxidation pathway in the faba bean.

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┌─────────────────────────────┐
│      FABA BEAN LIPIDS       │   phospholipase A
│  Phospholipids (polar)      │ ─────────────────────→      free fatty acids
│                             │                             especially 18:2,
│  Triglycerides (neutral)    │      lipase                 18:3
│                             │ ─────────────────────→
└─────────────────────────────┘
```

β-carotene
(provitamin A)

coupled nonenzymic
reaction   lipoxygenase
                                          O_2

colourless breakdown
(oxidation) products

oxidation products   ←──────────────── hydroperoxides
(rancidity, off-flavours)
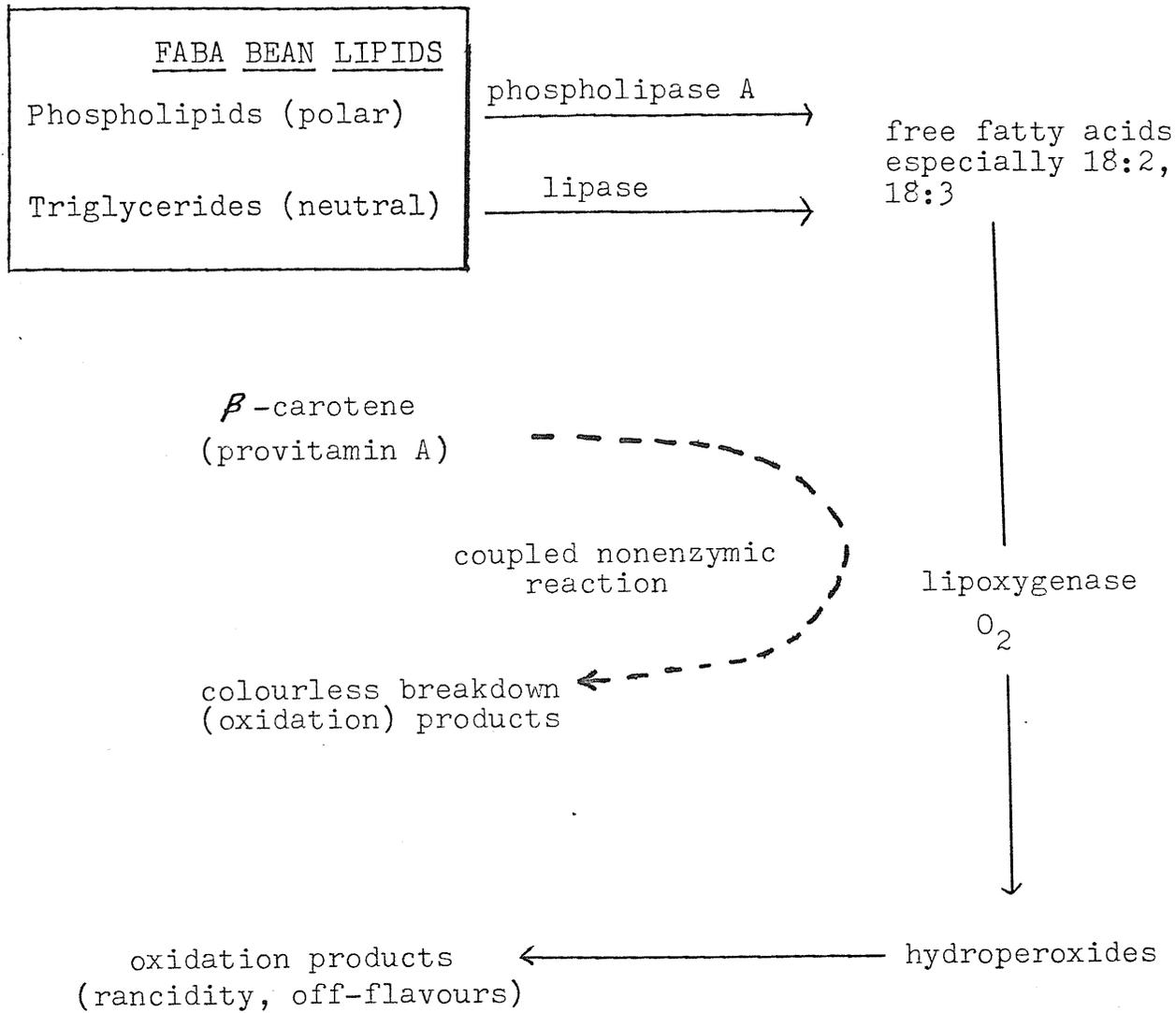
ginous seeds where lipase activity generally is manifest upon germination. Seed lipases have received relatively little attention, and have been almost exclusively concerned with lipases that exhibit exceptional characteristics for example, the acid lipase of castor bean. In the literature, studies on lipase are very entangled and confusing, indicating the difficulty in studying the enzyme, the major drawback being the limitations imposed by the water-insoluble substrate, lipase being active only at the lipid-water interface.

The object of this study was the detection of lipase in faba bean, and the purification and characterization of the enzyme. The determination of the effect of certain conditions (pH, temperature, activators and inhibitors) on faba bean lipase activity was undertaken. The determination of the molecular weight of the enzyme, along with comparison of lipolytic activity with commercial hog pancreatic lipase, was undertaken using polyacrylamide gel electrophoresis.

CHAPTER 2

REVIEW OF LITERATURE

The enzyme lipase belongs to the widely-distributed group of enzymes known as the esterases, which are involved in the splitting of ester linkages by the addition of water. Enzymes hydrolysing triglycerides have studied for well over a hundred years, but in the literature, studies on lipase are entangled with studies on esterases. The diversity of data seems to be partly due to the use of unpurified enzyme preparations and partly to the wide choice of substrates, assay conditions and methods used to determine lipase activity. The first workers in the field assumed that lipase hydrolysed only natural triglycerides, while more extensive investigations of specificity showed that lipases were very unspecific enzymes, and that there appeared to be considerable overlap with the equally unspecific esterases. The nature of either the fatty acid or the alcohol moiety has a secondary effect on the role of lipolysis. The real difference between ordinary esterases and lipases appears to reside in the physical nature of the substrates, with lipases being unable to attack substrate molecules fully dispersed in water, acting only at the water-lipid interface (Sarda and Desnuelle, 1958). The minimum degree of molecular aggregation of the substrate compatible with lipase action is still unknown.

Thus, lipases form a rather indefinite section of the ester-
ase group of enzymes, but it is useful to distinguish
lipases from other esterases by the definition recommended
by the International Union of Biochemistry (1961), namely,
that lipases hydrolyse emulsified esters of glycerol,
whereas other water-soluble esterases hydrolyse water-
soluble substrates.

All lipolytic enzymes are hydrolases and therefore,
belong to class 3 within the classification recommended by
the Enzyme Commission (Florkin and Stotz, 1965). Lipases
are currently classified among the hydrolases and are
ester hydrolases, enzyme group 3.1. No bonds other than
carboxyl ester bonds have ever been found to be hydrolysed
by lipases and they are therefore, defined as carboxyl
ester hydrolases, and as lipase acts on esters of glycerol
the enzyme is fully classified as glycerol ester hydrolase
(EC 3.1.1.3).

Lipases were considered to be enzymes hydrolyzing
glycerol esters according to the equation:

$$A\ Triglyceride + H_2O \longrightarrow Diglyceride + fatty\ acid\ ion$$
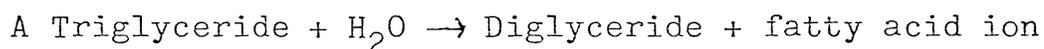
Present information shows that the equation above is
not quite correct, with triglyceride hydrolysis by most
lipases now known to go beyond the diglyceride stage and
to form substantial amounts of monoglycerides and sometimes
of free glycerol (Entressangles and Desnuelle, 1968). Also,
lipases have been recently shown to rapidly hydrolyse ester
substrates other than glycerides (Seneriva and Dufour, 1972).

2.1 DISTRIBUTION OF LIPASES

Lipases are widely distributed in animals, plants and micro-organisms. Although lipases from many different sources have been described, only relatively few have been investigated in detail. A comprehensive list of lipases definitely or tentatively identified in animals, plants or micro-organisms will be found in the review of Wills (1965).

2.1.1 Animal Lipases

Mammalian lipases have received greatest attention in recent years. Three groups of enzymes may be distinguished in mammals: the lipases discharged into the digestive tracts by specialized organs, tissue lipases, and milk lipases.

Among the digestive lipases the enzyme synthesized by the pancreas is the best known and most often investigated, as well as being one of the earlier enzymes to be recognized (Claude Bernard, 1856). Despite its low level when compared to that of other pancreatic enzymes (1.2% of the total proteins in cattle pancreatic juice, 2.5% in pig and 3.4% in rat)(Marchis-Mouren, 1965), this lipase plays an essential role during the intraluminar digestion of dietary triglycerides. In addition, the existence of gastric and intestinal lipases, often disputed, now appears to be definitely proved. Lipases have been reported to be present in a number of tissues or organs of mammals such as heart, brain, muscle, adipose tissue and serum, where they are known as lipoprotein lipases. They have been identified in milk.

2.1.2 Plant Lipases

Few studies have been made so far on the distribution of lipases in whole plants except in seeds and fruits. Most of the effort in this area has been devoted to seed lipases. Seeds are generally rich in triacylglycerols, which serve as a compact source of energy for the newly-emerging plant. During germination of the seed, the triacylglycerol stores disappear. Since the fatty acids cannot be oxidised to provide energy until they are released from the triacylglycerols, lipolytic enzymes are probably rate-controlling during germination. Germination is usually rapid and lipolytic activity is relatively high at that time.

Crushing or storage generally activates dormant lipases in a seed, and the resulting accumulation of free fatty acids can cause an industrially-important oil to become unacceptable or to require additional processing to remove the acids. Nevertheless, investigators have neglected the lipases in the most important food oil-seeds, for example, soybean, cottonseed, corn, safflower, coconut, sesame, and other industrial seeds. Very few reports on the lipases of these seeds are available. Most attention has been paid to seed lipases that exhibit some unusual property, for example, the acid lipase of castor bean (Ory et al, 1960). Lipase activity has been reported in wheat (Sullivan and Howe, 1933), palm (Savary et al, 1957), barley and malt (Lowy, 1945), coconut (Sadasivan, 1951), peanuts (Sanders and Pattee, 1972), cotton (Olcott and Fontaine, 1941) wheat

germ (Singer and Holfstee, 1948; Stauffer and Glass, 1966),
and oats (Martin and Peers, 1953).

2.1.3 Microbial Lipases

In the past, interest in microbial lipases re-
sulted from investigation of food spoilage, especially of
dairy products. The short-chain fatty acids are directly
responsible for flavour defects, while the long-chain fatty
acids could presumably be converted more readily to carbonyls
and other volatile compounds such as free acids. In con-
trast, free fatty acids in some dairy products, notably
cheese, contribute to desirable flavour, such as the action
of lipase in Penicillium roqueforti contributing to the
flavour of Roquefort cheese (Eitenmiller et al, 1970).
The production of lipases may assist in the classification
of micro-organisms, and the detection of those that are
pathogenic (Lawrence et al 1967). Lipases are present in
many strains of bacteria and fungi. Most of the lipases
are intracellular, but some species, such as Stapylococcus,
(Davis, 1954) secrete extracellular lipases.

2.2 SUBSTRATES

Lipases hydrolyse emulsified triglycerides, which are
saturated or unsaturated fatty acid esters of glycerol.
The reaction of lipolytic enzymes should apply to the rate
constants of kinetic equations and it has been demonstrated
that the hydrolysis of triglycerides by pancreatic lipase
obeys the fundamental Michaelis-Menten equation (Sarda and
Desnuelle, 1958). Nevertheless, this agreement is very