ISOLATION AND CHARACTERIZATION OF *XANTHIUM* LIPASE

THESIS

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By

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This work is dedicated to Dr. David Calvin Whitenberg for his constant forbearance, understanding, and superior strength in giving guidance, trust, and encouragement during the experimental work and the preparation of this thesis which made the completion of my degree possible.

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CHAPTER I

INTRODUCTION

The fruit of the Xanthium sp. (cocklebur) contains two seeds; one is a large, non-dormant seed and the other is a small, dormant seed. Dormancy in the small seed can be broken and germination promoted by the presence of kinetin and light (9). Because an early event in the germination of seed involves the hydrolysis of stored foods, light and kinetin possibly interact to promote the synthesis or activation of specific hydrolytic enzymes, which in turn initiate the degradation of food reserves to supply energy for the germination process. Khan (9) has shown that inhibition of DNA-dependent RNA synthesis reverses the effect of kinetin and light, so synthesis of one or more enzymes may be required for the breaking of dormancy in cocklebur seeds. Since preliminary investigation showed that the principle food-storage material in cocklebur seeds is lipid, and because there is some evidence that light is important in the synthesis of lipase, it was decided to compare the development of lipase activity in germinating large and small seeds to determine whether lipase synthesis or activation is a primary event in the breaking of cocklebur dormancy.

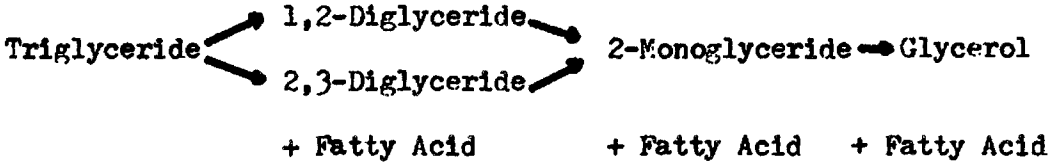
Published procedures for the isolation of seed lipase were found not to be applicable to cocklebur seed. As a result, it was necessary to develop analytical methods for the study of cocklebur lipase. This paper will present isolation methods and characteristics of the lipase

found in germinating large cocklebur seed.

CHAPTER II

LITERATURE REVIEW

During the germination of oil seeds, the catabolism of storage lipid usually takes place within a week. The breakdown of fat reserves, primarily triglycerides, provides the main source of energy for the germination process (24). Lipase is the enzyme that catalyzes the hydrolysis of triglycerides to free fatty acids and glycerol.



The rate of lipase reactions may be determined by measurement of either the rate of disappearance of the substrate (the triglyceride) (23), or the rate of production of the free fatty acids (14). Plant lipases have been isolated from many kinds of germinating seed, some of which are castor bean (11,14,20), peanut (20), cotton (12,20), wheat (8), lettuce (16), Cannabis and Cucurbita (23), and Vernonia (13). Castor bean lipase has been intensively investigated, but other seed lipases have not been purified or well characterized.

Several studies have been made on the effect of fatty acid chain length on the rate of hydrolysis of triglycerides. There is general agreement that short-chain glycerides are hydrolyzed at the maximum rate.

Sobotka and Glick (19) have determined the ability of pancreatic lipase to hydrolyze triglycerides that contained fatty acids ranging from 4 to 18 carbons in length, and have found tributyrin to be utilized most efficiently (Table I).

Sarda and Desnuelle (17) have clearly established that lipase hydrolyzes only water-insoluble substrates in a heterogeneous system. The emulsification of the triglyceride in a heterogeneous system is usually desirable in order to provide intimate contact between the enzyme and the substrate. As cited by Ory *et al.* (14), Wills has proposed albumin and gelatin as emulsifying agents. Other emulsifying agents that have commonly been used are: sodium taurocholate (3), and a mixture containing the fatty acid ester of polyethylene glycol, soy bean phosphatides, sorbitan monolaurate, sodium cholate and a polyglycerol ester of fatty acids (7). More recently, Desnuelle *et al.* (6) suggested 10% gum acacia for the emulsification of tributyrin. The use of these emulsifying systems with lipases from various sources has shown that the substrate specificity of a lipase is influenced by the nature of the emulsifying system. If the substrate is not emulsified or if the emulsification is inadequate, the rate of shaking becomes an important factor that affects the rate of hydrolysis (23).

Another factor that will influence the rate of hydrolysis of fats by lipase is the effect of pH on the lipase enzyme, the properties of the emulsified substrate, and the substrate:aqueous phase interface. Determinations of castor bean lipase activity by Ory *et al.* (14) have shown the pH range over which the enzyme is active to be between 4.0 and 5.0, with an optimum of 4.3. *Cannalis* and *Cucurbita* seeds both have lipases with an optimum pH of about 5.0 (23). Lettuce lipase has an

TABLE I

Comparison of Rates of Hydrolysis of Triglycerides by Pancreatic Lipase

(relative rates, maximum = 100)

Substrates	C atoms of fatty acids	rates
Tributyrin	4	100.00
Trivalerin	5	39.20
Tricaproin	6	47.60
Triheptylin	7	70.00
Tricaprylin	8	62.30
Tristearin	18	0.07
Triolein	18	2.40

acid optimum pH of about 3.5 to 4.0 (16), whereas most other seeds investigated have pH optimums in an alkaline range of 7.0 to 8.0. Sullivan (21) found that wheat, for example, has a pH optimum of 7.3 to 8.2, but Drapron (8) reported a wheat lipase optimum of 9.0 using the method described by Desnuelle et al. (6). Olney et al. (13) reported that the maximum release rate of free fatty acids for Vernonia anthelmintica seed lipase was around pH 7.5 to 8.0, and Ching (2) found that there were two pH optimums for Douglas fir: an acid lipase, pH 5.2 and a neutral lipase, pH 7.1.

Temperature also plays an important role in lipase hydrolysis, with the most effective temperature range being between 30° and 40°C. The optimum temperature for castor bean lipase activity was 40°C (14), Vernonia lipase was incubated in a range between 37° and 43°C with no apparent variance in activity (13), and lipase of soy bean was most active at 30°C (23). Desnuelle (5) has found that the rate of lipid hydrolysis, in a heterogeneous system, may not be affected by temperature in the range of 30° to 36°C, but hydrolysis becomes markedly dependent when bile salts are added. He has interpreted this effect to result from increased interfacial area produced by the detergent action of bile salts.

Desnuelle et al. (6) have suggested that the velocity of lipase action may depend upon the surface area of the emulsion where lipase is adsorbed at the fat:water interface. These workers have reported that lipases act only in heterogeneous systems, and are adsorbed at the fat: aqueous phase interface. Bile salts can increase the hydrolytic activity of lipase by acting as surface-active agents, and the increased rate of triglyceride hydrolysis may primarily be due to an emulsification

effect (5). The effect of emulsifying agents is complex and depends on the chemical nature of the agent and the source of lipase. Agents such as gum acacia, albumin, soaps, and synthetic detergents may increase the hydrolytic rate of some lipases, but inhibit the activity of others (23). Another factor that affects lipase activity is the rate of shaking of the reaction mixture. If the mixture is not shaken, then degrees of emulsification become important. If the triglyceride suspension is rapidly shaken then a fresh interface is constantly being made available to the enzyme.

Wills (23), in his review of lipase activators, has pointed out that lipases in general are activated by calcium ions. The mechanism of action of calcium as an activator is still in question, but the generally accepted explanation is that calcium ions remove fatty acids formed during hydrolysis, as insoluble calcium soaps. Wills (22) has further proposed that calcium ions also function to maintain lipase stability. He has shown that purified lipase is more sensitive to heat when calcium is removed by chelation. Other lipase activators mentioned by Wills (23) include Al^{+++}, Fe^{+++}, Sn^{++}, Cr^{++}, Fe^{++}, Mg^{++}, Mn^{++}, Pb^{++}, Co^{++}, Zn^{++}, Ba^{++}, NH_4^+, K^+, and Na^+. Chloride, bromide and oxalate anions have also been found to activate lipase, but NO_3^- and F^- anions were inhibitory.

Like many other hydrolytic enzymes, lipase is inhibited by several heavy metals (23), but the extent of inhibition depends on the experimental conditions. The buffer or the emulsifying agent may protect against inhibition. Also, oxidizing agents tend to destroy lipase activity. Castor bean lipase is inhibited by atmospheric oxygen and by H_2O_2, and the enzyme is protected by glutathione or by cysteine (23). This

inhibition may be the result of the oxidation of -SH groups. By the use of several specific sulfhydryl reagents, many attempts have been made to establish whether lipases are -SH enzymes. Singer (18) concluded that -SH groups were involved but were not part of the active site, and that different inhibitions observed were a result of the different molecular sizes of the substrates that caused different steric blocking of the active sites. Wills (22) studied the effect of several -SH reagents on lipase and found that in the case of wheat lipase, inhibition did in fact depend on the substrate used. Experiments with -SH reagents lead to the general opinion that lipases do not contain -SH groups as part of their active site, because were this true, much greater inhibition by -SH reagents would be observed. Difficulty arises in the correct explanation of the moderate inhibitory effect observed with various -SH binding reagents, but it is possible that these reagents react, less readily, with groups other than -SH, e.g. the -S- of methionine, or that -SH groups are part of the enzyme but not part of the active site. If the latter be true, then combination with -SH groups may cause interference with enzyme activity, possibly by a steric blocking effect.

Wills (23) has reviewed the literature concerning buffer systems that have been used in the extraction or assay of seed lipases. Phosphate buffers that contained one or more activating and/or protective agents have been used most frequently, although Tris-HCl and acetate buffers have been used in some cases. Other buffer systems have not been reported.

CHAPTER III

MATERIALS AND METHODS

Plant Material: Cocklebur seed (Xanthium pensylvanicum, Wallr.) were gathered locally. Seeds used in this investigation had been stored one year at room temperature at the time of the recorded data. Six large seeds were removed from the burs, sterilized with 10% Clorox solution, placed in 9 cm petri dishes on 2 layers of filter paper, moistened with 10 ml deionized water and grown in the dark (petri dishes were wrapped in foil) for up to 7 days.

Enzyme Preparation: The cotyledons were excised from 5 seeds, weighed and then ground in 4 times their weight of cold buffer. A Sorvall microhomogenizer was used and the plant material was ground for 2 minutes. The homogenate was transferred to a 30 ml Corex centrifuge tube and then centrifuged for 10 minutes at 8000 x g ($0^{o}C$). The supernatant contained no measurable enzyme activity and was subsequently discarded, while the pellet was resuspended in 6 ml of cold deionized water. A 1-ml aliquot of this suspension was removed and frozen for a later protein determination. The remaining sample was again centrifuged for 10 minutes at 8000 x g ($0^{o}C$), the supernatant discarded, and the pellet suspended in cold buffer. One-ml aliquots of this preparation were used for the enzyme assays and the controls.

A number of buffer systems of various pH values were employed in an attempt to obtain an active lipase preparation. Phosphate buffers used included: 0.1 M phosphate, pH 8.5; 0.1 M phosphate buffer containing 1 mM 2-mercaptoethanol, pH 7.3; 0.1 M phosphate buffer containing 1 mM 2-mercaptoethanol and 0.01 M EDTA, pH 8.8; 0.05 M phosphate buffer containing 0.01 M EDTA and 0.05 M cysteine-HCl, pH 5.0; and 0.1 M phosphate buffer containing 0.3 M KCl. Tris-HCl buffers employed included 0.05 M Tris-HCl, pH 7.0, alone or in combination with the following additives: 0.1 M $CaCl_2$, pH 8.2; 0.1 M $CaCl_2$ and 0.1 M ascorbic acid, pH 6.4; 0.1 M ascorbic acid, pH 7.2; 0.01 M 2-mercaptoethanol, pH 7.5; and 0.1 M ascorbic acid plus 0.01 M 2-mercaptoethanol, pH 7.5. Other buffer systems used were: 0.1 M citrate-PO_4, pH 5.0; 0.25 M acetate, pH 6.0; 0.5 mM HEPES, pH 8.0; and 0.5 mM HEPES plus 0.5 mM cysteine-HCl. Seed were also extracted with deionized water or 0.3 M KCl in an effort to obtain an active lipase preparation.

Substrate: The stock substrate consisted of an emulsion of 82.5 ml of 10% gum acacia, 7.5 gm crushed ice, and 10 ml tributyrin (1). This mixture was homogenized with a Sorvall Omni-Mixer for 2 minutes in the cold.

Assay: Ten ml of the stock substrate mixture and 19 ml deionized water were combined to form the final assay medium. This made a total volume of 30 ml after the enzyme preparation was added. The emulsion was put into a 50 ml beaker and placed in a thermostated water bath at 30°C, then a pH electrode was inserted into the solution and a small stream of CO_2-free nitrogen gas was bubbled through the reaction mixture. The pH of the substrate mixture was adjusted to the desired pH range with 0.1 N NaOH, a 1-ml aliquot of the enzyme preparation was added,

the pH was readjusted to the desired range, a stop watch was started, and a solution of 0.001 N NaOH was used to maintain a constant pH for 3 minutes. Milliequivalents of NaOH were then determined for the 3 minute lipase hydrolysis of the tributyrin substrate.

As a general procedure, enzyme preparations made with a given buffer were assayed over the range of pH 4.0 to pH 8.0 at intervals of 0.5 pH unit. In some cases, enzyme activity was determined at values up to pH 11.5.

Inhibition Studies: Because of the possibility that cocklebur seed contain a natural lipase inhibitor, the effect of cocklebur lipase concentration on reaction rate was studied with enzyme preparations obtained from HEPES:cysteine-HCl buffer. The effect of buffer concentration in the assay mixture on reaction velocity was also studied. To determine whether the supernatant liquid of the fractionated crude homogenate contained an inhibitor, portions of the particulate fraction and the supernatant liquid were recombined prior to assay. In addition, one series of experiments was conducted in which lipase obtained from cotton seed was added to the cocklebur homogenates in order to determine whether a general lipase inhibitor was present.

Protein Determination: Insoluble protein from the particulate matter of the 1-ml frozen water aliquot was hydrolyzed in 5 ml of 1.0 N NaOH for 15 minutes at 100°C, and the protein was determined by a modified version of the Lowry method described by Potty (15). Absorbance measurements were made at 500 nm with a Beckman Model 25 spectrophotometer, and the amounts of protein present in these samples were calculated on the basis of a standard curve made by the use of bovine serum albumin.

Lipid Utilization: To determine lipid utilization in the germin-

ating cocklebur seed, an extraction of carbohydrates and lipids was made with a Sorvall Omni-Mixer to grind weighed seeds in 20 ml hot 80% ethanol. Grinding was performed at top speed for 3 minutes, then the homogenate was transferred quantitatively to a centrifuge tube, centrifuged at 2000 x g for 10 minutes, the supernatant was decanted and saved, and the precipitate was suspended in 20 ml hot 80% ethanol. The sample was centrifuged again and the supernatant was added to the previous supernatant. This was repeated twice, all 80% ethanol extractions were combined and evaporated to dryness in a flash evaporator at 70°C. The residue was washed twice with 5 ml portions of petroleum ether and placed into a 60 ml separatory funnel. The evaporating flask was then washed twice with 5 ml portions of water: methanol (3:1) and once with 5 ml deionized water, and all washings were combined in the separatory funnel. The separatory funnel was swirled gently to prevent emulsion formation and the lower layer was drawn off. The petroleum ether layer was washed with 5 ml of deionized water, then the water was drawn off and the petroleum ether layer was poured into a tared weighing bottle. The separatory funnel was washed with two 3-ml portions of petroleum ether and these washings were added to the weighing bottle. The liquid was evaporated in a hood and dried at 70°C overnight in an oven. The bottle was then weighed for total lipid present from the weighed seeds.

Lipids: To determine the types of lipids present in the germinating cocklebur seed, thin-layer chromatography was used to fractionate the fats according to lipid classes. The method was that described by Mangold (10), using Silica Gel G for the adsorbent, heptane:diethyl

ether:glacial acetic acid (80:20:1) as the solvent and phosphomolybdic acid to detect the spots.

CHAPTER IV

EXPERIMENTAL RESULTS

Isolation of Crude Lipase: No lipase activity could be found in the crude homogenate of the cotyledons of the cocklebur seeds. When the crude homogenates were centrifuged, fractionation of layers was observed: a fatty layer was found at the top of the tube, a second fraction consisted of the supernatant liquid, and a pellet of particulate matter comprised the third fraction. There seemed to be no enzyme activity in either the fatty layer or the supernatant, but an alkaline lipase was present in the particulate fraction.

Buffers: Results of the effectiveness of the different buffer systems for preparation of an active cocklebur lipase extract are summarized in the Tables II, III, and IV. Most buffers used for preparation of this enzyme extraction did not prove successful even in combination with supposed activators and protective agents that had been used effectively with other lipases.

The phosphate buffer extract did show some apparent lipase activity when potassium chloride and 2-mercaptoethanol were added (Table II). The activity appeared greater at alkaline pH values, but was still not great enough to measure accurately. Cotton seed lipase can be extracted with phosphate buffer (20); therefore, cotton seeds were extracted and used for lipase analysis as a system for purposes of comparison. The

TABLE II

Activity for Buffer Extracts of Cocklebur Lipase

(activity in pH units/minute)

Assay pH Values	0.1 M PO_4 Buffer (pH 8.5)	0.1 M PO_4 Buffer + 0.3 M KCl (pH 4.65)	0.1 M PO_4 Buffer + 0.001 M 2-SHEtOH (pH 7.3)
4.0	none	none	none
4.5	none	none	none
5.0	none	none	none
5.5	none	none	none
6.0	none	none	none
6.5	none	none	none
7.0	none	none	none
7.5	none	none	.02
8.0	none	-	.01
10.0	-	-	-
10.5	-	-	none

Assay pH Values	0.1 M PO_4 Buffer + 0.001 M 2-SHEtOH + 0.01 M EDTA (pH 8.8)	0.05 M PO_4 + 0.1 M Vit. C (pH 6.5)	0.05 M PO_4 Buffer + 0.01 M EDTA + 0.05 M cysteine-HCl (pH 5.0)
4.0	none	none	none
4.5	none	none	none
5.0	none	none	none
5.5	none	none	none
6.0	none	none	none
6.5	none	none	none
7.0	none	none	-
7.5	none	none	-
8.0	none	none	-

Abbreviations: PO_4: Phosphate buffer; EDTA: Ethylenediaminetetraacetic acid; Vit. C: Ascorbic acid; 2-SHEtOH: 2-mercaptoethanol.

cotton lipase extracted with phosphate-KCl buffer was active and when added to cocklebur homogenates, no inhibition was detected.

The data in Table III indicate that Tris-HCl buffer was more effective for preparing an active cocklebur lipase, and with the activating agent, $CaCl_2$, plus the antioxidant, ascorbic acid, activity increased, although the -SH protecting agent, 2-mercaptoethanol, had no effect. With the citrate-phosphate buffer there was no activity observed and similarly with the acetate buffer no activity was shown in the lipase assays.

Not until the HEPES buffer was used for the lipase extraction was there considerable hydrolysis of the tributyrin substrate (Table IV). HEPES buffer containing 0.5 mM cycteine-HCl showed an increase in the amount of lipolytic activity. A detergent extraction was also made with the use of 0.5% (w/v) sodium deoxycholate with the above buffer system, but apparently the lipase was inhibited by this extraction buffer. There appeared to be greater than a two-fold increase using the HEPES buffer containing the 0.5 mM cysteine-HCl than using the HEPES buffer alone; therefore, the former buffer was decided upon as the extraction buffer for the cocklebur lipase. Since the assays at alkaline pH values showed the greatest lipase activity, an extracting buffer of pH 8.0 was used.

Inhibition Studies: Recombination of the supernatant liquid with the particulate fraction that contained active lipase resulted in complete inhibition of the lipase. The inhibitor(s) seem to be specific for the cocklebur enzyme; at least, no inhibition of cotton seed lipase was obtained. Extraction of 1 volume of the supernatant liquid with 3 volumes of petroleum ether or ethyl ether did not reduce the inhibitory effect of the supernatant. This suggests that a water-soluble inhibitor

TABLE III

Activity for Buffer Extracts of Cocklebur Lipase

(activity in pH units/minute)

Assay pH Values	0.05 M Tris-HCl Buffer (pH 7.0)	0.05 M Tris-HCl + 0.1 M $CaCl_2$ (pH 8.2)	0.05 M Tris-HCl + 0.1 M $CaCl_2$ + 0.1 M Vit. C (pH 6.4)
4.0	none	none	none
4.5	none	none	none
5.0	none	none	none
5.5	none	none	none
6.0	none	none	none
6.5	none	none	none
7.0	none	none	none
7.5	none	-	.02
8.0	-	-	-
8.5	-	-	.01

Assay pH Values	0.05 M Tris-HCl + 0.1 M Vit. C (pH 7.2)	0.05 M Tris-HCl + 0.01 M 2-SHEtOH (pH 7.5)	0.05 M Tris-HCl + 0.1 M Vit. C + 0.01 M 2-SHEtOH (pH 7.5)
4.0	none	none	-
4.5	none	none	-
5.0	none	none	-
5.5	none	none	-
6.0	none	none	-
6.5	none	-	none
7.0	.02	-	none
7.5	.02	-	none
8.0	.03	-	none
8.5	.02	none	none
9.0	-	none	none
9.5	-	none	-
10.0	-	none	-
10.5	-	-	none
11.0	-	-	none
11.5	-	none	-

Abbreviations: Vit. C: Ascorbic acid; 2-SHEtOH: 2-mercaptoethanol

TABLE IV

Activity for Buffer Extracts of Cocklebur Lipase

(activity in pH units/minute)

Assay pH Values	0.1 M Citrate-PO_4 Buffer (pH 5.0)	0.24 M Acetate Buffer (pH 6.0)	Water	0.3 M KCl
4.0	none	none	none	none
4.5	none	none	-	none
5.0	none	none	none	none
5.5	none	none	-	none
6.0	none	-	none	none
6.5	none	-	-	none
7.0	none	-	none	none
7.5	none	none	-	none
8.0	none	none	none	none
8.5	none	none	-	none
9.0	none	-	none	none

Assay pH Values	0.5 mM HEPES Buffer (pH 8.0)	0.5 mM HEPES + 0.5 mM cysteine-HCl (pH 8.0)
4.0	-	-
4.5	-	-
5.0	.02	-
5.5	.01	-
6.0	.01	-
6.5	-	-
7.0	.02	-
7.5	.10	.31
8.0	.22	.64
8.5	.25	.52
9.0	.12	.33

Abbreviations: HEPES: N-2Hydroxyethylpiperazine-N'-2-ethanesulfonic acid

is involved.

Figure 1 shows that if reaction velocity is plotted versus enzyme concentration, a sigmoid curve is obtained. Thus enzyme inhibition is observed at lower enzyme:assay medium ratios, but activation of the enzyme is seen at higher ratios. Such an effect is not due to the presence of HEPES:cysteine-HCl buffer in the assay medium since the reaction velocity for a given enzyme concentration remained constant as the buffer concentration was varied from 0 to 1.0 ml.

Optimum pH: The optimum pH for lipolytic activity was determined by an assay of the enzyme over a pH range of 4.0 to 11.0. Maximum lipase activity was observed between 8.0 and 8.2 (Figure 2: A and B).

Temperature: Besides the effect of pH and types of buffers employed, it has been shown that temperature also shows an effect on the activity of lipases. Incubations carried on at temperatures between 20° and 50°C indicated an optimum temperature near 30°C (Figure 3). The lipase was essentially inactive above 45°C, indicating destruction of the enzyme.

Lipid Utilization and Lipase Activity: It seemed apparent that lipase was active during the first week of germination since the lipid content decreased from 31% to 7% within the first five days (Figure 4). This indicated that a considerable amount of stored fatty material was used during the germination process. Lipase activity in germinating cocklebur seeds tends to increase during the first week of initiated metabolic activity. The relationship between lipase activity and lipid content is shown in Figure 4.

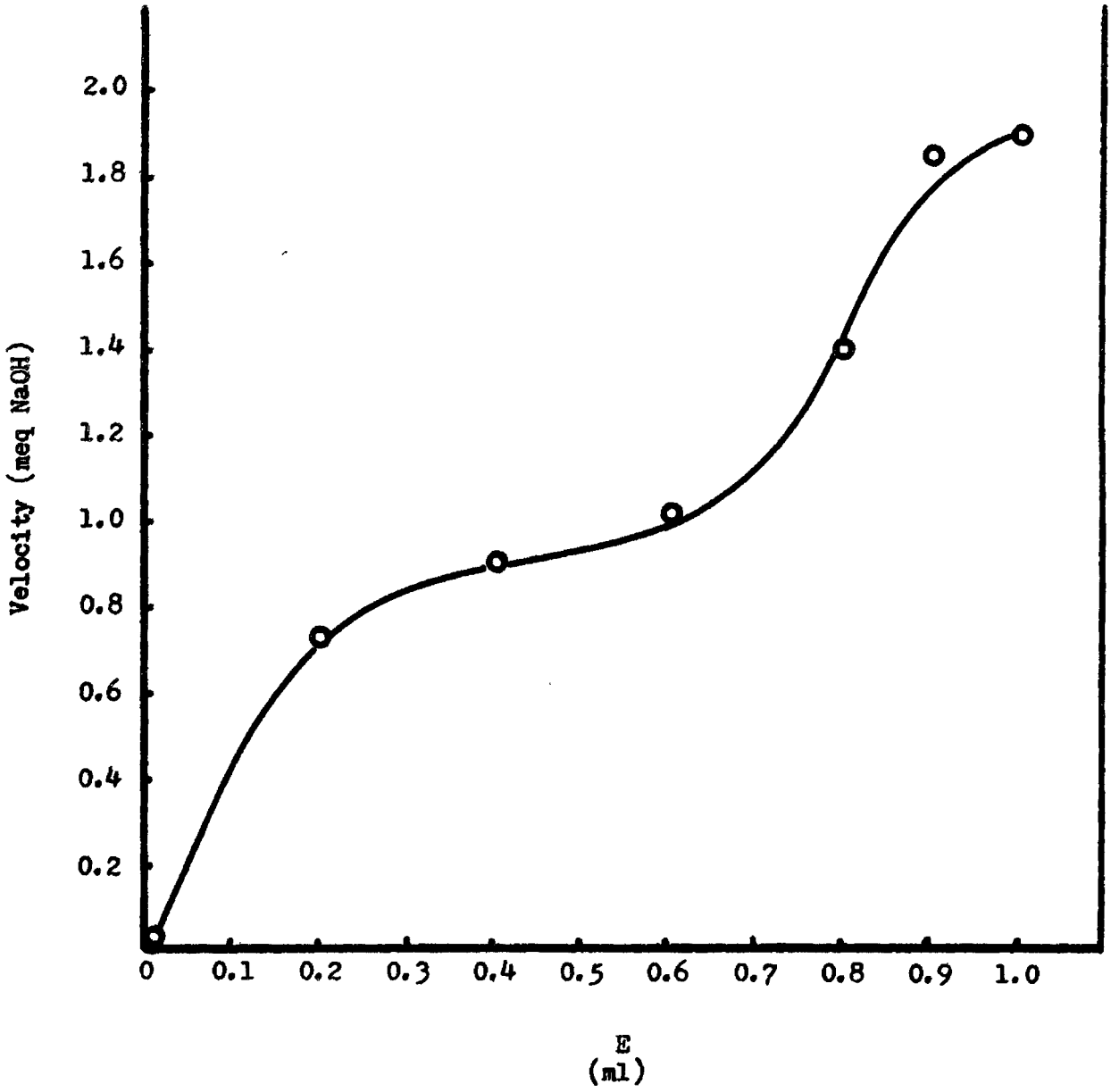


Figure 1. Effect of Enzyme Concentration on Reaction Velocity

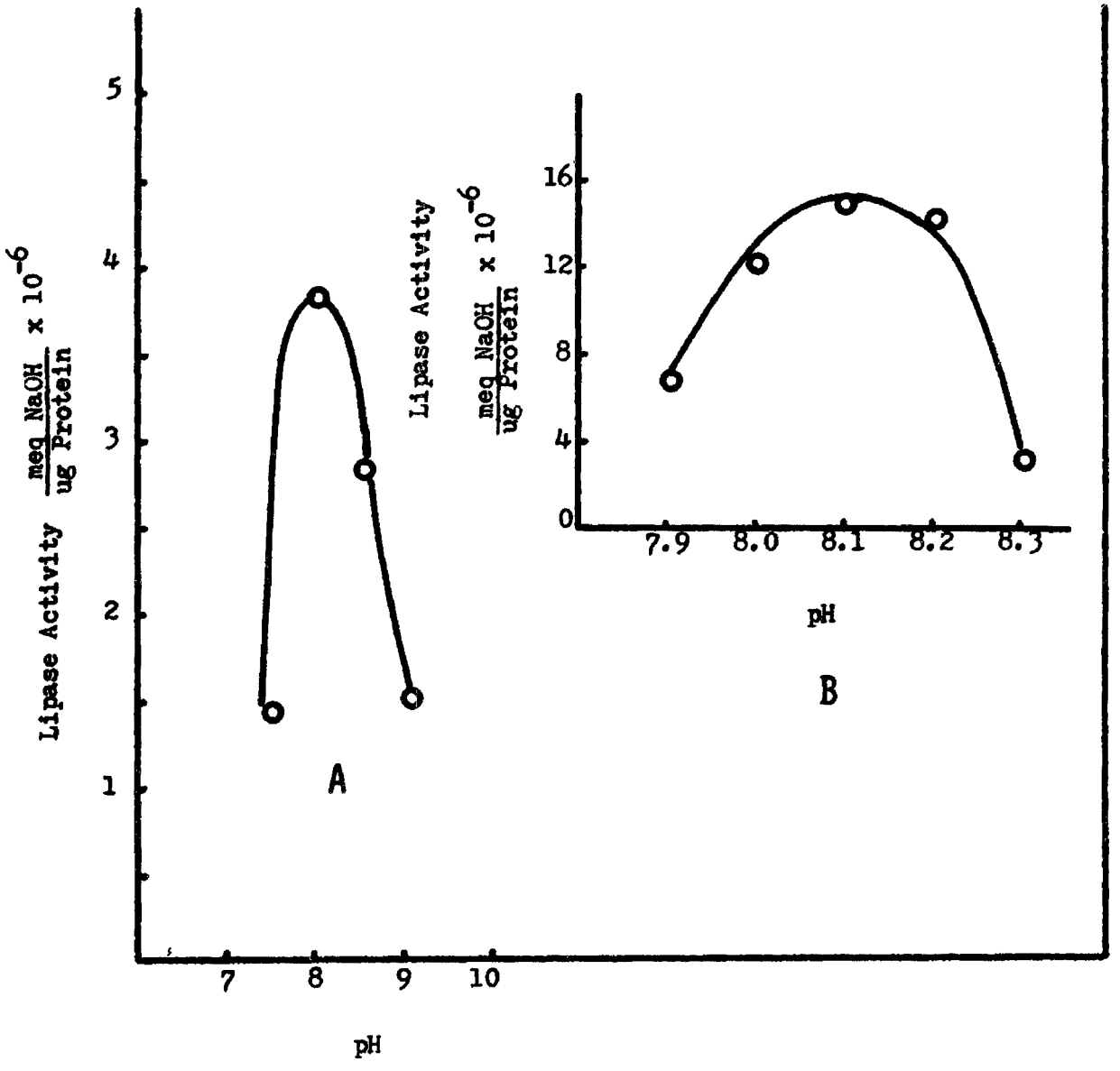


Figure 2. Effect of pH on the Activity of Cocklebur Lipase

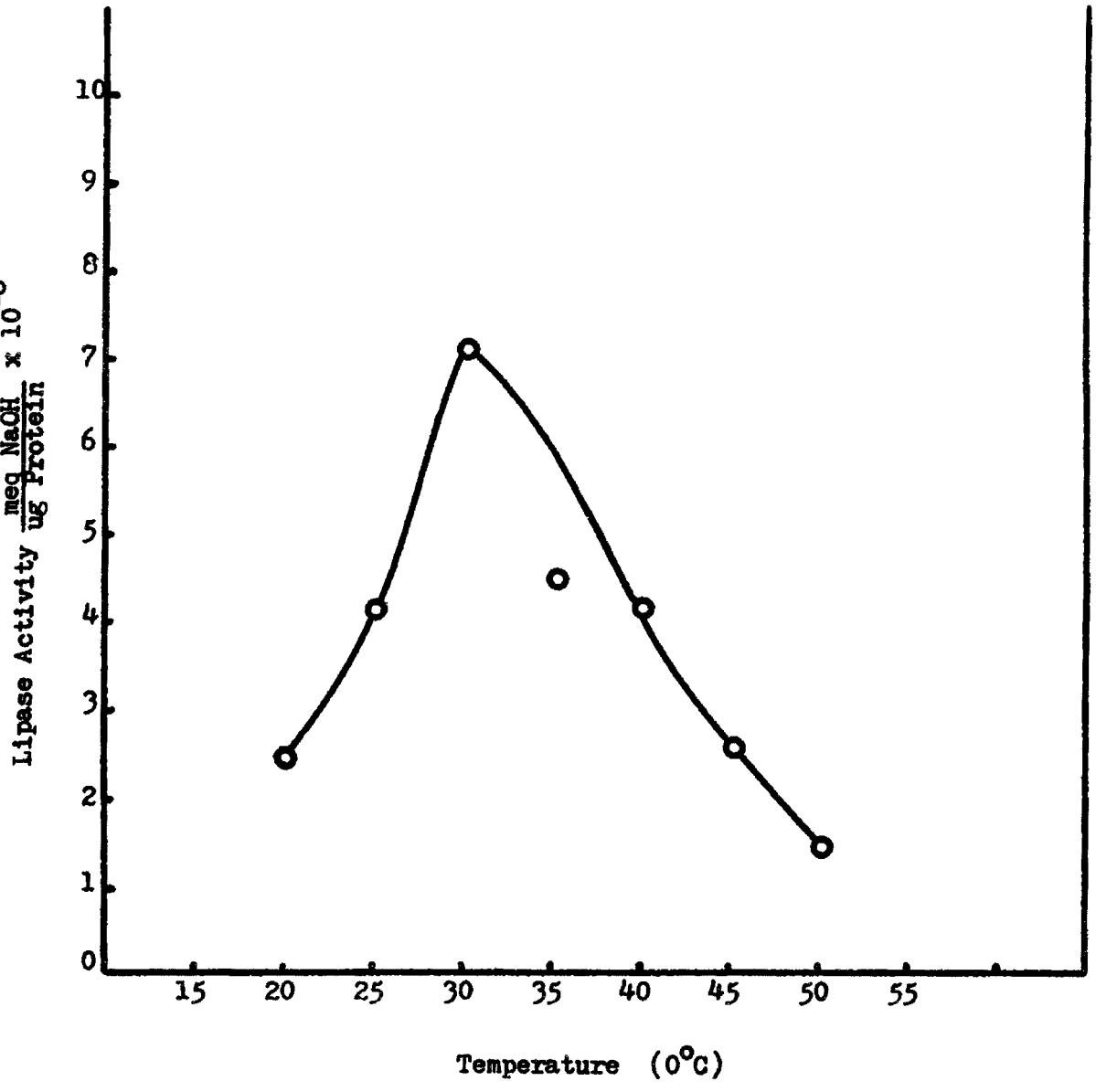


Figure 3. Effect of Temperature on the Activity of Cocklebur Lipase

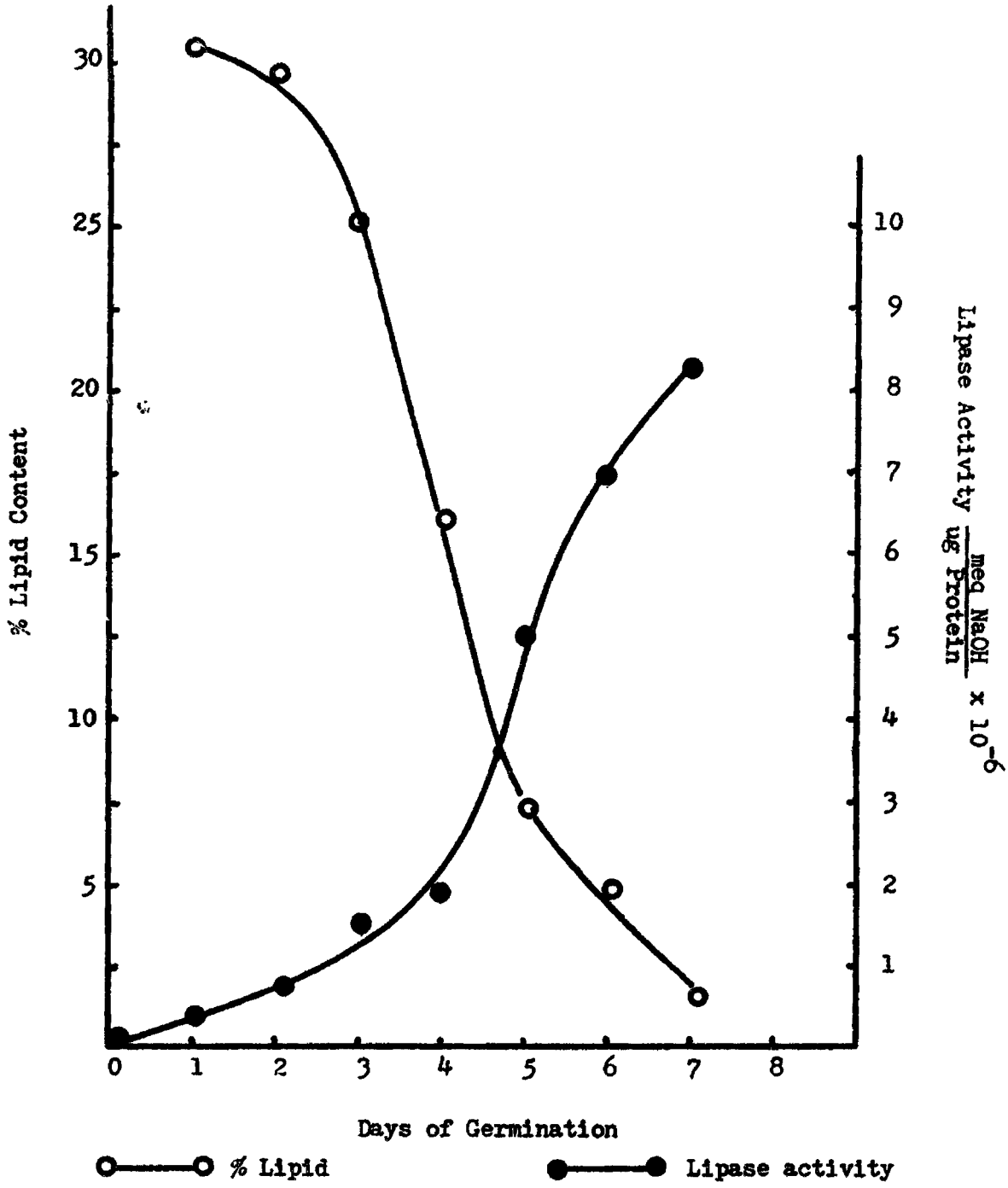


Figure 4. Lipid Utilization and Lipase Activity During a Seven-Day Germination Period

CHAPTER V

DISCUSSION

Results of this study show that germinating large seed of cocklebur contain an alkaline lipase with a pH optimum of 8.0 to 8.2 that is tightly bound to a particulate fraction of the seed. Such results are in agreement with those reported for several other kinds of seeds. An acid lipase, which has been found in other seeds, was not detected. Cocklebur lipase seems to be somewhat unique when compared to other seed lipases in that it is extremely sensitive to the buffer system employed for the homogenization of the seed. Phosphate buffers, which have been used routinely in the study of seed lipases, appear to be completely ineffective for the preparation of a crude lipase from cocklebur. Even the addition of activators, antioxidants, and -SH group protectants which have been reported to be effective in the preparation of active lipase extracts does not improve the usefulness of phosphate buffer as an homogenizing medium for cocklebur seed. Tris buffer, which has been used in some studies on seed lipase, was generally ineffective. Slight lipase activity was observed if cocklebur seeds were homogenized in 0.05 M Tris-HCl that contained 0.1 M ascorbic acid, but the activity was so low that the data obtained with this buffer system are considered to be unreliable. HEPES buffer has not been reported as an homogenizing medium for seed lipase studies, but it has proved useful for the isolation of

tobacco enzymes that are particulate in the native state (4), and was therefore employed in this study. Of the buffer systems used in this study, HEPES alone was effective for the preparation of an active cocklebur lipase. The addition of 0.5 mM cysteine-HCl increased the activity of the crude lipase by a factor of 2 to 3 over its optimum pH range.

The effectiveness of HEPES for the preparation of an active crude lipase from cocklebur cannot be explained adequately from the present data. However, at least 4 possibilities exist: (a) HEPES, possibly because of the sulfonic acid group present in its structure, unmasks the active site of the enzyme although the enzyme remains bound to particulate matter; (b) HEPES may function in a manner similar to some native activator that is present in the seed but which is removed by other buffers in the extraction procedure; (c) HEPES may act as an allosteric activator; or (d) HEPES may antagonize a natural lipase inhibitor that is present in the seed and released during the homogenizing process. The fact that a natural inhibitor is present can be seen from the fact that crude homogenates of cocklebur seed in HEPES buffer show no lipase activity, but the particulate fraction is active when removed from the supernatant solution. If the supernatant solution is added with the particulate fraction to the assay medium, inhibition of lipase again occurs. Although the nature of the inhibitor was not determined, it appears to be a water-soluble material since extraction of the supernatant liquid with petroleum ether or ethyl ether does not remove the inhibitor. The data presented in Figure 1 suggest that perhaps a combination of the first, third, and fourth possibilities listed above is involved. The initial part of the curve is indicative

of an internal inhibitor, but as enzyme concentration in the assay medium is increased, apparent activation results. Reaction velocity decreases again at the highest concentrations of enzyme employed, and this may indicate that there is some optimum ratio of enzyme, inhibitor, activator, and substrate that permits a maximum reaction rate. No change in reaction rate is obtained if enzyme concentration is held constant and buffer concentration in the assay medium is varied. Such results indicate that the buffer effects occur at the time the seed are homogenized and the particulate fraction is isolated. Possibly, then, HEPES acts as a mild detergent to release the inhibitor in a soluble form and to expose the active site of the enzyme. Some allosteric binding of HEPES during homogenization of the seed may also occur, and this may result in further activation of the lipase.

The literature review points out that -SH protectants often increase the activity of lipase preparations although there is general agreement that -SH groups are not a part of the active site. In view of the foregoing, one may speculate that cysteine is acting as an allosteric activator or is antagonizing the effect of the natural lipase inhibitor present in cocklebur seed. The arguments, of course, do not rule out the possibility that cocklebur lipase does contain -SH groups as part of the active site. Studies to elucidate the reason for activation of cocklebur lipase by cysteine were beyond the scope of this investigation.

Attempts to solubilize and purify the enzyme proved futile. The addition of deoxycholate inhibited the enzyme, but some other detergent might prove to be an effective solubilizer. Further homogenization with a Ten Broeck homogenizer in an attempt to obtain a finer particulate

fraction resulted in inactivation of the enzyme, and the NH_4OH fractionation procedure of Olney *et al.* (13) also destroyed the lipase activity.

Other characteristics of cocklebur lipase tend to be in general agreement with those of lipases from various seeds. The temperature optimum of 30°C falls within the general range of temperature optimums for seed lipases, and cocklebur lipase exhibits greater activity with triglycerides that contain short-chain rather than long-chain fatty acids. As might be expected, an increase in lipase activity is correlated with the decrease in lipid content of the seed during the 7-day germination period covered in this study.

Since seed lipases have not been well characterized, the unique characteristics of cocklebur lipase could form the basis for further studies to provide fundamental information about the nature and activity of plant lipases.

CHAPTER VI

SUMMARY

An active crude lipase tightly bound to a particulate fraction was obtained from germinating large cocklebur seed by extraction with 0.5 mM HEPES buffer that contained 0.5 mM cysteine-HCl. Various phosphate, Tris, citrate-PO_4, and acetate buffers were ineffective, as were deionized water and 0.3 M KCl.

The lipase was found to have an optimum pH of 8.0 to 8.2, a temperature optimum of 30°C, and to hydrolyze triglycerides that contain short-chain fatty acids. Lipase activity was found to increase in the germinating seed throughout the 7-day experimental period, and such increase was correlated with a decrease in lipid content of the seeds.

The difficulty in obtaining active lipase preparations from cocklebur seed appears to be due to the presence of a natural lipase inhibitor(s) present in the seed. The inhibitor seems to be water-soluble. Possible reasons for the ability of HEPES buffer and cysteine to overcome the action of the inhibitor are discussed.

CHAPTER VII

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