J. Lipid Research
April, 1960

Castor bean lipase: action on its endogenous substrate*

Robert L. Ory, Allen J. St. Angelo, and Aaron M. Altschul

Southern Regional Research Laboratory,
Southern Utilization Research and Development Division,
Agricultural Research Service, U. S. Department of Agriculture,
New Orleans 19, Louisiana

[Received for publication November 24, 1959]

SUMMARY

A method is described for obtaining a lipase emulsion from castor beans which rapidly hydrolyzes all of its endogenous substrate at an optimum pH of 4.0 to 4.2. The course of hydrolysis can be described by a first-order reaction; no additives are required for activity and the reaction goes to completion in less than 1 hour. The enzyme emulsion (oil-in-water) is stable for at least 2 weeks in a pH 7.0 phosphate buffer containing added cysteine and ethylenediaminetetraacetic acid; it is rather heat stable, but the activity is lowered by mechanical shaking. Reagents that react with sulfhydryl groups inactivate the enzyme; the inhibition by mercuric ion and *p*-chloromercuribenzoic acid can be reversed with an excess of cysteine. The lipase emulsion is not inhibited by diisopropylfluorophosphate in concentrations lower than 0.01 M, nor by protamine sulfate and heparin in relatively high concentrations. It is inhibited by 0.01 M fluoride.

Although there is an extensive literature on the action of seed lipases on their own substrates *in situ* (1, 2, 3) and of partially purified enzymes on a variety of substrates (4), there is no information on the behavior of the partially purified lipases on their own natural substrates. In most instances the source of lipase was oil-free tissue (5), but even when the original tissue was used, an excess of additional substrate was added; it is the hydrolysis of this added substrate which was followed.

Seed lipases might well be studied by starting first with crude systems and endogenous substrates. Thereafter any modifications in the enzyme system during purification can be detected by comparison of its behavior with that of the crude enzyme on its endogenous substrate. The lipase in castor beans *(Ricinus communis)* is particularly suitable for study of the crude system since this enzyme is reported to have its optimum activity at acid pH. Hence the operations of isolation of the enzyme and substrate can be carried out at neutral pH with no hydrolysis taking place.

One of the difficulties in studying this enzyme has been the presence of allergens in the pomace (6, 7). Instances are known in which investigators became allergic to this material and were required to terminate their research on the lipase at an early stage. It is possible to minimize this problem by extraction of the kernel with water or buffer followed by high speed centrifugation: the oil and a small amount of protein float to the top and the bulk of the proteins remain in the underlayer which is discarded. The fatty layer contains the lipase: this can easily be demonstrated by adjusting the pH to around 4, whereupon there is rapid and complete hydrolysis of the oil.

This is a report of the properties of the crude lipase of castor bean acting on its own endogenous substrate. The hydrolysis can be described as a first-order reaction throughout the range of substrate concentration with a maximum rate between pH 4 and 4.2. The enzyme in the presence of substrate is relatively heat stable; it is inhibited by reagents which react with sulfhydryl groups. Adjustment of pH is all that is necessary for activation of the enzyme; no additional calcium ion, other salts, or serum albumin are required for activity.

MATERIAL AND METHODS

Seed. Castor beans *(Ricinus communis)*, Cimmarron variety, were used unless otherwise indicated. The seeds were two years old when obtained; they were stored at 15°C until used.

* Presented in part at the 134th National Meeting, American Chemical Society, Chicago, September 7–12, 1958.

Preparation of Lipase Emulsion. The castor beans were soaked in water overnight at 4°C; all subsequent operations in preparing the lipase emulsions were also carried out at this temperature. The swollen seeds, containing about 20 per cent water, were dehulled, ground with sand in 20 ml. of extraction medium (0.05 M cysteine, 0.1 M phosphate buffer, pH 7.0, 0.01 M EDTA[1] per 20 to 25 g. of beans), and filtered through cheesecloth. This operation was repeated twice; the extracts were combined and centrifuged at 11,000 × *g* for 30 minutes. The thick fatty layer was removed by spatula, mixed with 2 volumes of the same buffer, and stored at 4°C until used. These lipase emulsions contain about 24 per cent lipid and 1 per cent protein. It is interesting to note that the ratio of protein to fat is 0.04, which is in the same range as that reported for chylomicrons (8).

Measurement of Activity. Two ml. of the lipase emulsion and 4 ml. of water were stirred by magnetic stirrer for 10 minutes at room temperature (24°C) in order to bring the test mixture into temperature equilibrium. This treatment did not impair the enzyme activity. An aliquot was withdrawn for analysis of initial ester (glycerides) content and 4 ml. of buffer at the same temperature was added to adjust to pH 4 (unless stated otherwise), thus starting the reaction. Samples for analysis were withdrawn at stated intervals and were pipetted into 10 ml. alcohol-ether (1:3, v/v), which was then boiled to coagulate the protein. These solutions were filtered through sintered glass to remove precipitated protein and evaporated on a steam cone to dryness. The dry fat was then taken up in 15 ml. of dry peroxide-free ether (the ether was always freshly purified by passage over a column of activated alumina), 0.3 ml. each of 2.5 per cent solutions in alcohol of hydroxylamine hydrochloride and sodium hydroxide were added, and the flasks placed in a water bath at 60°C until the ether was evaporated, and then held there for an additional 10 seconds to complete hydroxamate formation. Five ml. of ferric perchlorate (0.32 per cent in alcohol, prepared fresh daily) was added and, after waiting at least 20 minutes for maximum color development, the absorption of the samples was read in a spectrophotometer at 520 mμ.

This procedure for ester content is based on the formation of a ferric hydroxamate-fatty acid complex (9, 10). Methyl ricinoleate was the standard used for calibration of the analytical procedure.

[1] The following abbreviations are used in the text: EDTA, ethylenediaminetetraacetic acid; NEM, N-ethylmaleimide; PCMB, *p*-chloromercuribenzoic acid; and DFP, diisopropylfluorophosphate.

The relative rate of hydrolysis was obtained from the first-order rate constant as is described in the next section.

RESULTS

Rate of Hydrolysis. The course of hydrolysis in a typical reaction is given by the experimental points in Figure 1. When the logarithm of the ester concentration is plotted against time elapsed from the start of the reaction, the experimental points fall on a straight line (*A*, Fig. 1). The relative lipase activity (k/2.303) is given by the first-order rate constant,

$$\frac{k}{2.303} = \frac{\log S_o - \log S_t}{t},$$

where S_o is the original ester concentration and S_t is the ester concentration at time, t; the initial rate of hydrolysis may be calculated by multiplying the rate constant by the initial ester concentration. For the data in Figure 1 the initial rate is 6.2 mg. of ester per ml. per minute. Line *B* (Fig. 1) was calculated from the rate constant obtained from the data in curve *A* (Fig. 1); the open circles are the experimental points.

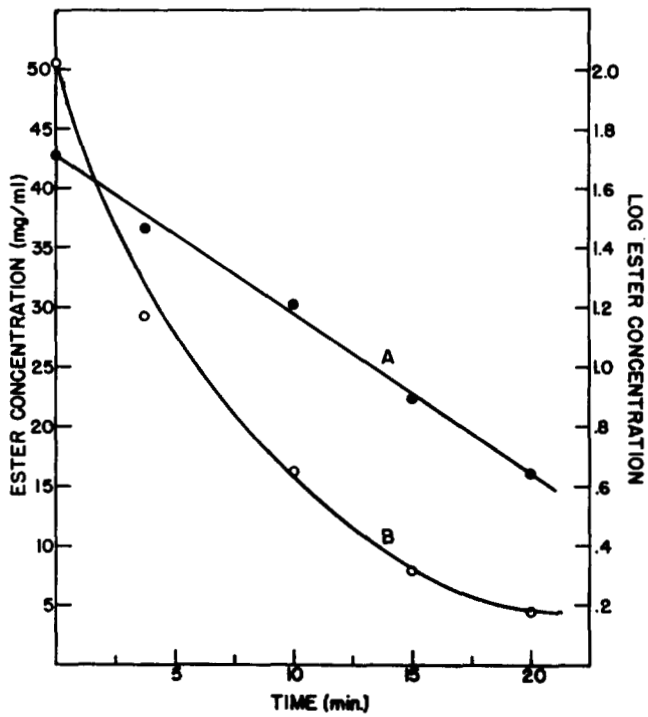

FIG. 1. Hydrolysis of endogenous castor oil by crude castor lipase at pH 4.5 in acetic acid-sodium acetate buffer. Reaction flask contained 2 ml. lipase emulsion, 4 ml. water, and 4 ml. buffer. The scale at the left is for curve *B*.

From these data it is evident that hydrolysis in this system is rapid and complete; no added fatty acid acceptor is needed, nor was there any evidence of reversal of hydrolysis. The course of hydrolysis is described by first-order kinetics over practically the entire range of substrate concentration. Such behavior differs from hydrolysis of fats by pancreatic lipase in which the relative rate of the reactions sharply decreases in the order: triglyceride → diglyceride; diglyceride → monoglyceride; and monoglyceride → glycerol (11). It is possible that the rate-limiting reaction in the hydrolysis by castor bean lipase is the step: triglyceride → diglyceride. Under such circumstances the reaction would have the same type of kinetics throughout the entire range of substrate concentration.

pH Optimum. The effect of pH on the relative rate of hydrolysis was obtained by using a number of different acetate and phosphate buffers. The optimum pH is in the range of 4.0 to 4.2. This is a little lower but in general agreement with the work of previous investigators who listed the optimum pH to be in the range of 4.5 to 5.0 (12, 13, 14).

Stability. The problem of stability in a system containing both enzyme and substrate must take into account both the hydrolysis of the substrate prior to testing and the stability of the enzyme. A comparison was made of the stability of substrate in two different media used for extraction and preparation of the lipase emulsion. Hydrolysis of endogenous substrate was rather rapid in an unbuffered medium adjusted initially to pH 7 with sodium hydroxide; the ester content was reduced from 22 to 5 mg. per ml. in 6 days. The substrate was stable for over a week in a cysteine-sodium hydroxide buffer.

The enzyme activity is stable in buffered media for at least 14 days; there does not seem to be any advantage in adding cysteine to a buffered solution. The combination of phosphate buffer (0.1 M, pH 7), cysteine (0.05 M), and EDTA (0.01 M), however, gave a somewhat higher rate; hence this was used as the standard medium in preparing the lipase emulsions.

The inclusion of cysteine was suggested by reports that some other plant lipases (15) are sensitive to sulfhydryl reagents and are stabilized by cysteine. The combination of EDTA and cysteine was prompted by its successful use in stabilizing papain (16).

Mechanical Stability. Each determination of lipase activity in these experiments was made on a separate vial of lipase emulsion which was stored undisturbed until assayed. This same stability was not observed when, in the course of experiments on successive days, aliquots of lipase emulsion were removed from a single preparation kept in a stoppered glass cylinder and shaken well before withdrawal of each aliquot. This suggested that the lipase was unstable to even this small amount of shaking. There was also the possibility that the renewal of oxygen which took place when the aliquot was withdrawn was a factor in reduction of activity.

To test these points, a freshly prepared standard lipase emulsion was divided into three parts: One-third was distributed as 2 ml. portions in 5 ml. capacity vials, stoppered, and stored at 4°C without further shaking; one-third was distributed into similar vials and placed on a slowly rocking apparatus at 4°C for continuous agitation; the remaining third was retained in the graduated cylinder at 4°C and shaken and opened twice or more daily to simulate normal sampling procedure. The results showed conclusively that the enzyme preparation is sensitive to shaking with or without renewal of the air above the emulsion. The enzyme in vials which were undisturbed retained activity for 18 days, whereas the other two groups kept for the same length of time decreased in activity 46 and 61 per cent, respectively. In view of this finding, samples from freshly prepared lipase emulsions were distributed into separate vials and stored undisturbed until tested.

Stability to Heating. Flasks containing the castor bean lipase emulsion, still at pH 7.0, were set in water baths at designated temperatures and time intervals, then immediately immersed in ice baths to reduce the temperature quickly. Afterward the flasks were placed on a magnetic stirrer, the buffer was added to initiate the reaction, and the rate of hydrolysis was measured in the usual way. The effect of length of heating at various temperatures on inactivation of the enzyme is shown graphically in Figure 2. It is noteworthy that

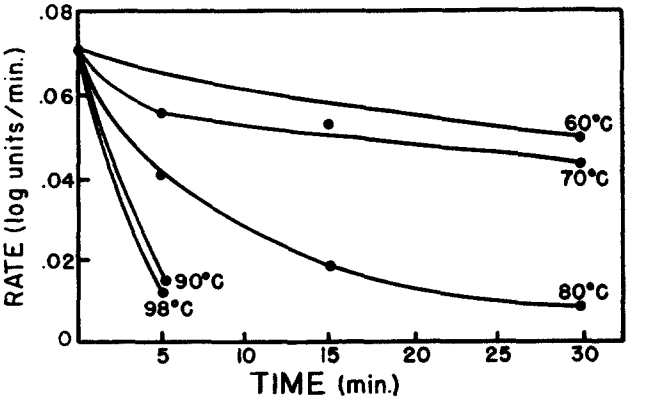

FIG. 2. Heat stability of crude castor lipase emulsion. The abscissa indicates the length of time heated at each temperature.

the castor lipase in the fatty layer seems rather heat stable: only about 20 per cent of the activity was lost by heating at 60°C for 30 minutes at pH 7.0. No activity was lost on standing for 60 minutes at 24°C.

Effect on Activity of Soaking of Castor Beans. Since some seeds, e.g., peanut and cottonseed (17), exhibit little or no lipolytic activity *in vitro* but assume such activity on germination, the question arose whether the presoaking of the castor beans in water even at 4°C might be inducing germination with a subsequent increase in lipase activity. Lipase emulsions prepared from presoaked and unsoaked beans were tested for activity in the usual way. There was little difference in relative activity, the rates being 0.092 and 0.076 log units per minute, respectively. Presoaked beans were much easier to dehull; hence presoaking was included as part of the standard procedure.

Effect of Variety and Age of Seed. There is a decrease in lipase activity of castor beans on long storage: the relative activity of the enzyme from two-year-old beans was 0.064 log units per minute, and after another year of storage of the beans, it was 0.036. A fresh sample of castor beans (less than one year old), Baker 296 Inbred variety, had a relative enzyme activity of 0.065 log units per minute.

Effect of Metal Ions. Various metals, as the acetate salts, adjusted to pH 6.0 to 7.0, were incubated with the lipase emulsion containing no cysteine or EDTA for 10 minutes. Then the pH was lowered and the activity measured with the results as summarized in Table 1. Ferric iron, zinc, and magnesium ions in the concentrations listed had no effect on the enzyme. Calcium ion inhibited but slightly at the high concentration. It accelerated the reaction at lower concentrations but not to the extent reported for other lipases, for example, pancreatic lipase (18). Copper ion (Cu^{2+}) inhibited at the higher concentration; lead produced slight inhibition. The most powerful inhibitor of the group tested was mercuric ion, which produced 67 per cent inhibition at a concentration of 6×10^{-5} M.

Effect of Additives. The influence of various other additives on the activity of the lipase emulsion was studied in a similar manner with results as given in Table 2. Serum albumin, which acts as a fatty acid acceptor for lipoprotein lipase (19), had no effect on the rate or course of hydrolysis over a wide range of concentration. Though it had no effect on the rate of reaction, serum albumin did have a visible effect on the appearance of the emulsion; when it was present in the reaction mixture, the emulsion appeared to be more stable physically. Protamine and sodium heparinate inhibit lipoprotein lipase of adipose tissue (20), but neither had any influence on the activity of this enzyme.

Sodium fluoride (0.01 M) inhibited the activity of castor bean lipase. This behavior is in accord with the reported inhibition of other lipases by fluoride (21, 22). Cyanide did not inhibit in the concentration used.

PCMB produced complete inhibition at 0.001 M concentration. Inhibition by NEM, much less potent in this instance than PCMB, seemed to depend upon the time of incubation. Cysteine completely reversed the inhibition by PCMB; it also reversed inhibition by mercuric ion. This evidence is suggestive that at least one —SH group is involved in the functional activity of the lipase.

DFP, a powerful inhibitor of some hydrolases, does not inhibit pancreatic lipase and in some concentrations even appears to stabilize the enzyme (23), presumably protecting it from destruction by other enzymes present in the crude preparation. DFP inhibited the castor bean lipase emulsion, but only incompletely and in much higher concentrations than needed normally; 0.02 M and higher as compared to 10^{-6} M and lower reported for other enzymes.

TABLE 1. EFFECT OF METAL IONS ON HYDROLYSIS OF ENDOGENOUS SUBSTRATE BY LIPASE EMULSION *

Metal	Concentration	Relative Activity
	M	*Per cent*
None		100
Ca^{++}	1×10^{-2}	76
	6×10^{-3}	100
	1×10^{-3}	137
	6×10^{-4}	124
Zn^{++}	6×10^{-4}	99
Fe^{+++}	6×10^{-4}	94
Mg^{++}	1×10^{-3}	101
Pb^{++}	6×10^{-4}	86
Cu^{++}	1×10^{-3}	54
	6×10^{-4}	91
	6×10^{-5}	107
Hg^{++}	1×10^{-4}	28
	6×10^{-5}	33
	3×10^{-5}	87
	6×10^{-6}	111

* Each flask contained 2 ml. of lipase emulsion prepared in 0.1 M Tris buffer (pH 7.2), a solution of the metal ion, and water to make a total of 9.5 ml.; this mixture was incubated for 10 minutes; the reaction was initiated by addition of 0.5 ml. of 0.5 M acetate buffer (pH 4.0).

TABLE 2. EFFECT OF ADDITIVES ON ENDOGENOUS HYDROLYSIS BY LIPASE EMULSION *

Additive	Concentration	Length of Incubation	Relative Activity
			per cent
None			100 †
Serum albumin	0.5–2.0%	10 min.	100
Protamine sulfate	150–300 μg./ml.	10 min.	100
Heparin, sodium salt	1 mg./ml.	10 min.	100
N-Ethylmaleimide	0.002 M	15 min.	79
	0.002 M	18 hrs.	53
	0.004 M	18 hrs.	46
p-Chloromercuri-benzoic acid	0.001 M	30 min.	0
p-Chloromercuri-benzoic acid ‡	0.001 M	30 min.	100
Diisopropylfluoro-phosphate	0.2 M	18 hrs.	31
	0.025 M	18 hrs.	50
	0.01 M	18 hrs.	106
Cyanide §	0.005 M	10 min.	92
Fluoride §	0.01 M	10 min.	41

* Each flask contained 2 ml. of lipase emulsion prepared in 0.1 M phosphate buffer (pH 7.0), a solution of additive, and water to make a total of 6 ml.; this mixture was incubated for the length of time indicated; the reaction was initiated by addition of 4 ml. of 0.1 M acetate buffer (pH 4.0).

† Appropriate controls with no additives, incubated for the same lengths of time, were used in calculating relative activity.

‡ After 30 minutes of incubation, 0.005 M cysteine was added; 30 minutes later buffer was added to initiate the reaction.

§ Enzyme prepared in Tris buffer.

DISCUSSION

The procedure for isolation of the enzyme, together with its substrate, seems to provide a complete lipase system. This is reflected in the rapidity of the reaction and its completeness without additives. In much of the literature on seed lipases, reactions are carried out for periods of 2 hours to as long as 10 days. It is questionable whether the hydrolytic changes that take place in some of the longer periods are mediated by physiological enzyme systems; more likely these are vestiges of enzyme systems. This system also seems to have a greater stability to water. Moulé (24) has reported that incubation of castor bean pomace in water at 20°C for 1 hour destroyed 90 per cent of its lipase activity; but in the experiments presented here there is no evidence of such extraordinary instability to water, either during storage of the lipase emulsion or during measurement of rate of hydrolysis. Whether the stability of our preparation is due to the presence of substrates or to the method of preparation, which does not involve prior removal of fat, cannot be stated from this work.

Examination of the active emulsions under the microscope and with the use of water- and oil-soluble stains, before and after adjustment to acid pH, indicated that the emulsions are of the oil-in-water type. A freshly prepared lipase emulsion will "cream" after standing for several hours. Nevertheless, this will quickly reconstitute on mixing. When the lipase emulsion has been shaken for several weeks or heated as described previously, it will take on an oily appearance. We have not been able to associate directly this weakening in the emulsifying activity with lowering of lipase activity. The two seem to go hand in hand in experiments on shaking or heating, but it is possible to inhibit the lipase activity entirely by addition of mercuric ion or of PCMB without interfering with its emulsifying properties.

There seems to be every indication that sulfhydryl groups are part of the active system of the enzyme. Although it is not necessary to have cysteine or other reducing agents present for full activity of the enzyme, there is more activity in its presence and with EDTA. It is entirely possible that when the substrate is removed, cysteine or some other reducing agent will be needed to maintain activity, just as similar reducing agents are needed for maintenance of activity of papain (16). The effect of EDTA might be to remove interfering ions, such as copper, which were demonstrated to have an inhibitory effect. The fact that the enzyme is not inhibited by EDTA is also presumptive evidence that no metal coenzymes are part of the system.

The lipase system described herein can obviously serve only as a guidepost for further purification of the enzyme. Preliminary experiments (25) have shown that it is possible to remove the substrate from the lipase emulsion by extraction with ether in the presence of saturated salt solution. The resulting preparation catalyzes hydrolysis of glycerides of long-chain fatty acids without addition of emulsifiers or fatty acid acceptors; here too the course of the reaction can be described by first-order kinetics.

The authors wish to thank Mr. Don S. Bolley, of The Baker Castor Oil Company, for the supply of castor beans.

REFERENCES

1. Altschul, A. M. In *Cottonseed and Cottonseed Products*, edited by A. E. Bailey, New York, Interscience Publishers, Inc., 1948, p. 157.
2. Browne, Jr., C. A. *J. Am. Chem. Soc.* **25**: 948, 1903.
3. Lewkowitsch, J., and G. H. Warburton. *Chemical Technology and Analysis of Oils, Fats and Waxes*, 6th ed., New York, Macmillan, 1922, vol. 2, p. 558.
4. Bamann, E., and E. Ullmann. In *Handbuch der Pflanzenphysiologie*, edited by W. Ruhland, Berlin, Springer-Verlag, 1957, vol. 7, p. 109.
5. Longenecker, H. E., and D. E. Haley. *J. Am. Chem. Soc.* **57**: 2019, 1935.
6. Bolley, D. S., and R. A. Holmes. In *Processed Plant Protein Foodstuffs*, edited by A. M. Altschul, New York, Academic Press, Inc., 1958, p. 840.
7. Rose, W. G. *J. Am. Oil Chemists' Soc.* **28**: 47, 1951.
8. Rodbell, M. *Science* **127**: 701, 1958.
9. Hill, U. T. *Anal. Chem.* **19**: 932, 1947.
10. Bauer, F. C., Jr., and E. F. Hirsch. *Arch. Biochem.* **20**: 242, 1949.
11. Marchis-Mouren, G., L. Sarda and P. Desnuelle. *Arch. Biochem. Biophys.* **83**: 309, 1959.
12. Bamann, E., and E. Ullmann. *Biochem. Z.* **312**: 9, 1942.
13. Haley, D. E., and J. F. Lyman. *J. Am. Chem. Soc.* **43**: 2664, 1921.
14. Guillemet, R. *Compt. rend. soc. biol.* **108**: 781, 1931.
15. Luchsinger, W. W., L. S. Cuendet, P. D. Boyer, and W. D. Geddes. *Cereal Chem.* **32**: 395, 1955.
16. Kimmel, J. R., and E. L. Smith. *J. Biol. Chem.* **207**: 515, 1954.
17. Olcott, H. S., and T. D. Fontaine. *J. Am. Chem. Soc.* **63**: 825, 1941.
18. Sarda, L., and P. Desnuelle. *Biochim. et Biophys. Acta* **30**: 513, 1958.
19. Korn, E. D. *J. Biol. Chem.* **215**: 1, 1955.
20. Korn, E. D., and T. W. Quigley, Jr. *J. Biol. Chem.* **226**: 833, 1957.
21. Rothschild, P. *Biochem. Z.* **206**: 186, 1929.
22. Lipmann, F., and L. C. Tuttle. *Biochim. et Biophys. Acta* **4**: 301, 1950.
23. Desnuelle, P., M. J. Constantin and L. Sarda. *Bull. soc. chim. biol.* **38**: 625, 1956.
24. Moulé, Y. *Oléagineux* **8**: 561, 1953.
25. Altschul, A. M., R. L. Ory and A. J. St. Angelo. *Federation Proc.* **18**: 180, 1959.