[CONTRIBUTION FROM THE LABORATORY OF THE DEPARTMENT OF AGRICULTURAL AND BIOLOGICAL CHEMISTRY, THE PENNSYLVANIA STATE COLLEGE]

Further Studies on the Nature of Ricinus Lipase and its Action[1]

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Considerable recent progress has been made along special lines of enzyme research, especially in the elucidation of the nature and mode of action of enzymes. This progress has been witnessed in connection with nearly all except the fat splitting enzymes. Ricinus lipase, the lipolytic principle of the castor bean (*Ricinus communis*) is the most highly active of this particular group. It, however, has received the least attention during the recent advances in our knowledge of enzymes. This fact is probably due to a variety of underlying reasons chief of which is the discouraging sensitivity of most workers to the dried fat free castor bean preparations.

The present communication represents an attempt to contribute to the existing knowledge of the nature of the lipolytically active principle of the castor bean and the mode of its action.

Experimental

A. Materials and Methods.—The experimental procedures and techniques followed in these studies were essentially the same as those previously reported.[2] The considerable husk on the castor beans[3] was removed prior to extraction in all cases except where a specific experiment was conducted to redetermine the necessity of this operation[4-6] (*cf.* Table II).

Method of Calculation of Lipolytic Activity.—A formula for the calculation of the percentage hydrolysis of an oil is given by Longenecker and Haley.[2] Through a typographical error in the original paper, the term (× 100) in the formula was omitted. The correct expression of the formula is

$$\frac{\text{ml. 0.1 } N \text{ alkali (sample)} - \text{ml. 0.1 } N \text{ alk. (blank)}}{\text{Saponification no.}^{7} - \text{Free fatty acid no.}^{7}} \times 100 = \% \text{ hydrolysis}$$

A standard procedure was adopted for the estimation of lipolytic activity. This was followed unless mention is made of a special procedure in some of the following experiments. This "standard determination" consisted of introducing definite weights of olive oil and lipase material into 5-ml. hard glass bottles which were then brought to the proper temperature (37–38°). Acetic acid (0.1 *N*) was next added to provide the necessary water for the reaction and to bring the *p*H of the mixture to the optimum.[8] The same sample of olive oil was used throughout the work reported in this paper.

While the formula mentioned above permits the calculation of the percentage hydrolysis, it is desirable at times to know the relative activity of different preparations as compared to each other. To express this activity an arbitrary measure, the lipase unit (L. U.) has been derived.[9] The formula $1000/WT$ = L. U., in which T is the time of reaction in minutes required to effect a 40% hydrolysis of olive oil (saponification no. 190), and W is the weight of the enzyme preparation in grams.

The time required for a 40% hydrolysis may best be determined by plotting the course of the reaction against time and determining the point at which 40% of the material is hydrolyzed. The point of 40% hydrolysis was chosen as the basis of calculations after a number of observations indicated that the cumulative effects of contact with the digestion mixture (discussed later) were not appreciable until some time after 50–60% of the olive oil had hydrolyzed. Typical results from the calculations of the lipase units in two active preparations are shown in Table I.

TABLE I

ENZYME UNITAGE OF TWO TYPICAL RICINUS LIPASE PREPARATIONS

Wt. lipase material, g.	Time for 40% hydrolysis, min.	Lipase units, L. U.
Preparation 13a, passed 20-mesh sieve		
0.100	16	625
.080	19.5	636
.070	23	621
.060	26	641
.050	38	526
.040	51	500
Preparation 13b, passed 40-mesh sieve		
0.100	11	909
.080	15	890
.060	19.5	900
.050	22	910
.040	30	825

Effect of Decortication on Lipase Activity.—A large number of whole beans was subdivided into two samples: from one the outer husk was removed prior to the extraction, while the other was treated in the usual manner except that the husk was not removed. The relative activities of the two samples are shown in Table II.

(1) Authorized for publication on January 16, 1937, as paper No. 757 in the Journal Series of the Pennsylvania Agricultural Experiment Station. Submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy at the Pennsylvania State College.

(2) Longenecker and Haley, THIS JOURNAL, **57**, 2019 (1935).

(3) We are indebted to The Baker Castor Oil Co. of Newark, N. J., for a generous supply of large size Manchurian beans.

(4) Nicloux, *Mem. Soc. Biol.*, **56**, 701, 839, 868 (1904).

(5) Armstrong and Ormerod, *Proc. Roy. Soc.* (London), **78B**, 376 (1906).

(6) Noyes, Lorberblatt and Falk, *J. Biol. Chem.*, **68**, 135 (1926).

(7) Expressed in ml. 0.1 *N* alkali.

(8) Haley and Lyman, THIS JOURNAL, **43**, 2664 (1921).

(9) The suggestions of Dr. E. S. Flinn have been helpful in this portion of the work.

TABLE II

COMPARISON OF ACTIVITIES OF RICINUS LIPASE FROM WHOLE AND DECORTICATED BEANS

Time of digestion, min.	Hydrolysis, % A	B
6	..	28.3
10	39.7	40.0
15	33.5	53.0
30	76.3	72.9
45	82.1	81.7
60	85.8	86.0
120	89.0	92.6
180	90.7	..
300	92.7	..

Effect of Fat Solvent on Lipase Activity.—Since it was observed that the extraction of the beans proceeded more rapidly with some solvents than with others, the activity of bean preparations extracted with ethyl ether, acetone and a carefully fractionated (b. p. 32–34°) petroleum ether were compared with those extracted with ordinary petroleum ether (b. p. 20–40°). In all cases 1.0 g. of olive oil, 0.100 g. of lipase and 0.6 ml. of 0.1 *N* acetaldehyde were used. The results are shown in Table III.

TABLE III

PERCENTAGE HYDROLYSIS OF OLIVE OIL WITH RICINUS LIPASE PREPARED WITH VARIOUS SOLVENTS

Solvent used	Time of digestion, hrs. 1	4	24
Petroleum ether (20–40°)	59.0	81.3	90.2
Petroleum ether (32–34°)	64.2	82.4	95.6
Ethyl ether	22.1	52.3	72.0
Acetone	20.7	41.1	57.0

Effect of Different Amounts of Water and Oil on Apparent Lipase Activity.—To determine the optimum conditions for lipase activity, the amounts and proportions of olive oil and water in the digestion mixture were varied, as shown in Table IV. The quantities of lipase material (0.100 g. of 417 L. U.), and acetic acid (equivalent to 0.6 ml. of 0.1 *N*) were kept constant in this experiment, and the e. m. f. of the solutions determined with a quinhydrone electrode to assure the proper *p*H.

TABLE IV

ACTION OF RICINUS LIPASE IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF OLIVE OIL AND WATER

Composition digestion mixture Oil, g.	Water, g.	E. m. f.$_{25°}$, −mv.	Hours 1	5	24 Hydrolysis, %
1.00	0.02	...	4.4	14.0	..
1.00	.03	...	16.9	30.0	37.6
1.00	.05	170	30.6	49.3	60.5
1.00	.06	172	35.5	49.8	59.7
1.00	.10	172	43.0	65.9	74.3
1.00	.20	173	50.6	74.0	86.6
1.00	.30	170	53.6	81.8	91.5
1.00	.60	172	55.8	73.9	94.2
1.00	1.00	172	50.1	75.6	93.4
1.00	2.00	180	42.8	74.7	92.9
1.00	3.00	190	40.2	68.0	90.7
0.50	0.60	175	78.1	100.0	..
1.00	0.60	172	56.1	73.9	94.2
1.50	0.60	172	40.0	68.0	..
2.00	0.60	172	34.8	60.7	..
3.00	0.60	178	18.7	52.0	..

Effect of Water on Lipase Activity.—To demonstrate the inactivation of lipase preparations by water two series of samples were treated under the following conditions. In series A, 0.100 g. of lipase preparation (910 L. U.) was placed in 0.5 ml. of carbonate-free distilled water at 37°, and held at this temperature for different lengths of time. At the end of the holding period 1.00 g. of olive oil and 0.1 ml. of 0.6 *N* acetic acid was added and the percentage hydrolysis determined. In series B the same amount of lipase preparation was placed in 1.00 g. of olive oil and 0.5 ml. of carbon dioxide-free distilled water and held at 37° for varying lengths of time. At the end of the holding period 0.1 ml. of 0.6 *N* acetic acid was added likewise, and the percentage hydrolysis determined. No correction was necessary for the action of the enzyme with water alone. Fig. 1 shows the results of this experiment.

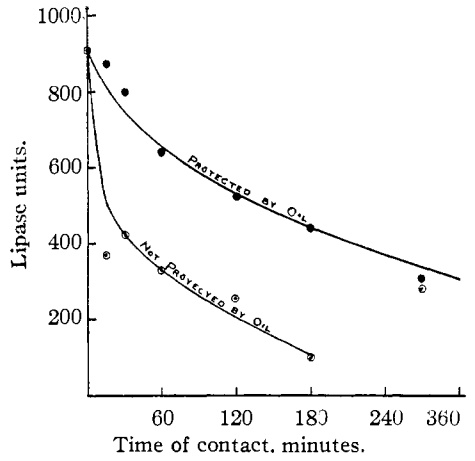

Fig. 1.—The effect of water on lipase activity.

Effect of Contact with Substrate and End-Products on Lipase Activity.—It has long been known that adsorption complexes may be formed between the enzyme and its substrate and between the enzyme and the end-products of the reaction. Such adsorption tends to inactivate the enzyme. In the case of Ricinus lipase and olive oil, the end-products are chiefly oleic acid and glycerol.

In preliminary experiments, the effect of oleic acid, olive oil and glycerol on the lipase were studied in the following manner: 2-g. samples of a lipase preparation (417 L. U.) were shaken at intervals during two days with 20 ml. each of the three materials. The undissolved material was filtered off and the residues from the oleic acid and olive oil were extracted separately with petroleum ether (b. p. 30–32°). The glycerol residues were not workable. The preparations thus obtained were used in standard determinations of five and one-half hours duration, together with a sample of untreated lipase preparation as a control. The results were as follows

Treatment	% Hydrolysis
Control	85
Oleic acid	52
Olive oil	75

The effect of oleic acid in inactivating the lipase preparation is thus apparent. A further test was made in which five series of samples were studied. The results are shown in Fig. 2. In all series, 0.100 g. of lipase preparation (670 L. U.) and 0.6 ml. of 0.1 *N* acetic acid were used. In series A, 1.00 g. of olive oil was used and constituted a standard determination; in series B, 2.00 g. of olive oil was used. In series C the reaction with 1.00 g. of oil was allowed to proceed for twenty-four hours, after which an additional 1.00 g. of oil was added, the bottles being shaken for three minutes, and the percentage hydrolysis determined in the usual fashion at intervals. Series D was similar in all respects to series C except that at the end of twenty-four hours a third portion of 1.00 g. of oil was added, and the mixture allowed to react for an additional twenty-four hour period, after which the percentage hydrolysis was determined as in the other series. The results are expressed on the basis of 1.00 g. of olive oil.

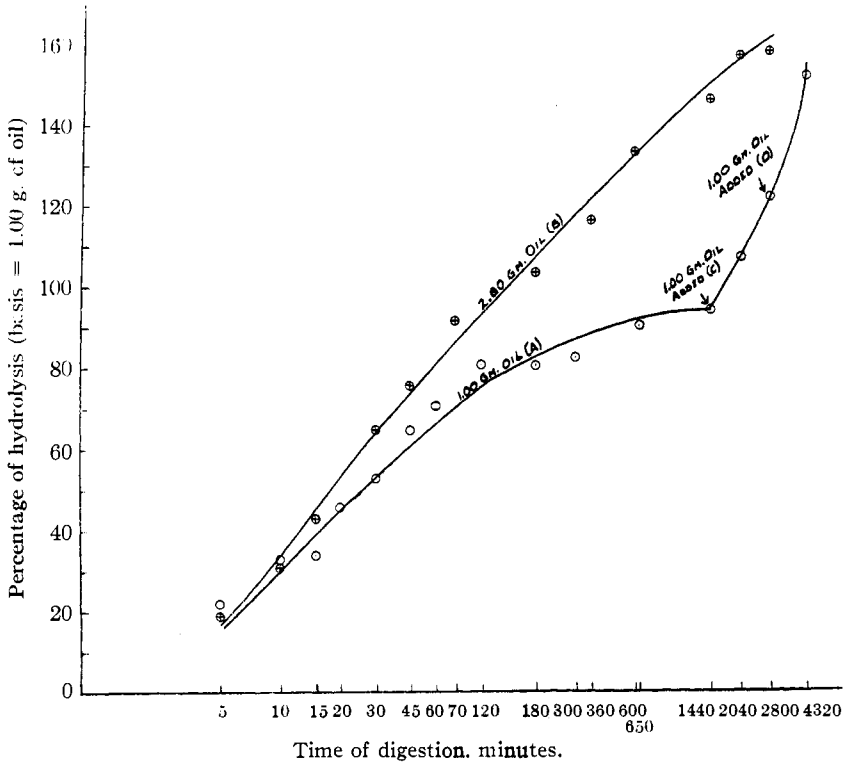

Fig. 2.—The effect on lipase activity of contact with substrate and end-product.

Discussion

The arbitrary method of calculating lipolytic activity suggested in this paper is an application similarly employed in the study of other enzymes.[10] It considerably simplifies the task of analyzing large numbers of data either for the comparison of rates of action of various preparations or the effect of a particular treatment. When small quantities of lipase preparation were used or when the time required for 40% hydrolysis was greater than twenty-five to thirty minutes the results appeared to be low, otherwise the method gives consistent results.

Preparations of the same high degree of activity have been obtained from whole and decorticated beans. This fact is of considerable importance in the large scale preparation of lipase-containing material, since the removal of the seed coats is a tedious process. It has been found that lipase preparations with an activity of 900–1000 lipase units could be prepared readily when extraction of the oil from the testing beans was carried out in glass equipment with low boiling petroleum ether. It is to be noted that the toxic nature of the castor bean[9-11] is intensified in the fat free preparations described here. It was necessary to observe constant caution to avoid physical contact with the dried extracted powder. All grinding and sieving operations were carried out in a closed box and a dust mask was worn whenever the preparations were handled.

Water was found to be necessary in quantities slightly exceeding the theoretical (0.06 g. per gram of olive oil) for maximum hydrolysis. The striking fact revealed by this set of experiments (Table IV) is that the fat hydrolysis is catalyzed to a considerable extent in the presence of the theoretical amount of water or less. There was hydrolysis in the presence of only a trace of water. This suggests the utility of the lipase to the germinating seed which usually has only small quantities of water for its divers reactions.

The percentage hydrolysis was higher in those cases where there was less oil per unit weight of enzyme. This supports the argument that a definite combination of enzyme and substrate does take place with Ricinus lipase. It is clear from these data that the rate of reaction is dependent upon the substrate concentration and not on the concentration of water in the digestion

(10) Northrup, *J. Gen. Physiol.*, **16**, 41 (1932).

(11) Dowgard, *J. Am. Pharm. Assoc.*, **12**, 116 (1923).

mixture. Such evidence is of importance in a consideration of the kinetics of the reaction, a report upon which will be made in a future communication.

The nature of the lipase material used in these experiments has been called to attention.[2] The preparations were solid mixtures consisting largely of protein and ash. It would be preferable to confine the term Ricinus lipase to the chemical individual which shows intensive and extensive lipolytic activity; as it is used here, it refers only to a sieved preparation of fat-free, castor beans. Attempts to isolate or even concentrate more active materials from these preparations yielded no positive results. The chief difficulty in these attempts seemed to lie in the marked effect of water on the activity of the enzyme, as is shown above (*cf.* Fig. 1).

While no evidence has been obtained to indicate the nature of this action of water on the enzyme, the presence of proteolytic as well as oxidative enzymes in our preparations may offer the clue. It is proposed to investigate these and other possibilities.

The effect of contact with the digestion mixture on the active enzyme probably represents the cumulative effects of water and the end-products formed during the reaction. The slight increase in hydrolysis on addition of substrate indicates that the enzyme has been inactivated but not entirely so after twenty-four hours contact with these substances. There was little additional hydrolysis of more substrate added after a second twenty-four hour period. These figures are interesting in contrast with the results obtained on the same amount of substrate in contact with the enzyme from the beginning of the reaction. Lack of a preparation of comparable unitage prevented following the course of this inactivation as would have been desirable after thirty, forty-five, sixty and one hundred and twenty minutes of contact with the digestion mixture.

Summary

In a continuation of studies on Ricinus lipase obtained from castor beans it has been found unnecessary to shell the beans prior to their extraction, extraneous material being removed while sieving at a later stage during the preparation. Low boiling petroleum ether proved the most satisfactory solvent for the preparation of very active samples. Reference has been made to dangers in handling these fat-free castor bean preparations.

An arbitrary expression for comparing the activities of preparations or the effect of various treatments has been presented. It is based on the time required for 40% hydrolysis. Limitations in the use of this lipase unitage basis are discussed.

Variation of the substrate concentration and the amount of water in the digestion mixture has shown the optimum conditions for Ricinus lipase action and the variance permissible for considerable action. Lipolytic action in the presence of a trace of water suggests the utility of the enzyme to the germinating seed. The dependence of the rate of reaction on the substrate concentration and not on the amount of water, except in quantities approximating the theoretical required, is discussed.

The inactivating effect of water and the end-products of the reaction have been shown. Inactivation of Ricinus lipase by water was found to take place in the presence and absence of oil.

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