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Ricinus Lipase, its Nature and Specificity[1]

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The lipolytic principle of the castor bean (*Ricinus communis*) has engaged the attention of many research workers since its presence was first proved in 1890 almost simultaneously by Green[3] and Sigmund.[4] However, a review of the available literature on the active substance, Ricinus lipase, has failed to reveal any general concordance of experimental results. Haley and Lyman's discovery[5] that with oils optimum conditions for activity were obtained only within narrow limits of hydrogen ion concentration, about *p*H 4.7–4.8, made it necessary to repeat most of the previous work.

The object of the present investigation was to prepare a highly active, dry and stable preparation of Ricinus lipase from resting castor seeds and to study the nature of its action and its relative specificity in the hydrolysis of various vegetable and animal oils. These studies were undertaken to elucidate the mode of action of Ricinus lipase and to contribute to the knowledge of enzyme action in general.

Experimental

A. Preparation of Lipase Material.—Ricinus lipase was prepared from large size castor beans[6] by hulling and macerating them, extracting the fat as completely as possible with low boiling petroleum ether (20–40°), and finally drying, pulverizing and sifting the finished product through a 60-mesh sieve. This is a modification of the method given by Haley and Lyman.[5] Other methods for the preparation of Ricinus lipase have been reviewed by Longenecker.[7] Most of them are less suitable.

B. Estimation of Activity.—In all experiments reported here, a standard procedure was adopted for the determination of lipolytic activity: 1.00 gram of oil was weighed into five-cc. hard-glass bottles and two drops of toluene added to prevent bacterial action. To this was added a weighed quantity of enzyme material and its activator, tenth normal acetic acid. The *p*H of the resulting mixture was 4.8. It was emulsified by vigorous shaking for three minutes. Digestion was carried on at a temperature of 37°. To prevent loss of any of the digestion materials, the bottles were stoppered with paraffined corks. The results reported represent averages of triplicate determinations. "Blanks" always accompanied the samples.

After a definite time of incubation, the reaction mixture was placed in 50 cc. of hot 95% alcohol and the free fatty acids in the digestion mixture were titrated with 0.1 *N* alkali using phenolphthalein as the indicator. The starting time for each experiment was that time when acetic acid was added to the digestion mixture; the final time, when the reaction mixture was placed in the alcohol. It was found by preliminary experiments that no hydrolysis took place until after addition of the acid and that hydrolysis was completely inhibited in the amount of alcohol used in the titration.

Titration results are of little value, *per se*, in studies of the relative specificity of lipolytic catalysis of oils although several workers have reported their findings in this way. A more significant expression is the percentage of oil hydrolyzed or the number of moles of triglyceride hy-

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(2) Submitted in partial fulfilment of the requirements for the degree of Master of Science at The Pennsylvania State College.

(3) Green, *Proc. Roy. Soc.* (London), **48B**, 370 (1890).

(4) Sigmund, *Monatsh.*, **11**, 272 (1890).

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

(6) Courtesy of The Baker Castor Oil Co., Newark, N. J.

(7) Longenecker, M. S. Thesis, The Pennsylvania State College, 1934.

drolyzed. For the calculation of the percentage hydrolysis, the formulas suggested by Sudborough, Watson and Varma and Wilson and Merrill[8] have been used as a basis. The difference between the volume of alkali required for the sample and that required for the blank is a measure of the fatty acids resulting from the direct cleavage of glycerides due to the action of the lipase; the ester number is a measure of the total fatty acids in glyceride combination. To obtain the percentage hydrolysis, the following formula has been used

$$\frac{\text{cc. 0.1 } N \text{ alkali (sample)} - \text{cc. 0.1 } N \text{ alkali (blank)}}{\text{Saponification no.}^a - \text{Free fatty acid no.}^a} = \% \text{ hydrolysis}$$

[a] Expressed in cc. of 0.1 *N* alkali.

C. Activity of Old and New Preparations.—The ability of Ricinus lipase to retain its activity over a long period was demonstrated in the following experiment in which the activity of a sample similarly prepared by one of us in 1924 was compared with that of a freshly prepared sample: 1.00 gram of olive oil and 0.6 cc. of 0.1 *N* acetic acid were used; the amount of enzyme material was varied; one set of samples (A) was shaken for three minutes at the start, then allowed to stand for twenty-four hours at 29–30°; another set of samples (B) was shaken continuously by machine for fourteen hours then allowed to stand for ten hours at 29–30°. The results are given in Table I.

TABLE I

COMPARISON OF ACTIVITIES OF TEN-YEAR OLD AND NEW PREPARATIONS

Treatment	Percentage hydrolysis ten-year old sample 0.050 g.	0.100 g.	Percentage hydrolysis new sample 0.050 g.	0.100 g.
A	6.79	17.82	77.83	86.00
B	14.03	49.36	85.43	86.70

TABLE II

TYPICAL RATE OF REACTION AND THE EFFECT OF TEMPERATURE AND SHAKING

In each experiment, 1.00 g. of olive oil, 0.6 cc. of 0.1 *N* acetic acid, 0.100 g. of lipase material and two drops of toluene were shaken together and incubated at 37–38°. A, shaken for three minutes at the start. B, shaken for three minutes at the start, let stand for thirty minutes and shaken again for two minutes. C, same as A except that temperature was 27–28°.

Time of digestion	Percentage hydrolysis A	B	C
5 min.	11.02	...	...
10 min.	19.35	...	25.88
15 min.	31.91	...	...
20 min.	38.33	...	44.70
30 min.	39.22	...	...
40 min.	50.63	...	55.88
45 min.	...	57.93	...
50 min.	51.91	...	...
60 min.	59.60	66.39	59.66
2 hrs.	65.37	74.85	67.80
3 hrs.	71.14	79.34	71.90
5 hrs.	72.42	80.50	...
10 hrs.	74.21	...	...
24 hrs.	77.80	77.42	...

(8) Sudborough, Watson and Varma, *J. Indian Inst. Sci.*, **2**, 241 (1919); Wilson and Merrill, *J. Am. Leather Chem. Assocn.*, 1 (1926).

D. Rate of Hydrolysis of Olive Oil.—Olive oil was selected as a substrate to demonstrate, first, typical rates of reaction and second, some of the factors influencing it. In Table II, data are given which express (A) a normal rate of reaction, (B) the effect of shaking the digestion mixture after the reaction has proceeded for thirty minutes and (C) the effect of temperature.

E. Relative Specificity of Ricinus Lipase Action.—A study of the rates of hydrolysis of various members of a general group of substances should yield information as to the relative specificity or affinity of an enzyme for a particular substrate. A variety of oils of vegetable and animal origin were selected for specificity studies. Rates of hydrolysis of these oils are found in Table III.

Discussion

In analyzing and interpreting these results the nature of the active material must be considered. "Ricinus lipase" has been used here to represent the hulled, fat-free, resting castor seeds. Proteins, carbohydrates and salts originally present have not been removed from the final product.

It is significant that a ten-year old preparation retained a fairly high activity compared with a fresh sample. Enzymatic activity is known to decrease considerably in short periods, especially when the enzymic material is in solution. Dry preparations are usually more stable. So far as the authors can ascertain, however, there is no report in the literature showing the retention of activity of any enzyme preparation over a period of ten years.

One condition that appears to be a *sine qua non* factor for Ricinus lipase activity is the colloidal state. One may assume that an emulsion arises in a heterogeneous system such as the digestion mixture of oil, enzyme and dilute acid. Sudan III and conductivity tests indicated in this work that it is of the water-in-oil type under the experimental conditions. This type of emulsion was favorable for the action of Ricinus lipase. In fact, there is every reason to believe that the enzyme is not as active in an emulsion of the opposite type.

The production of a suitable emulsion was aided decidedly by shaking the digestion mixture. Shaking by hand for three minutes ensured that approximately the same degree of emulsification was obtained at the beginning of each experiment. Once formed, however, the emulsion was not stable. It tended to separate into layers of water and oil within one-half hour, when the rate of hydrolysis began to decrease. Shaking the digestion mixture at this point restored the emul-

TABLE III

RATES OF HYDROLYSIS OF VARIOUS OILS OF VEGETABLE AND ANIMAL ORIGIN

In each experiment, 1.00 g. of oil, 0.100 g. of lipase material, two drops of toluene and 0.6 cc. of 0.1 *N* acetic acid were shaken together for three minutes at the start, then let stand at 37–38°.

	Percentage hydrolysis								
	Minutes					Hours			
Oil	15	30	45	60	120	3	5	10	24
Castor	33.33	47.48	59.15	59.15	72.70	83.30	...	...	...
Coconut	30.67	43.26	48.96	50.73	54.27	59.58	61.37	65.81	67.05
Corn	35.37	53.86	60.74	67.16	71.64	72.09	81.79	87.01	90.75
Cottonseed	36.96	52.90	62.75	63.33	72.61	71.30	83.33	86.52	87.25
Fish	17.66	22.24	26.81	27.33	31.48	33.24	36.06	37.00	41.67
Linseed	21.44	34.25	38.78	42.88	52.48	56.61	71.12	72.59	72.90
Neat's-foot	28.93	32.43	43.11	47.96	57.86	63.69	68.31	69.33	70.68
Olive	31.91	39.22	50.63	59.60	65.31	71.14	72.42	74.21	77.80
Peach kernel	24.36	33.35	47.57	52.20	57.57	61.20	65.54	70.18	69.60
Peanut	37.55	55.86	63.55	67.40	77.73	83.70	94.40	97.24	...
Rape	39.52	48.36	54.95	62.02	71.49	69.89	77.44	81.14	83.45
Sperm	10.18	13.71	16.83	20.36	18.28	18.89	23.68	19.53	23.47
Soybean	17.66	35.05	53.13	61.30	77.64	73.99	84.35	84.94	89.17
Whale	16.77	25.33	26.26	28.98	32.82	35.14	37.67	41.81	41.71

sion and with it there was an increased rate and extent of reaction in a given time, as is seen in Table II.

A higher percentage of hydrolysis in cases where a lower temperature was used, as shown also in Table II, can be correlated with the stability of the emulsion. In each case it was observed that the tendency for the emulsion to break was much less at the lower temperature.

That Ricinus lipase exhibits a differential rate of attack for various oils appears obvious from Table III. Previous investigators have reported similar experiments and results.[9-13] In the majority of cases, it has been emphasized that the Ricinus lipase exhibited a relative specificity for those glycerides of high molecular weight. The results above seem to offer support for this argument, with a few exceptions. Thus, for the various oils used, listed in the order of decreasing percentage hydrolysis, the mean molecular weights are:

Oils	Mean mol. wt.	Oils	Mean mol. wt.
Peanut	870	Linseed	786
Castor	921	Neat's-foot	995
Corn	895	Peach kernel	785
Cottonseed	870	Coconut	589
Soybean	869	Whale	590
Rape	956	Fish	616
Olive	765		

However, if another line of reasoning be adopted, this apparent specificity disappears almost entirely. When the number of moles of glyceride hydrolyzed after a given time is considered, no relative specificity of Ricinus lipase is observed. The results are all of the same order of magnitude and the probability that any individual variations are due to an actual difference in specificity is very low indeed.

(9) Connstein, *Ergebnisse der Physiol.*, **3**, 194 (1904).
(10) Tanaka, *J. Coll. Eng. (Imp. Univ. Tokyo)*, **5**, 25, 152 (1912).
(11) Takamiya, *J. Agr. Chem. Soc.* (Japan), **5**, 595 (1929).
(12) Falk, THIS JOURNAL, **35**, 616 (1913).
(13) Sudborough and Watson, *J. Indian Inst. Sci.*, **5**, 119 (1922).

Summary and Conclusions

A dry, stable and highly active preparation of Ricinus lipase has been obtained from hulled castor beans by extracting the fat with low boiling petroleum ether, pulverizing and sifting the residue of the bean through a 60-mesh sieve. The activity of this fresh preparation was compared with that of one ten years old. Considerable activity was obtained with the latter.

A water-in-oil emulsion appears to be desirable for Ricinus lipase action. The formation of this emulsion was aided by shaking and by lower temperatures of incubation.

Rates of hydrolysis of a variety of oils of vegetable and animal origin were obtained for studies on the nature of action and specificity of Ricinus lipase. Under the experimental conditions, Ricinus lipase catalyzed the hydrolysis of the following oils, listed in order of decreasing percentage hydrolysis after a given time: peanut, castor, corn, cottonseed, soybean, rape, olive, linseed, neat's-foot, peach kernel, coconut, whale, fish and sperm. An analysis of the data on the basis of the number of moles hydrolyzed revealed that the lipase showed no specificity in its attack on glyceride molecules containing carbon chains of different length.

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