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The Protein Nature of Enzymes. An Investigation of Pancreatic Lipase

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The purification of enzymes by absorption methods has been elaborated by Willstätter and co-workers, and applied by them to the separation and purification of pancreatic enzymes.[1] During the course of their investigation the conclusion was reached that the enzyme itself was not protein in nature since the finally dialyzed preparations no longer gave qualitative protein tests. That this conclusion may be invalid has been shown by Sherman and co-workers[2] who have made a critical study of Willstätter's adsorption purification technique as applied to pancreatic amylase. In addition, apparently pure crystalline enzymes have been shown to possess the characteristics of typical proteins.[3]

The present communication gives evidence of the protein nature of pancreatic lipase, and tends to confirm the viewpoint that the digestive enzymes are protein substances. A method has been developed for the purification of lipase by protein precipitating agents, which enables a given crude enzyme preparation to be concentrated more readily than any previous method.

It has been shown previously that lipase can be removed from pancreatic juice by precipitation with 55–60% ammonium sulfate.[4] In the present investigation the enzyme was found to have the characteristics of a globulin.

Experimental Part

Preparation of Pancreas Powder.—Fresh lamb pancreas was dried and defatted with acetone and ether according to the method of Willstätter and Waldschmidt-Leitz.[1] This treatment caused practically no loss in activity, and the material retained its activity when stored in an ice box during a four-months period.

Measurement of Activity.—To determine activity, the enzyme solutions were placed in 20-cm. test-tubes with 2 cc. of substrate emulsion (4 volumes of olive oil + 1 volume of 1% sodium oleate), 1 cc. of hexylresorcinol solution (0.5×10^{-6} mole per cc.) to prevent bacterial growth, 1 cc. of buffer (P_H 8.0, 9.5 cc. $M/15$ Na_2HPO_4 + 0.5 cc. $M/15$ NaH_2PO_4), and distilled water to a final volume of 10 cc. The test-tubes were stoppered and shaken for twenty-four hours at room temperature. At the end of the run 25 cc. of alcohol was added to each tube, which was then titrated (phenolphthalein) with 0.1380 N sodium hydroxide. Each tube was accompanied by two blank tubes. One contained 2 cc. of substrate emulsion, 1 cc. of buffer and 2 cc. of water. The other contained 1 cc. of hexylresorcinol solution, the same volume of enzyme solution as used in the run, and water to a total volume of 5 cc. At the end

(1) Willstätter and Waldschmidt-Leitz, *Z. physiol. Chem.*, **125**, 132 (1923).

(2) Sherman, Caldwell and Adams, THIS JOURNAL, **48**, 2947 (1926).

(3) Sumner, *J. Biol. Chem.*, **69**, 435 (1926); Northrop, *J. Gen. Physiol.*, **13**, 739 (1930); Caldwell, Booher and Sherman, *Science*, **74**, 37 (1931); Northrop and Kunitz, *ibid.*, **73**, 262 (1931).

(4) Gyotoku and Terashima, *Biochem. Z.*, **217**, 292 (1930).

of 24 hrs., 25 cc. of alcohol was added to the blank containing the enzyme, after which it was mixed with the other blank and titrated.

The activity expressed as lipase units was determined according to the method of Willstätter and co-workers.[5] They define the lipase unit as that quantity of lipase which will hydrolyze 24% of 2.5 g. of olive oil (saponification No. 185.5) in 1 hour at 30° in the presence of 2 cc. of *N* ammonia–ammonium chloride buffer (P_H 8.9), with 10 mg. of calcium chloride and 15 mg. of albumin as activators, in a volume of 13 cc. The titrations were carried out with 0.773 *N* alcoholic potassium hydroxide after the addition of alcohol to a volume of 125 cc., followed by 20 cc. of ether, and twelve drops of 1% thymolphthalein.

Purification.—Pancreas powder was extracted with 0.025 *N* ammonium hydroxide, 10% sodium chloride solution, and a combination of 10% sodium chloride in 0.025 *N* ammonium hydroxide. One gram of pancreas powder was treated with 30 cc. of extracting agent for three hours at 37°, after which the liquid was filtered through paper, made neutral to phenolphthalein with dilute ammonium hydroxide or sulfuric acid, and 5% of the total volume tested for activity as recorded in Table I.

TABLE I

ACTIVITY OF PANCREAS POWDER EXTRACTS

Extracting agent	Total NaOH, cc.	Blank NaOH, cc.	Activity NaOH, cc.
0.025 *N* NH_4OH	11.75	2.80	8.95
10% NaCl	16.25	1.05	15.20
10% NaCl in 0.025 *N* NH_4OH	11.40	1.30	10.10

It was previously found that ammonia extracts were more active[6] than either water or glycerol extracts. Ten per cent. sodium chloride solution repeatedly removed more lipase from the powder than any of the agents heretofore used for this purpose.

An ammonia extract was made neutral to brom thymol blue with dilute sulfuric acid, and separate portions were saturated with sodium chloride and magnesium sulfate, respectively, while a third was half saturated, filtered, and then saturated with ammonium sulfate. The precipitates were filtered off, redissolved in distilled water, made neutral to phenolphthalein with ammonium hydroxide, and their activity determined. The quantity of precipitate tested in each case (Table II) was equivalent to that which would be obtained from 1.5 cc. of the original ammonia extract.

TABLE II

ACTIVITY OF PROTEIN FRACTIONS PRECIPITATED BY INORGANIC SALTS

Preparation	Total NaOH, cc.	Blank NaOH, cc.	Activity NaOH, cc.	Yield, %
NaCl ppt.	4.55	0.90	3.65	41
$MgSO_4$ ppt.	16.55	6.20	10.35	115[a]
$(NH_4)_2SO_4$ globulin ppt.	12.87	5.70	7.17	80
$(NH_4)_2SO_4$ albumin ppt.	7.00	6.50	0.50	5

[a] The apparent yield of over 100% was due to the activating effect of magnesium sulfate, which was demonstrated in independent tests.

A rapid loss in activity was observed when these preparations were dialyzed or dried over calcium chloride in a desiccator. Preparations made by adsorption methods are also known to lose activity on dialysis. Active preparations precipitated with 80% alcohol or 70% acetone had practically no activity.

The P_H of the enzyme solution when saturated with magnesium sulfate was 4.5 as

(5) Willstätter, Waldschmidt-Leitz and Memmen, *Z. physiol. Chem.*, **125**, 93 (1923).

(6) Glick and King, *J. Biol. Chem.*, **97**, 675 (1932).

measured by quinhydrone and saturated calomel electrodes. The P_H when half-saturated with ammonium sulfate was 5.5, and 5.3 when fully saturated. The solubility of magnesium sulfate and the acidity of its saturated solution probably accounts for its efficient precipitation of the enzyme. A preliminary investigation of its solubility characteristics indicated that the isoelectric point of the enzyme was in the range of P_H 4.5 to 4.7.

To compare the degree of purification of the best preparations made by these precipitation methods with those of Willstätter and co-workers made by the adsorption technique, the activities, expressed in terms of lipase units, were determined for the pancreas powder, the saline extract, the magnesium sulfate precipitate from the saline extract, and the latter preparation after reprecipitation with magnesium sulfate.

The degree of purification (Table III) was calculated both on the basis of the number of lipase units per mg. of nitrogen, and as the number of lipase units per cg. total solids (the lipase value of Willstätter, Waldschmidt-Leitz, and Memmen).

The nitrogen content of the preparations was determined by the Folin-Wright method.[7]

The total-solids values were determined by dialyzing in collodion bags 10 cc. of each preparation for five days against 2 liters of distilled water, changed daily, and finally evaporating the dialyzed liquids to dryness at 100° in platinum crucibles. The weight of the dry solids was measured on a micro balance.

TABLE III

DEGREE OF PURIFICATION OF LIPASE IN SUCCESSIVE STEPS

	Material tested	Hydrolysis, %	Lipase units	Total solids, mg.	N, mg.	Lipase value	Lipase units mg. N
(a)	Pancreas powder	6.4	0.15	10	1.487	0.15	0.101
(b)	10% NaCl extract of (a)	10.7	.30	0.0936[a]	1.632[b]	32.0	.184
(c)	$MgSO_4$ precipitate of (b)	18.6	.70	.0550[a]	0.812[b]	127.3	.862
(d)	Reprecipitation of (c) by $MgSO_4$	11.9	.35	.0261[a]	.323[b]	134.1	1.084

[a] Determined after dialysis. [b] Determined before dialysis.

To confirm the superiority of 10% sodium chloride solution as an extraction agent over the 87% glycerol solution commonly used, 10 cc. of each was placed with 0.25 g. of pancreas powder and kept at 37° for three hours. After filtration, the activity of 1 cc. of each of the liquids was measured. The saline extract produced 10.7% hydrolysis, while the glycerol extract produced only 6.7% hydrolysis under identical conditions. The amount of enzyme in the saline extract represented 80% of that in the original dry powder. By longer extraction and washing with glycerol, Willstätter and co-workers obtained 65 to 70% of the enzyme from powdered pig pancreas.

Discussion

That pancreatic lipase is itself a globulin, or essentially associated with a globulin, is strongly indicated by the following facts: 10% sodium chloride solution is the best extraction solvent; it is precipitated by half saturated ammonium sulfate, saturated magnesium sulfate, and to some extent by saturated sodium chloride solution; and dialysis causes precipitation from its most highly purified solutions. Before dialysis the purest solutions obtained gave positive ninhydrin, biuret and xanthoproteic tests in addition to the typical protein precipitations.

(7) Folin and Wright, *J. Biol. Chem.*, **38**, 461 (1919).

Starting with dried pig pancreas powder having a lipase value of 0.81, Willstätter and co-workers succeeded in concentrating the enzyme to a lipase value of 207.0. They employed two adsorptions with alumina and one with kaolin, giving their most concentrated preparation, which represented a purification of 256 times. By extracting sheep pancreas powder, having a lipase value of 0.15, with 10% sodium chloride solution and precipitating the enzyme[8] twice by saturation with magnesium sulfate, the final preparation possessed a lipase value of 134.1, representing a purification of 894 times.

The dialysis method of determining total solids is not likely to give reproducible results because of variation in the permeability of dialyzing bags and in details of carrying out the dialysis. That the method gives a falsely high value for the degree of purification has already been pointed out by Sherman and co-workers,[2] and is clearly confirmed by the data in Table III. It will be seen that the weight of the total solids after dialysis amounts to less than one-tenth of the weight of the nitrogen alone before dialysis. This indicates that about 98 or 99% of the nitrogenous material was lost in the course of dialysis. Our dialysis was much less vigorous than that employed by Willstätter, although we were using different membranes.

If we base the degree of purification upon the lipase units per mg. of nitrogen, the actual number of times the enzyme was concentrated is seen to be only 10.7 instead of 894. It is very unlikely that large amounts of non-protein nitrogen would be precipitated by magnesium sulfate under the conditions of the experiment. Further work on the problem of purification is being continued. We do not claim that the preparation herein described represents a pure substance.

Summary

The use of ten per cent. sodium chloride solution as an extracting agent for lipase from pancreas powder has been shown to produce more active preparations than can be obtained by other solvents previously used.

Pancreatic lipase has been found to be precipitated quantitatively by saturation with magnesium sulfate. An activating effect of magnesium salts upon the enzyme has also been observed.

The term "lipase value" as now used in the literature to indicate enzyme purification has been shown to be unwarranted. Instead, the expression of activity in terms of lipase units per milligram of nitrogen has been supported.

The extraction and precipitation technique herein described for pancreatic lipase permits greater purification than other methods previously recorded.

(8) It is well known that the pancreas powder of sheep possesses less lipolytic activity than that of the pig.

The solubility characteristics and general behavior of the enzyme point strongly toward its being essentially a globulin.

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The Reaction of a Free Radical, Triphenylmethyl, with Ethers, Esters and Acetone[1]

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This paper will present work showing that under certain conditions triphenylmethyl reacted with ether, ethyl acetate, and acetone, forming triphenylcarbinol in yields of 44, 31 and 14%, respectively, together with smaller amounts of other substances. During the course of some experiments on triphenylmethyl an ethereal solution of the compound, prepared *in situ* from triphenylchloromethane and copper, was decolorized when heated overnight in a sealed tube at 80°. Upon opening the tube a considerable pressure of gas, later identified as carbon monoxide and methane, was revealed. Acetaldehyde, triphenylcarbinol, triphenylmethane, and an uncrystallizable oil were found in the solution. In the absence of copper or cuprous chloride the decolorization of a solution of the free radical required heating to 110° for a longer time. Gases were still found in the tube after the reaction and the aldehyde was detected but the solid products could not be identified. With ethyl acetate the behavior in general was similar to that with ether except that no gaseous nor aldehyde products were found. A reaction with acetone was not as smooth as that with ether or with ethyl acetate.

This interesting discovery extends the list of substances with which a free radical, triphenylmethyl, reacts to include some of the most common solvents[2] used. It is also another illustration of the great tendency of triphenylmethyl to saturate itself at the expense of other molecules which are relatively stable compounds.[3]

The mechanism of the reaction is not known but a likely inference, in the cases of ether and ethyl acetate, at least, is that triphenylmethyl ethyl ether is formed as an intermediate which then breaks[4] down into triphenylcarbinol and triphenylmethane.

(1) From a portion of the thesis of Lawson V. Peakes, Jr., presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy, 1932.

(2) Schmidlin [*Ber.*, **39**, 632, 4189 (1906); **40**, 2316 (1907)] observed that the yield of triphenylmethyl from triphenylchloromethane and magnesium in ether was less when the solution was heated for a long time. He attributes his results to an alpha and beta form of triphenylmethylmagnesium chloride. He also found considerable quantities of triphenylcarbinol in his product at times, a fact which he believes was due to the least trace of moisture.

(3) As another example of this type the reaction with *o*- and *p*-xylene may be cited, Wieland and Müller, *Ann.*, **401**, 236 (1913).

(4) Norris and Young, THIS JOURNAL, **52**, 753 (1930).