PRELIMINARY CHARACTERIZATION OF THE LIPASE OF MYCOTORULA LIPOLYTICA[1]

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In the preceding paper the factors influencing the production of lipase by *Mycotorula lipolytica* were discussed (Peters and Nelson, 1948). The considerable increase in lipase production under proper conditions of growth of the culture warranted a study of the characteristics of the enzyme. This lipase may be of particular interest because of the possibility of its useful application in the cheese-making industry, especially in the manufacture of blue or Roquefort type cheese from pasteurized milk.

Although considerable information is available dealing with the characterization of animal and vegetable lipases, little work seems to have been done on microbial lipases. Avery and Cullen (1920) found that pneumococcus lipase hydrolyzed tributyrin most actively at pH 7.8, whereas Stevens and West (1922) reported that hemolytic streptococci showed greatest lipase activity at pH 7.9, with ethyl butyrate as substrate. The enzyme produced by streptococci was inactivated by exposures to temperatures above 55 C for 5 minutes. Gorbach and Guentner (1932) found the lipase of beer yeast to be most active at pH 6.6 to 6.8. This lipase showed optimum activity at 30 C, with higher temperatures proving detrimental to the enzyme. The same workers also observed that the rate of lipase activity decreased with time and that the amount of olive oil hydrolyzed was not directly proportional to the amount of beer-yeast lipase present, although there was a point of optimum activity per unit of lipase. Thibodeau and Macy (1942) reported that lipase from *Penicillium roqueforti* showed optimum activity at pH 5.3 to 7.5.

The concentration and purification of lipases from various sources has been accomplished by several methods, of which precipitation of the enzyme with certain salts seemed most satisfactory. Gyotoku and Terashima (1930) were able to precipitate lipase of blood, pancreas, and stomach by the use of a 55 to 60 per cent concentration of ammonium sulfate, whereas Glick and King (1933) used, with varying success, magnesium sulfate, half and fully saturated ammonium sulfate, and 10 per cent sodium chloride as precipitating agents. Ostwald and Mischke (1940) were able to concentrate pancreatic lipase from solutions at pH 4.5 by continuous bubbling of nitrogen gas through the solution and the collection of the foam.

METHODS

The medium for lipase production, the growth conditions, the determination of pH, and the quantitative determination of lipase activity were the same as reported in an earlier paper (Peters and Nelson, 1948).

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Cell-free preparations were obtained by first centrifuging 3-day-old nutrient broth cultures of *M. lipolytica* at 3,500 rpm for 15 minutes and then filtering the supernatant through an ultra-fine sintered glass (pyrex) filter with the aid of suction.

The lyophilized material was prepared by placing a 200-ml quantity of a 3-day-old broth culture of *M. lipolytica* in a 500-ml round-bottom flask with ground-glass stopper. The culture was frozen while the flask was being rotated in an acetone dry-ice bath. The flask containing the frozen culture was evacuated by the use of a vacuum pump, resulting in sublimation of the ice in the frozen culture. The water vapors were extracted by continuous suction and condensed and frozen in another flask, which was held in an acetone dry-ice bath.

In the concentration of lipase by foaming, 3-day-old broth cultures were centrifuged and the supernatant was placed in 100- or 200-ml quantities into 225- or 500-ml gas-washing bottles, respectively. Foaming was produced by bubbling compressed carbon dioxide gas through the supernatant at from 50 to 75 mm mercury pressure. The foam was collected in graduated cylinders of a size convenient to permit the collection of the desired volume of foam. In the experiments on repeated foaming, the third and fourth fractions were diluted by adding distilled water and acidified with hydrochloric acid to pH 4.0, to obtain the desired volume of 200 ml for further fractionation.

The precipitation of lipase with ammonium sulfate and ethyl alcohol was performed on 3-day-old broth cultures, which were centrifuged and the supernatant used in 400-ml quantities. To 400 ml of supernatant in a 1-liter separatory funnel was added ammonium sulfate to the saturation point (about 200 g). Next, 130 ml of 95 per cent ethyl alcohol were added and the mixture was well shaken. The flocculent precipitate gathered at the water-alcohol interface. After the aqueous layer was drawn off, the precipitate and the alcohol layer were decanted onto a previously weighed hardened filter paper, supported in a Buchner funnel. Suction was applied to the flask and the alcohol filtered off. A 10-ml quantity of diethyl ether was added to remove the residual alcohol. The filter paper was freed from the last traces of ether by placing it at 37 C for 2 hours. The dry precipitate on the filter paper was weighed and its lipase activity measured.

RESULTS

The data in table 1 show that the pH of the emulsion upon which the enzyme acts has a definite effect upon the activity of the lipase. Although hydrolysis of fat took place over the pH range from 4.0 to 8.0, greatest lipase activity took place at pH 6.2 to 6.5. The decrease in lipase activity was more abrupt on the alkaline side of the optimum than on the acid side.

In five trials in which pasteurized homogenized milk was substituted for butterfat-agar emulsion as substrate (and for which data are not shown), acid degree values ranging between 47 and 87 were obtained, causing a lowering of the reaction of the milk by as much as 0.9 of a pH unit in some cases. These results demonstrate the activity in milk of lipase from *M. lipolytica*.

The influence of various incubation temperatures upon the activity of the lipase from *M. lipolytica* is shown in tables 2 and 3. Optimum activity was demonstrated at 28 and 33 C. Inactivation at 43 C is apparent after 30 hours of incubation time, but at 37 C a 54-hour period was required to produce a

TABLE 1

The influence of pH upon M. lipolytica lipase activity

pH OF SUBSTRATE	LIPASE ACTIVITY* PER ML OF ENZYME PREPARATION	
	Trial 1	Trial 2
4.0	16	—
4.5	22	—
5.0	24	—
5.5	26	—
6.0	32	36.5
6.2	40	40
6.4	—	34.5
6.5	55	—
6.6	—	25.5
6.7	37	—
7.0	31	22.5
7.5	15	—
8.0	12	—

* Expressed in acid degrees.

TABLE 2

The influence of temperature and time upon M. lipolytica lipase activity

(Average of two trials)

REACTION TIME IN HOURS	LIPASE ACTIVITY* AFTER VARIOUS INCUBATION PERIODS AT TEMPERATURES OF			
	28 C	33 C	37 C	43 C
6	11.0	10.5	9.0	12.0
18	20.0	19.5	14.0	15.0
30	22.5	27.5	19.5	16.5
42	35.5	32.5	28.5	18.0
54	43.0	41.0	31.0	18.0
66	45.0	43.0	30.0	17.0
78	49.0	48.5	31.5	20.0
102	62.5	63.0	34.0	20.5
126	70.0	72.5	36.0	23.0
150	74.0	73.5	33.0	19.5

* Expressed as acid degrees per milliliter of enzyme preparation.

distinct inactivating effect. Other trials showed that temperatures above 43 C resulted in earlier inactivation of the enzyme. A temperature of 52 C was sufficient to inactivate the lipase within 8 hours. The initial rate of lipase activity at 10 C was slightly lower than at 21 to 33 C (table 3). However,

results obtained after an incubation period of 810 hours at 10 C showed acid degree values comparable to those of samples held at 21 C. The temperature of 10 C was chosen because blue cheese ripening takes place at approximately this temperature.

The data presented in table 4 show that, when various dilutions of a concentrated enzyme preparation (broth culture concentrated to one-fourth original

TABLE 3

The influence of lower temperatures and prolonged holding time upon M. lipolytica lipase activity

TIME IN HOURS	LIPASE ACTIVITY* AFTER VARIOUS INCUBATION PERIODS AT TEMPERATURES OF			
	10 C	21 C	28 C	33 C
18	8	12	11	16
66	—	22	23	23
114	13†	27	26	25
162	20	33	29	26
306	27	40	—	—
450	28	29	—	—
810	40	46	—	—

* Expressed as acid degrees per milliliter of enzyme preparation.
† At 90 hours.

TABLE 4

The influence of varying amounts of M. lipolytica lipase preparation (concentrated by lyophilizing) upon lipase activity
(Average of two trials)

AMOUNT OF CONCENTRATE USED (ML)	LIPASE ACTIVITY* AS	
	Determined per increment used	Calculated per ml of concentrate used
0.01	5	500
0.03	7	233
0.05	9	180
0.1	15	150
0.2	20	97.5
0.4	32.5	81
0.8	42	52

* Expressed as acid degrees per milliliter of enzyme preparation.

volume by lyophilizing) were used in the quantitative determination of lipase activity and the results calculated back to a basis of total activity per milliliter of original preparation, the calculated values for lipase activity were considerably higher when the smaller quantities of preparation were used.

Storage in well-filled screw-cap test tubes of cell-free filtrates containing lipase showed that a temperature of 3 to 5 C was more satisfactory for the preservation of the enzyme activity than was a temperature 23 to 25 C (table

5). The results of two trials were closely comparable. Other studies showed that the addition of cysteine did not prove advantageous for the preservation of lipase activity during storage.

Concentration of *M. lipolytica* cultures by lyophilizing, either partially or to complete dryness, resulted in considerable concentration of the lipase enzyme, as shown in table 6. However, lyophilized portions equivalent to 1 ml of the original culture yielded acid degree values about one-half of the values of the original culture in two cases, and only one-third of the original value in one case. Continued lyophilizing up to the change from liquid to solid state of the culture resulted in a sticky, gluey mass which was difficult to handle but which

TABLE 5

The influence of storage temperature, time, and the presence of cysteine upon M. lipolytica lipase activity

STORAGE CONDITION	LIPASE ACTIVITY* AFTER STORAGE TIME IN DAYS					
	Trial 1			Trial 2		
	0	4	106	0	4	96
3– 5 C	25	22.5	22	32	26	28
23–25 C	25	17.5	13	32	26	16
23–25 C and cysteine	25	21.0	14	32	18	16

* Expressed as acid degrees per milliliter of enzyme preparation.

TABLE 6

The effect of lyophilizing treatment of M. lipolytica cultures upon its lipase activity

ITEM STUDIED	TRIAL NUMBER		
	1*	2*	3
Acid degrees per ml of original culture†	36	70	54
Original weight of culture†	200 g	183 g	190 g
Weight of culture after lyophilizing	50 g	31 g	12 g
Acid degrees of 1 ml equiv. of orig. culture	19	24	26
Acid degree ratio of orig. to lyoph. culture	2:1	3:1	2:1

* Incompletely dried. † Calculated as 1 ml = 1 g.

dissolved readily in distilled water or in phosphate buffer solutions, and was possessed of high enzyme activity per unit mass.

Preliminary trials on the concentration of lipase by the foaming procedure at 0.5-unit intervals from pH 4.0 to pH 7.0 gave the highest concentration of lipase in the foam portion and the lowest concentration in the residual portion at pH 4.0. Accordingly, this pH level was used in further experiments on repeated foaming. The results of two trials are shown in table 7. Although the first fractionation did not result in an increase in lipase concentration, as judged by the acid degree values obtained, further fractionations resulted in increases in lipase concentration. Either the removal of the lipase by 50 per cent fraction-

ation was practically complete, or the lipase in the residue was destroyed, since the residues showed little or no lipase activity. The increase in acid degree values in the two trials was approximately 250 per cent in each case. The lipase remaining in the final foam fraction represented about 8 per cent of the initial lipase activity.

In preliminary trials in which magnesium sulfate, ammonium sulfate, and sodium chloride were used as precipitating agents, precipitation of a lipase-active

TABLE 7

The influence of repeated foaming upon the concentration of lipase produced by M. lipolytica

NUMBERS OF FRACTIONATION	VOLUME OF FRACTION (ML)	LIPASE ACTIVITY* PER ML OF			
		Liquid foam		Residue	
		Trial 1	Trial 2	Trial 1	Trial 2
Original	800	27	24	27	24
1	400	20	24	3	4
2	200	26	34	1	3
3	100	40	40	2	0
4	50	50	50	0	4
5	25	67	60	0	1

* Expressed as acid degrees per milliliter of enzyme preparation.

TABLE 8

The influence of temperature during the salting-out with ammonium sulfate and ethyl alcohol upon the lipase activity of the precipitate

ITEMS STUDIED	TRIAL 1		TRIAL 2	
	6 C	24 C	6 C	24 C
Acid degrees per ml of original supernatant	25	25	23	23
Total weight of precipitate (g)	0.2	0.2	0.16	0.18
Acid degrees per 0.03 g of precipitate	21	6	44	6
Calculated acid degrees per total precipitate	140	40	300	36
Calculated per cent recovery of original total lipase activity	1.4	0.4	3.0	0.36

substance occurred only in saturated solutions of ammonium sulfate. The data in table 8 show the results of two trials in which the lipase-active substance was precipitated by means of ammonium sulfate and ethyl alcohol, as described under "Methods," using temperatures of 6 and 24 C. Although an active preparation was obtained at each temperature, the results are much in favor of the lower temperature. The lipase activities of the precipitate obtained at 6 C were 250 and 730 per cent greater than the activities of the corresponding precipitates prepared at 24 C. In either case the enzyme recovered represented 3 per cent or less of the original lipase activity.

DISCUSSION

The lipolytic enzyme system of *M. lipolytica* has its optimum activity at pH 6.2 to 6.5, a range that relates the enzyme to the lipases of the yeasts and molds that have been studied in this respect, rather than to the bacterial lipases that are most active at somewhat alkaline reactions. The lipase of *M. lipolytica* is active over the entire range in which blue cheese ripening takes place, namely, pH 4.7 to 6.5 (Coulter, Combs, and George, 1938), and thus might be expected to be able to bring about considerable hydrolysis of butterfat during the ripening process of cheese of this type.

Although the optimum activity of the enzyme upon butterfat is at 28 to 33 C, the considerable activity at 10 C demonstrates the probability that the enzyme would be active in cheese curd at that temperature. The use of temperatures of approximately 32 C, as commonly maintained during the cheese-making process until hooping of the curd, would favor early enzyme activity.

Inactivation at temperatures of 42 C and above should result in no difficulty in cheese making as long as proper temperature levels are maintained during the cheese-making operation following the addition of the enzyme to the pasteurized milk. In a similar manner, the storage of cell-free enzyme preparations at low temperatures, such as 3 to 5 C, should aid in maintaining the strength of the lipase in solution, although a slight drop in activity may be expected on prolonged holding.

With respect to the concentration and purification of the enzyme system, all three methods employed resulted in large losses of total lipase activity. Lyophilizing was least detrimental to the enzyme, whereas fractionation by either foaming or salting-out at room temperature resulted in the greatest losses of lipase. The use of lower temperatures during foaming might very possibly decrease the extent of inactivation during the process, as was observed in the trials on salting-out of lipase with ammonium sulfate and ethyl alcohol. Further work would be necessary in order to obtain a more complete picture of this situation.

The observed characteristics of the enzyme indicate that it may be of considerable value in the manufacture of blue or Roquefort-type cheese from pasteurized milk as a replacement for the normal milk lipase destroyed in pasteurization.

SUMMARY AND CONCLUSIONS

Lipase activity on butterfat over a range of reaction from pH 4.0 to pH 8.0 was demonstrated for lipase obtained from *Mycotorula lipolytica*, with pH 6.2 to 6.5 being optimum.

Lipase activity on butterfat was demonstrated at temperatures from 10 to 52 C; temperatures of 37 C and above inactivated the enzyme, the reaction being more rapid at the higher temperatures studied.

A storage temperature of 5 C was much superior to 25 C for preserving the enzyme in cell-free preparations, activity being maintained at a high level for at least 3 months when storage was at the lower temperature in closed containers.

Lyophilized enzyme preparations retained about half their original total lipase activity.

Considerable concentration of lipase by foaming was demonstrated; large losses of enzyme activity occurred during the process.

The salting-out of lipase with saturated ammonium sulfate plus 30 per cent ethyl alcohol resulted in a white precipitate high in lipase activity.

REFERENCES

AVERY, O. T., AND CULLEN, G. E. 1920 Studies on the enzymes of pneumococcus. II. Lipolytic enzymes: esterase. J. Exptl. Med., **32**, 571–582.

COULTER, S. T., COMBS, W. B., AND GEORGE, J. S. 1938 The pH of blue or American Roquefort cheese. J. Dairy Sci., **21**, 273–274.

GLICK, D., AND KING, C. G. 1933 The protein nature of enzymes. An investigation of pancreatic lipase. J. Am. Chem. Soc., **55**, 2445–2449.

GORBACH, G., AND GUENTNER, H. 1932 Ueber Hefe Lipase. Monatsh. Chem., **61**, 47–60.

GYOTOKU, K., AND TERASHIMA, S. 1930 Studien ueber Lipase. V. Die Lipase und Eiweisskoerper. Biochem. Z., **217**, 292–305.

OSTWALD, W., AND MISCHKE, W. 1940 Untersuchungen ueber Zerschaeumung mit besonderer Ruecksicht auf Fragen der angewandten Chemie. III. Kolloid Z., **90**, 205–220.

PETERS, I. I., AND NELSON, F. E. 1948 Factors influencing the production of lipase by *Mycotorula lipolytica*. J. Bact., **55**, 581–591.

STEVENS, F. A., AND WEST, R. 1922 The peptase, lipase and invertase of hemolytic streptococcus. J. Exptl. Med., **35**, 832–845.

THIBODEAU, R., AND MACY, H. 1942 Growth and enzyme activity of *Penicillium roqueforti*. Minn. Agr. Expt. Sta., Tech. Bull. 152.