The Milk Lipases. III. Comparison of Milk Lipases in Supernatant and in Sediment Separated by Centrifugation of Skim Milk

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The fractional centrifugation of skim milk reported in a previous paper¹⁾ revealed that more than half of the original lipase activity still remained in the supernatant after centrifugation at $25,000\times G$ for 195 minutes in total. This result suggested the possible existence of two types of lipase system, one sedimented and the other not sedimented, in skim milk. Therefore, some properties of the milk lipases in the supernatant were reported in this paper to give some information on the two types of lipase system. Particularly, the effect of added formaldehyde on the lipase activities in the supernatant and sediment was studied in some detail.

In this connection, HERRINGTON and KRUKOVSKI²⁾ divided milk lipases into two groups, one inhibited by formaldehyde and the other not affected with it. They added from 1 to 27 drops of formalin to 160 ml of 20 per cent non-homogenized cream and found that, in all cases, the lipase activity had been reduced greatly in the cream. It was also shown that no lipolysis had been inhibited at all even by 27 drops of formalin as well as a single drop of it. They concluded rather prematurely that milk contained two types of lipases.

ROAHEN and SOMMER³⁾ added 0.05 or 0.15 ml of formalin containing 37 per cent formaldehyde to 110 g of a mixture of a sample and substrate, and demonstrated that lipolysis had been reduced by about one-third in the mixture, no distinction being apparent between the two levels of addition. PETERSON et al.⁴⁾ reported that 2.0 g of formaldehyde in one liter of milk had reduced the splitting of tributyrin by 75 per cent. GOULD⁵⁾ found that no formalin had inhibited lipase in homogenized cream when added at the following ratios of formalin to the cream: 1:1350, 1:450, and 1:250. TARASSUK and RICHARDSON⁶⁾ reported that formalin accelerated lipase activity. SCHWARTZ et al.⁷⁾ proved that formaldehyde was a competitive inhibitor, observing that the lipase system was always inhibited in raw skim milk to which had been added formaldehyde before the addition of substrate. At a concentration of 8.6×10^{-3} M the optimum lipolytic activity was observed at pH 7.5 to 8.5, whereas at a concentration of 4.6×10^{-2} M it was observed only at pH 7.0. The inhibitory effect of formaldehyde on milk lipase was also reported by DEMOTT⁸⁾.

Comparison of lipase activity between the supernatant and the sediment was also undertaken on mastitis milk as well as normal milk in this paper.

Materials and Methods

Skim milk: Skim milk was prepared in the same manner as described in the previous paper¹⁾

Sediment: Dialyzed sediment was prepared in the same manner as described in the previous paper⁹⁾.

Supernatant: Skim milk was centrifuged at $12,600\times G$ for 90 minutes. The resulting supernatant was dialyzed against 25 volumes of de-ionized water at 4°C for 24 hours, with cellophane as dialysis membrane, before it was ready for use.

Supernatant of mastitis milk: A cow with the healthy quarters of the udder, except one which had been affected with mastitis, was found by chance. It was used as source of mastitis milk. Mastitis milk was confirmed as such on the ground of pH value, chloride content, cell count, and the results of Hotis test¹⁰⁾. The milk from each quarter was skimmed twice by centrifugation at $900\times G$ for 10 minutes at room temperature. The skim milk obtained was subjected to supercentrifugation at $145,000\times G$ for 30 minutes at 1°C , a Hitachi ultracentrifuge, model 40 P, being used.

Determination of lipase activity: The modified method described in the previous paper⁹⁾ was used. Chemicals were added in the same manner as mentioned in the previous paper⁹⁾.

Results

Effect of added sodium chloride, ammonium hydroxide, and calcium chloride on lipase activity: The results are presented in Table 1. These chemicals affected lipase in the supernatant as well as in the sediment at pH 8.6, except calcium chloride the effect of which was more emphasized in the supernatant. There were some differences, however, in the effect among these chemicals at pH 7.0. The lipase in the supernatant was not inactivated by sodium chloride, but was activated by ammonia. The lipase in the sediment, however, was inactivated by sodium chloride, but was not activated or very slightly activated by ammonia at pH 7.0. Calcium chloride in the concentration of 0.05 M slightly inactivated the lipase in the sediment, but slightly activated the lipase in the supernatant.

Effect of added formaldehyde on lipase activity: Some workers²⁾ divided the milk lipases into two groups, one inhibited by formaldehyde and the other not affected with it. Then formaldehyde was examined for effect on lipase activity. The results, as shown in Fig. 1, reveal that the lipase system in the supernatant is similar to that in the sediment, at least, insofar as the effect of added formaldehyde is concerned. They also suggest that lipase was relatively stable against added formaldehyde at pH 7.0, but not at pH 8.6. It was not achieved, however, to classify the milk lipase system according to the sensitivity against added formaldehyde at a single level of pH.

Lipase activity in supernatant obtained from mastitis milk: Since mastitis is quite common and has a drastic effect on the composition and properties of milk, it is reasonable to

Table 1. Comparison of added chemicals for effect on lipase activities of sediment and supernatant

Chemical	Concentration M	pH 7.0		pH 8.6	
		Sediment	Supernatant	Sediment	Supernatant
None	0	100	100	100	100
NaCl	0.3	69.6	102.2	100.9	92.5
NH ₄ OH	0.03	107.8	154.8	117.2	109.6
CaCl ₂	0.005	—	100.8	—	113.6
CaCl ₂	0.05	90.9	116.0	182.6	271.0

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Fig. 1. Relationship between concentration of added formaldehyde and its inhibitory effect on lipase activities of sediment and supernatant at various pH levels.

Table 2. Comparison of lipase activities of skimmed mastitis milk and skimmed normal milk, and their supernatant

Udder quarter	Condition	Mastitis test				Total lipase activity* per 100 ml			
		pH	Cl %	Cell $\times 10^3/\text{ml}$	Hotis test	pH 7.0		pH 8.6	
						Skim milk	Super-natant	Skim milk	Super-natant
Right fore	Normal	6.58	0.084	60	—	51.8	12.2	69.4	14.7
Right hind	Mastitis	7.15	0.280	6850	—	12.1	12.2	14.9	9.2
Left fore	Normal	6.72	0.100	240	—	47.1	12.6	68.5	18.9
Left hind	Normal	6.76	0.082	25	—	54.5	16.4	72.2	17.2

* The quantity in ml of normal base necessary to neutralize 100 g of fat in the reaction mixture for 1 hour.

assume that mastitis may have some influence on the lipase activity of milk. As presented in Table 2, the milk obtained from the right hind quarter was considered to be mastitis milk

because of its high pH, chloride content, and cell count. Total lipase activity of skim milk obtained from mastitis milk was much less than that of nonmastitis skim milk. However, all of the lipase activity at pH 7.0 and over 60 per cent of that at pH 8.6 contained in mastitis skim milk were retained in the supernatant after centrifugation. On the other hand, only 20 to 30 per cent of the total lipase activity of nonmastitis skim milk remained in the supernatant.

Discussion

Investigation on the effect of added chemicals has given information on two lipase systems, one present in the sediment and the other in the supernatant. The effect of added sodium chloride, ammonia, and calcium chloride on lipase activity suggested that a lipase with pH 8.6 as optimum pH for lipolysis (pH-8.6 lipase) in the supernatant was similar to pH-8.6 lipase in the sediment. On the other hand, a lipase with pH 7.0 as optimum pH for lipolysis (pH-7.0 lipase) in the supernatant was not considered to be similar to pH-7.0 lipase in the sediment. The following facts, however, should be noted: (1) The lipases in the sediment are in a rather isolated form, but those in the supernatant are not. (2) Casein, which is labile to calcium ion, is assumed to be the major constituent of the sediment, but it is a minor constituent of the supernatant. There was a marked difference between the effect of sodium chloride on the lipase activity at pH 7.0 of the supernatant and that of the sediment. Such difference is of great interest, even when the two facts mentioned above are taken into consideration. The author suggested in his previous paper¹⁾ that the lipase in skim milk differed from QUIGLEY's lipoprotein lipases which had been separated from butter milk based on the effect of 0.3 M sodium chloride. By using 0.3 M sodium chloride, pH-7.0 lipase in skim milk also appears to be classified into two groups, which may be separated from each other by centrifugation at a certain number of G. The effect of added formaldehyde found in this study supports the similarity of the two lipase systems at pH 8.6, but does not support the dissimilarity at pH 7.0. There was, however, a slight difference in the effect between the two lipase systems at pH 6.5, which is very close to the average pH of milk. The lipase activity of the sediment was less inactivated by added formaldehyde than that of the supernatant at pH 6.5. The effect of added formaldehyde does not support either the classification of the milk lipases by formaldehyde proposed by HERRINGTON and KRUKOVSKY²⁾, even when the inhibitory effect of added formaldehyde is greatly influenced by pH value. All the milk lipases appeared to be inactivated by formaldehyde in the same rate at any single level of pH, except at pH 6.5. By an addition of 6×10^{-2} M formaldehyde, the lipase activity of the supernatant was reduced to 17 per cent of the original activity, but not to zero as in the case of that of the sediment. Since the effect of formaldehyde at a higher concentration than 6×10^{-2} M was not investigated, a possibility still remained that formaldehyde at any concentration higher than 6×10^{-2} M might cause no increase in inhibitory effect. HERRINGTON and KRUKOVSKY²⁾ added 1 to 27 drops of formalin to 160 ml of cream. This concentration, which was presumed to be equivalent to approximately 0.2 to 4.1×10^{-2} M, of formaldehyde was not useful for the classification of lipases at any single level of pH investigated. HERRINGTON and KRUKOVSKY²⁾ used non-homogenized cream as substrate and applied long-term incubation. They also determined free fatty acids by titration of butter fat separated by churning. The differences in assay procedure and in method of free fatty acid determination probably

contributed to the difference in results between their early work and the present experiment.

The lipase activity of skim milk prepared from mastitis milk was of interest. TARASSUK et al.¹¹⁾ concluded that spontaneous lipolysis occurred more often in mastitis milk than in non-mastitis milk, but that non-mastitis milk was much more susceptible to induced lipolysis on activation by shaking than mastitis milk. Their conclusions were supported, inasmuch as the lipase activity of skim milk measured in this experiment was considered to correspond to the "induced lipolysis". Since only one sample of mastitis milk was investigated in this experiment, no results might be conclusive. It is suggested, however, that this line of investigation may give further information on the origin of the milk lipases and on the milk lipase systems, if a sufficient number of samples are analyzed.

Summary

The supernatant and sediment were separated from milk by centrifugation at $12,600\times G$ for 90 minutes and at $9,600\times G$ for 60 minutes and subsequent dialysis. They were compared with regard to some properties of milk lipases contained in them.

Sodium chloride (0.3 M), ammonium hydroxide (0.03 M), and calcium chloride (0.05 M) affected lipase in the supernatant, as well as that in the sediment, at pH 8.6. There were some differences, however, in the effect of these chemicals at pH 7.0. Namely, sodium chloride lowered the lipase activity of the sediment to 69.6 per cent of the control trial, but did not affect the lipase in the supernatant. The lipase system in the supernatant was similar to that in the sediment at least at pH 7.0 and at pH 8.6, insofar as the effect of formaldehyde (0.3 to 6.3×10^{-2} M) was concerned. The lipase of the sediment was less inactivated by formaldehyde at pH 6.5 than that of the supernatant. Lipase was relatively stable at pH 7.0, but not at pH 8.6. It was not possible to achieve the classification of the milk lipase according to sensitivity to formaldehyde at a single level of pH.

The lipase activity of skim milk obtained from mastitis milk was one-fourth and one-fifth of normal milk at pH 7.0 and pH 8.6, respectively. The lipase activity of mastitis skim milk was recovered about 100 per cent at pH 7.0 and 61.7 per cent at pH 8.6 in the supernatant obtained by centrifugation at $145,000\times G$ for 30 minutes, but that of normal milk was recovered only 20 to 30 per cent under similar conditions.

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牛乳の脂肪分解酵素

III. 脱脂乳遠沈沈殿物および上澄液中の脂肪分解酵素の比較

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第 I 報において、脱脂乳遠沈沈殿物中の脂肪分解酵素 (LP と略す) の諸性質を報告した。それによつて、脱脂乳中の LP は、 $25,000 \times G$, 195 分間の遠沈を行なつても、50% 以上が上澄液中に残存することがわかつた。そこで、上澄液中の LP が、沈殿物中のそれと同じものであるかどうかを知るため、 $9,600 \times G$, 60 分間遠沈の沈殿物と、 $12,600 \times G$, 90 分間遠沈の上澄液を透析した後、それらの LP について、試薬添加の影響を調べた。

1) 塩化ナトリウム、アンモニアおよび塩化カルシウム添加の影響: 最適 pH 8.6 の LP については、沈殿物も、上澄液も、同じ結果を示した。最適 pH 7.0 の LP については、かなりの違いがあつた。沈殿物中の LP は、ある程度精製された形であり、また沈殿物は、主としてカゼインから成るものと考えられる。上澄液には、カゼインは比較的少なく、牛乳中の糖や塩類が、透析後も幾分残存しているものと考えられる。そこでこの結果だけで結論することはむずかしいが、上澄液中の最適 pH 7.0 の LP は、沈殿物中のそれと幾分異なるものと推察される。

2) ホルムアルデヒド添加の影響: 従来、牛乳中の LP には、ホルムアルデヒドによつて阻害されるものと、そうでないものがあるといわれていた。これによると、上澄液と沈殿物中の LP が、ホルムアルデヒドに対して異なる反応を示す可能性も考えられる。そこで両者

について、ホルムアルデヒド添加の影響を調べた。

その結果、沈殿物も、上澄液も、同じ傾向を示すことがわかつた。阻害の程度は、pH によつて著しく影響され、pH 7.0 では比較的安定であるが、pH 8.6 では、 $2.5 \times 10^{-2} M$ で活性を全く失う。ホルムアルデヒドにより、最適 pH 7.0 の LP は阻害されにくい、最適 pH 8.6 の LP は阻害されやすいと考えられる。しかし同一の pH においては、ホルムアルデヒドに対する反応によつて 2 種の LP を見分けることはできなかった。pH 6.5 においては、上澄液中の LP は、沈殿物中のそれよりも、ホルムアルデヒドによつて阻害されやすいと考えられる。しかしいずれも、ホルムアルデヒド添加濃度の増大に伴い、その活性を減ずる。

3) 乳房炎乳中の脂肪分解酵素: 1 分房が乳房炎、他の 3 分房が正常な 1 頭の乳牛の各分房から得た乳について、その脱脂乳と脱脂乳超遠沈 ($145,000 \times G$, 30 分) 上澄液に含まれる LP の活性を測定した。乳房炎乳の脱脂乳は、正常乳よりも LP が少なく、その 1/4 以下に過ぎなかつた。上澄液についてみると、最適 pH 7.0 の LP は 100%、最適 pH 8.6 の LP は 60% 以上が上澄液中に残存していた。これは、全 LP のわずかに 20~30% が上澄液中に見いだされるに過ぎない正常乳に比べて、著しく異なる点であつた。