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Inhibition of Microbial Lipases by Fatty Acids

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ABSTRACT

SMITH, J. L. (U.S. Department of Agriculture, Beltsville, Md.), AND JOHN A. ALFORD. Inhibition of microbial lipases by fatty acids. Appl. Microbiol. **14**:699–705. 1966.—Addition of lard or sodium oleate to the medium used for lipase production by *Pseudomonas fragi* resulted in a decreased accumulation of lipase in the culture supernatant fluid without affecting cell growth. The production and activity of lipase was inhibited by lard, sodium oleate, and the salts of other unsaturated fatty acids. Some divalent cations, Tweens, lecithin, and bovine serum prevented oleate inhibition, but did not reverse it. Similar inhibitory actions were observed with *Geotrichum candidum* lipase, but not with a staphylococcal lipase or pancreatic lipase. A protective effect by protein in crude enzyme preparations is indicated. The ability of oleate to lower surface tension does not appear to be related to its ability to inhibit lipase.

The ability of microorganisms to grow on and attack naturally occurring fats has been recognized for many years, but it is equally well known that most of the lipases involved also can be produced in media devoid of lipid material. In studies on the microbial lipases, the lipolytic activity of cultures grown in the presence of fat usually is determined by measuring the free fatty acids (FFA) that are liberated into the medium during growth (11, 22). When no lipid is included in the growth medium, the lipolytic activity is determined by adding a sample of the culture supernatant fluid or mycelial extract to a buffered fat substrate; the FFA released after a relatively short incubation period is then measured (3, 9, 16). However, Nashif and Nelson (17) observed that the lipolytic activity of the supernatant fluid from cultures grown in the presence of butterfat or triglycerides of long-chained fatty acids is considerably less than from cultures grown without fat. Similar observations in our earlier work (1), and, more recently, in unpublished studies on the action of microorganisms on carbonyls, led us to examine more closely this apparent inhibition by substrate. This paper describes the inhibitory effects of lard on the production and activity of *Pseudomonas fragi* lipase without concomitant effect on growth, as well as subsequent studies to determine the cause of the inhibition. Observations on pancreatic lipase and the lipases produced by *Geotrichum candidum* and *Staphylococcus aureus* also are reported.

MATERIALS AND METHODS

Production and assay of P. fragi lipase. The strains of *P. fragi* employed, the conditions of lipase production, and the methods of assay were as previously reported (3), with the following exceptions. (i) In some media, lard emulsions to give a final concentration of 0.5% lard were added to the culture medium. The supernatant fluid from these cultures contained FFA released from the fat as the lipase was produced. This titratable acidity was subtracted from the value obtained in the assay to give the net activity shown in the data. (ii) Assay samples were incubated for 1 hr. Unless noted otherwise, the lipase employed was the supernatant fluid from a 4- or 5-day culture of *P. fragi* grown in 1% Case peptone medium from Case Labs., Chicago, Ill.

Production and assay of G. candidum lipase. Maximal production of lipase by *G. candidum* was obtained in a high protein medium as previously described (4). The following synthetic medium gave good yield of lipase when incubated at 20 C for 4 days: 0.1% NH_4Cl, 0.15% KH_2PO_4, 0.012% $MgSO_4 \cdot 7H_2O$, 0.234% monosodium L-glutamate, 0.16% L-arginine·HCl, 0.07% L-lysine·HCl, and 0.001% each of $FeSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, and $MnSO_4 \cdot H_2O$. After the medium was autoclaved, sterile glucose was added to give a final concentration of 0.25%. Assay conditions were the same as for *P. fragi*.

Production and assay of S. aureus lipase. The lipase of *S. aureus* was produced in phosphate-buffered Trypticase (BBL) broth incubated on a shaker at 30 C for 24 hr, as previously described (4).

Source and assay of pancreatic lipase. The porcine pancreatic lipase was obtained from the Nutritional

Biochemicals Corp., Cleveland, Ohio. It was assayed at *p*H 8.0 in 0.05 M tris(hydroxymethyl)aminomethane containing 4% lard. A 2-ml amount of a solution containing 5 mg/ml of the dry enzyme was assayed in a total volume of 10 ml.

Measurement of growth. Standard plate counts of *P. fragi* were made on Case peptone medium (3) containing 1.5% agar. Plates were incubated at 20 C for 2 to 3 days.

Preparation of sodium salts of fatty acids. Fatty acids of > 99% purity (obtained from the Hormel Institute, Austin, Minn.) were dissolved in ethyl alcohol, then neutralized with alcoholic NaOH. The resulting precipitated salts were washed several times with alcohol, dried over sulfuric acid, and stored in a nitrogen atmosphere.

Surface tension. The surface tension was determined in dynes per centimeter with a Du Noüy tensiometer (Cenco Instruments Corp., Chicago, Ill.).

Determination of FFA. The FFA liberated from the lard were separated from the mono-, di-, and triglycerides by thin-layer chromatography (TLC) on silica gel, according to the procedure of Clément, Clément, and Bézard (5), and were methylated with HCl-methanol (14). The methyl esters then were separated on a column of 20% diethylene glycol succinate on GasChrom P (Applied Science Laboratories, Inc., State College, Pa.) in an F & M model 700 flame ionization gas chromatograph (F & M Scientific, Avondale, Pa.). They were identified by comparison of their retention times with known esters. Column temperature was 185 C.

RESULTS

The data in Fig. 1 indicate that, in the presence of lard emulsion, very little lipolytic activity de-

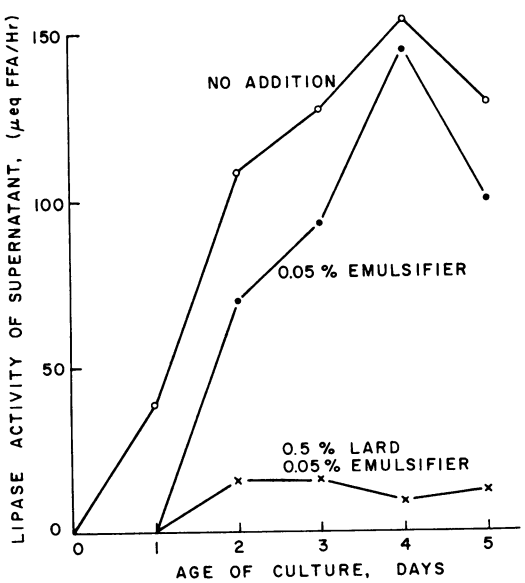

FIG. 1. *Effect of lard added to the growth medium on the production of lipase by Pseudomonas fragi.*

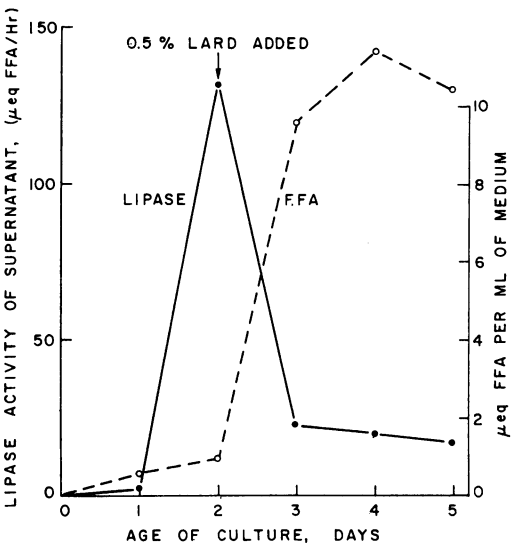

FIG. 2. *Effect of delayed addition of lard on lipase production during the growth of Pseudomonas fragi.*

velops in cultures of growing cells of *P. fragi*. The emulsifying agent, a soybean phospholipid, was only slightly inhibitory. Essentially identical growth curves were obtained in all three instances, indicating that the amount or rate of cell growth was not involved. In the cultures containing lard, the concentration of FFA in the supernatant fluid was found to be 5.0 μeq/ml at the 2nd day and remained at approximately that level throughout the experimental period. In the absence of lard emulsion, or in the presence of the emulsifier, little or no FFA were present in the culture supernatant fluid.

In another series of experiments, lard emulsion was added to the growth medium after 2 days instead of at zero-time. By this time, considerable lipase activity had appeared in the medium. From the data in Fig. 2, it can be seen that the addition of lard on the 2nd day caused approximately a sixfold decrease in lipase activity by the 3rd day. Concomitant with the decrease in lipase activity in the presence of lard was an increase in the fatty acid concentration in the medium. The emulsified lard had no inhibitory effect on growth.

To determine the effect of the emulsion on lipase activity directly, as differentiated from its effect on production of the enzyme, lard was added to cell-free supernatant fluids of 5-day cultures, and samples were removed at intervals for assay. Data in Fig. 3 show that the activity of the enzyme progressively decreased with time of exposure to lard, whereas the FFA in the supernatant fluid increased. Since there was no release of FFA in absence of lard, this indicates an in-

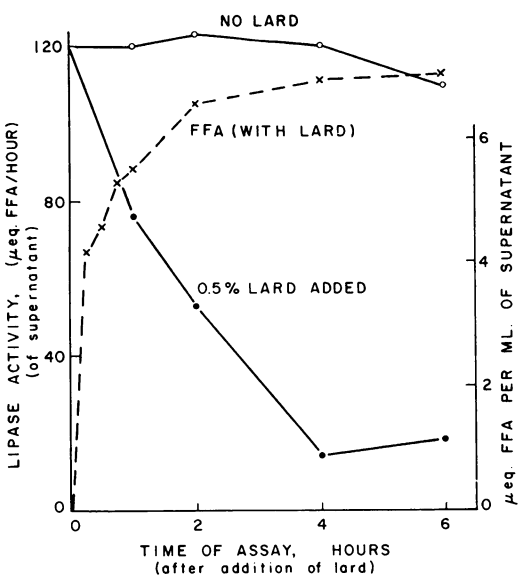

FIG. 3. *Effect of lard on the lipolytic activity of supernatant fluid from a 5-day culture o Pseudomonas fragi. Lard emulsion to give a lard concentration of 1.0% was added to the culture supernatant fluid. The flasks were incubated at 20 C, and samples were removed at intervals for lipase assay and determination of FFA content.*

verse relationship between free fatty acid accumulation and lipase activity.

There are many reports in which fatty acids and their salts have been shown to be inhibitory to enzymatic and biological systems: for example, lecithinase activity from kidney and lung extracts of mouse, guinea pig, and rabbit (10); esterase activity from sheep liver extracts (23); malic enzyme from whole cells of *Lactobacillus arabinosus* (6); trypsin and pepsin (13, 15, 19); and bacterial growth (18). Since the inhibition of lipase activity appeared to parallel the appearance of fatty acids, the sodium salts of various fatty acids were prepared and tested to determine whether they might have an effect on lipolytic activity similar to that reported for other enzymes. It was found that the salts of several unsaturated fatty acids were inhibitory, whereas saturated, and a few unsaturated, acids were without effect (Table 1). There also appeared to be some relationship between degree of inhibition and the ability to decrease surface tension to approximately 30 dynes per cm or lower.

Various divalent metallic ions (Ca^{++}, Sr^{++}, Ba^{++}, Cd^{++}, Mn^{++}, and Co^{++}) were found to eliminate the inhibition by sodium oleate (Table 2). Other cations (Sn^{++}, Hg^{++}, Pb^{++}, Cu^{++}, Mg^{++}, Fe^{++}, and Zn^{++}) were not effective. Addition of

TABLE 1. *Effect of sodium salts of fatty acids on the activity of lipase produced by Pseudomonas fragi*

Compound added	Inhibition of lipase after[a]				Surface tension
	0.5 hr	1 hr	2 hr	3 hr	
	%	%	%	%	*dynes/cm*
None	0	0	0	0	51.7
Na palmitoleate (16-1)[b]	59	88	94	93	25.3
Na oleate (18-1)	30	61	89	92	25.9
Na linoleate (18-2)	24	50	73	92	27.0
Na linolenate (18-3)	27	58	82	94	28.3
Na petroselenate (18-1)	23	54	84	93	24.8
Na arachidonate (20-4)	42	64	89	91	30.4
Na eicosapentenoate (20-5)	64	77	91	92	30.5
Na docosahexenoate (22-6)	48	67	75	71	31.1
Na elaidate (18-1)	—	—	—	6	42.1
Na erucate (22-1)	—	—	—	14	40.3
Na nervonate (24-1)	—	—	—	14	45.1
Na laurate (12-0)	—	—	—	0	42.6
Na myristate (14-0)	—	—	—	4	37.9
Na palmitate (16-0)	—	—	—	14	42.9
Na stearate (18-0)	—	—	—	10	44.6
Na arachidate (20-0)	—	—	—	20	53.6
Na behenate (22-0)	—	—	—	25	53.0
Distilled water	—	—	—	—	70.2

[a] After addition of the salts (final concentration, 1 μmole/ml) to the culture supernatant fluids, the flasks were incubated at 20 C, and samples were removed for assay after the times indicated.

[b] Number of carbon atoms and double bonds in molecule.

disodium ethylenediaminetetraacetate (EDTA) eliminated the protective effect of metallic ions. EDTA was not inhibitory to lipolytic activity.

The results in Table 3 indicate that a variety of chemically unrelated molecules, such as Tweens, bovine serum, Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.), and lecithins, also were effective in eliminating the inhibitory action of oleate. None of these compounds was inhibitory in the absence of oleate.

To determine the effect of the delayed addition of Ca^{++} on oleate inhibition, the cation was added to the enzyme 1 hr after the addition of oleate. The data in Fig. 4 show that Ca^{++} caused an immediate neutralization of further oleate inhibition, but there was no reversal of oleate inhibition that had already occurred. Similar results were obtained with Tween 80, soybean lecithin, and Triton X-100.

TABLE 2. *Effect of the addition of various metallic ions on the sodium oleate inhibition of the cell-free lipase of Pseudomonas fragi*

Metallic ion added	Inhibition of lipase activity[a]	
	Oleate[b]	Oleate and EDTA[b]
	%	%
None	98	96
Ca^{++}	4	86
Cd^{++}	0	84
Sr^{++}	0	89
Ba^{++}	2	41
Co^{++}	46	87
Mn^{++}	49	88

[a] Metallic ions, oleate, and EDTA were added simultaneously to the supernatant fluid and incubated at 20 C for 3 hr; a sample was then removed for assay.

[b] Oleate, EDTA, and metallic compounds each had a final concentration of 1 μmole/ml.

TABLE 3. *Effect of the addition of various organic compounds on the inhibition of the cell-free lipase of Pseudomonas fragi by sodium oleate*

Compound added	Inhibition of lipase[a]
	%
Sodium oleate[b]	91
Sodium oleate plus:	
Tween 20	0
Tween 40	0
Tween 80	0
Tween 81	0
Bovine serum	3
Triton X-100	22
Soybean lecithin	13
Animal lecithin	18
Egg lecithin	8
Astec 4135	26

[a] The compounds were added to the supernatant fluid and incubated at 20 C for 3 hr; then a sample was removed for assay.

[b] The final concentration of sodium oleate was 1 μmole/ml; other compounds, 2 mg/ml.

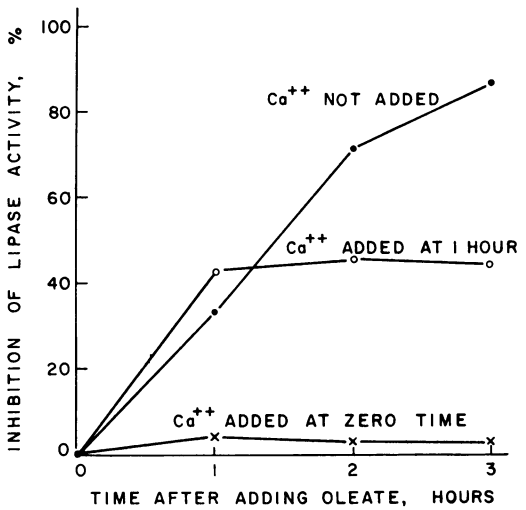

FIG. 4. *Effect of the delayed addition of Ca^{++} on oleate inhibition of the cell-free lipase of Pseudomonas fragi. Ca^{++} and sodium oleate were present in a concentration of 1 μmole/ml. After addition of oleate, supernatant fluid was incubated at 20 C, and samples were removed for assay at times indicated.*

The earlier suggestion of a correlation between surface tension and inhibitory activity was not supported by the data in Fig. 5. Increasing concentrations of sodium oleate showed increasing levels of inhibition of lipase activity and decreasing surface tensions. However, the addition of bovine serum eliminated the inhibitory effect, even though the surface tension still was depressed. The serum alone had no effect on surface tension, whereas, in other experiments, Triton X-100 and Astec 4135 decreased the surface tension to 29.8 and 29.9 dynes per cm, respectively, and also eliminated the inhibitory effect of the oleate.

As was shown with lard, inclusion of oleate in the growth medium limited the development of lipolytic activity in the supernatant fluid. Figure 6 indicates that this effect was not related to total cell population.

The data in Table 4 show that, when the lipase of *G. candidum* was produced in a high protein medium, its activity was not subsequently inhibited by sodium oleate. However, when it was produced in the synthetic medium described

above, it was inhibited in its activity to some extent by lard and almost completely by oleate. When *P. fragi* lipase was produced in a high protein medium such as that used for *G. candidum*, it was only partially inhibited in activity by sodium oleate, yet was completely inhibited if produced in 1% peptone or a synthetic medium. Staphylococcal lipase, for which no synthetic medium for production has yet been developed,

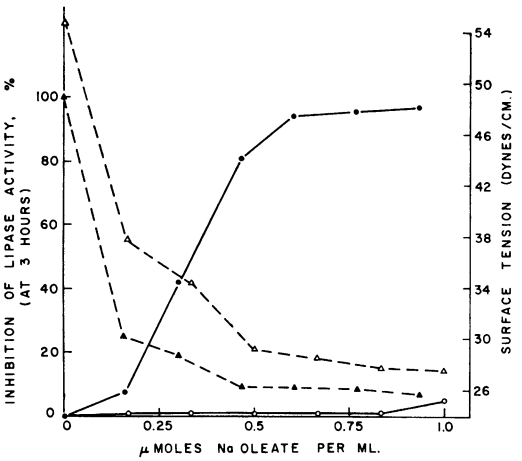

FIG. 5. *Relationship of surface tension to the inhibition by sodium oleate of the lipase activity of Pseudomonas fragi. Symbols:* ● = *inhibition by oleate;* ○ = *inhibition by oleate + 2 mg/ml of bovine serum;* ▲ = *surface tension with oleate;* △ = *surface tension with oleate + 2 mg/ml of bovine serum.*

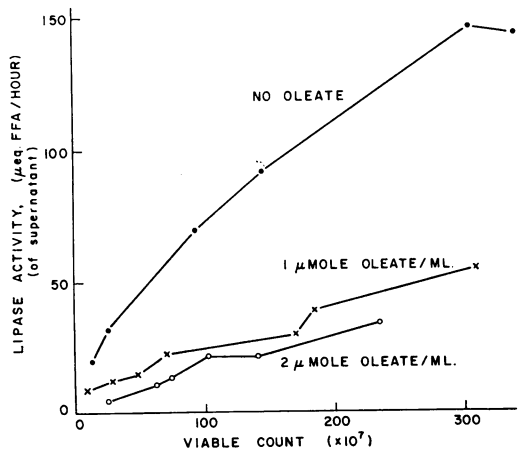

FIG. 6. *Production of lipase during the growth of Pseudomonas fragi in the absence and presence of sodium oleate. Sodium oleate was added to 1% peptone medium and the flasks were inoculated and incubated at 20 C. Samples were withdrawn at intervals for the determination of viable counts and lipase activity.*

TABLE 4. *Effect of lard and oleate on various lipolytic systems*

Source of lipase	Inhibition of lipase activity by[a]	
	Lard (1%)	Na oleate (1 μmole/ml)
	%	%
Geotrichum candidum (protein medium)[b]	29	0
Geotrichum candidum (synthetic medium)[b]	39	92
Pseudomonas fragi (high protein medium)[c]	76	42
Pseudomonas fragi (1% peptone medium)	85	91
Staphylococcus aureus (protein medium)[b]	31	0
Pancreatic lipase	8	0

[a] Lard or sodium oleate was added, the mixture was incubated at 20 C for 6 hr (lard) or 3 hr (oleate), and then a sample was removed for assay.

[b] As described by Alford and Smith (4).

[c] Standard Case peptone medium containing 4% Case peptone.

was not inhibited by oleate when produced in its recommended medium (4). Commercially available pancreatic lipase was not inhibited by oleate.

The FFA liberated from lard by lipase inhibited to 50% of its activity by sodium oleate were identified by gas chromatography and compared with those released by noninhibited lipase. The percentage distribution of fatty acids liberated from lard was approximately the same for both systems.

DISCUSSION

A number of unsaturated fatty acids have been shown to be inhibitory to the activity of *P. fragi* lipase. Among this group is oleic acid, which accounts for almost 70% of the fatty acids released from lard by this bacterial lipase (2). Thus, the inhibition of the lipase of *P. fragi* by lard is probably, at least in part, the result of unsaturated FFA accumulation following hydrolysis of the fat. This conclusion is supported by the slower rate of development of inhibition in the presence of lard when the fatty acid first had to be hydrolyzed from the fat, as compared with the more rapid rate when the fatty acid was added directly. The lower solubility and differences in configuration of some of the fatty acid salts (e.g., elaidic) may have been factors in their lack of inhibitory activity. However, salts of shorter chain fatty acids (C_2–C_{10}) were soluble, but not inhibitory.

The inhibition of lipase by oleate is irreversible, inasmuch as Ca^{++} prevents further inhibition but cannot reverse that which has already occurred.

In addition to this inhibitory effect of end product on the activity of the lipase, there also may be an effect on the synthesis of the enzyme. The presence of oleate during the growth of *P. fragi* and consequent production of lipase leads to a diminished accumulation of lipase in the culture. However, this could simply be another manifestation of inhibition of activity of the lipase after it has been synthesized and released from the cell. Inhibition of activity can be differentiated from inhibition of synthesis, but the reverse is not yet possible.

Although O'Leary (18) indicated that fatty acids exert a toxic effect on bacterial growth by their ability to lower surface tension, the concurrent decreases in lipase activity and surface tension when fatty acids were present probably were coincidental. Some compounds, such as Triton X-100, lowered surface tension but had no effect on lipase activity. Bovine serum, which alone has no effect on surface tension, neutralized the inhibition of sodium oleate without changing its effect on surface tension. No change in growth rate accompanied the drop in surface tension, as was reported by O'Leary (18). Gerstl and Tennant (10) concluded that lowered surface tension was not of primary importance in explaining fatty acid inhibition of animal lecithinases.

Peck (19) stated that Ca^{++} eliminated oleate inhibition of trypsin action by forming an insoluble salt with the fatty acid. Formation of insoluble soaps may explain the ability of Ca^{++}, Sr^{++}, Cd^{++}, and Ba^{++} to detoxify oleate activity against the lipase of *P. fragi*. Nevertheless, Fe^{++} and several other divalent ions are known to form insoluble soaps with oleate, yet they did not prevent inhibition of the lipase. However, these ineffective cations may be more strongly complexed by other components of the medium, such as phosphate or sulfhydryl groups, than are the effective detoxifying cations.

Coles and Lichstein (7) presented evidence that suggests that lecithin and Tween 40 may detoxify oleate for the malic enzyme from *L. arabinosus* by combining with the fatty acid. This may explain the ability of the Tweens, lecithin, and Triton X-100 to prevent the inhibition of lipase by oleate. In any case, the ability of proteins, particularly serum albumin, to combine with fatty acids may be responsible for the lipase protective action of bovine serum (8, 12, 20).

The ability of proteins to prevent the inhibition of lipase by oleate also may explain the apparent differences in sensitivity to oleate of lipases from different sources. Whereas *G. candidum* lipase produced in a protein medium is relatively insensitive to oleate, lipase produced by this microorganism in a synthetic medium is inhibited by the fatty acid. The same is true for *P. fragi*. Residual protein in the supernatant fluid of the protein medium cultures might function in the protective mechanism. Protein contamination of the enzyme preparation also could explain, at least in part, the lack of inhibitory effect of oleate on the activity of pancreatic lipase and staphylococcal lipase shown here and on the lipase produced by *Penicillium roquefortii* (21).

The protein and Ca^{++} in skim milk may be responsible for the relatively high level of staphylococcal lipase activity obtained by Vadehra and Harmon (22) in skim milk containing 10% butterfat. In addition, butterfat contains primarily saturated fatty acids which were shown in our study to have little inhibitory action. Thus, the presence of protein and possibly divalent metallic ions in various fat-containing foods may account for the ability of appreciable enzymatic lipolysis to occur, even though the enzyme is sensitive to very low concentrations of oleate and other unsaturated faty acids. Conversely, highly purified fats and oils may be less susceptible to extensive enzymatic hydrolysis, provided small amounts of an unsaturated fatty acid are present.

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