

Lipolytic Activity of Microorganisms at Low and Intermediate Temperatures. II. Fatty Acids Released As Determined by Gas Chromatography

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SUMMARY

Determination was made of the types and quantities of fatty acids released from lard, tallow, corn oil, and coconut oil by lipases from psychrophilic strains of *Pseudomonas*. The fatty acids were measured by gas chromatography. The rate of fatty acid liberation fell off sharply after the first few hours, but the ratios of the types of fatty acids were not affected until after 24 hours. Additional evidence is presented that much of the palmitic acid in lard is esterified at the beta position. Also discussed is the possible effect of differences in rate of lipolysis of fatty acids from natural substrates on interpretation of their degree of randomness, as measured by enzyme specificity.

Several workers have reported that microbial lipases attack the various natural and pure triglycerides at different rates (Goldman and Rayman, 1952; Mukherjee, 1951; Nashif and Nelson, 1953; Shipe, 1951; Wilcox *et al.*, 1955). Few direct measurements of the fatty acids released have been made, however. Richards and El-Sadek (1949), in an investigation on rancid butter, reported that bacteria produced less volatile acids but more solid acids than molds, and that the liquid acids constituted a majority of the fatty acids recovered. Wilcox *et al.* (1955) employed paper chromatography to measure the individual volatile fatty acids released from butterfat by microorganisms. No quantitative measurements were made.

Recent work by Mattson and co-workers (Mattson and Beck, 1955; Mattson and Lutton, 1958; Mattson *et al.*, 1952) and Savary *et al.* (1957), on pancreatic lipase and triglyceride structure, has indicated that pancreatic lipase is only specific for the fatty acid esterified at the α -position of the triglycerides. Using this position specificity, they presented additional evidence that the

fatty acids in natural triglycerides are not randomly distributed.

Improved techniques utilizing gas chromatography (Hornstein *et al.*, 1960) now make possible accurate determination of the individual nonvolatile fatty acids released from natural triglycerides. The present investigation was undertaken to determine how the rate of fatty acid liberation varied with time in bacterial lipolysis, whether the types of fatty acids released from natural fats varied with time, whether the temperature of enzyme production affected its specificity, and whether appreciable differences in strain specificity occurred among selected psychrophilic pseudomonads.

EXPERIMENTAL METHODS

The fat emulsions used were prepared as indicated in the first paper (Alford and Elliott, 1960), except that homogenization was carried out at 4000 lb, with a recycling period of 20 minutes.

Unless otherwise indicated, the bacteria producing the enzymes were grown for 3 days at 20°C in 1% peptone broth buffered at pH 7.0 with 0.05M phosphate. After centrifuging at 2500 × G, the supernatant was assayed for lipase activity as previously reported, with the following exceptions. In extraction of the fat and free fatty acids from the samples for titratable acidity and chromatographic

^a Deceased.

terminations, 10-ml aliquots were taken instead of 20-ml portions of petroleum ether. These slight modifications speeded up the procedure considerably without affecting its accuracy. The assay flasks, which contained 1 g of fat (as a 2% emulsion), were incubated 24 hr at 36°C unless otherwise indicated.

The method for separation of the free fatty acids from the fat and their subsequent methylation and determination by gas chromatography is described elsewhere (Hornstein *et al.*, 1960). The quantitative accuracy of this method varies somewhat with both the amount and type of fatty acid ester being measured. For the acids from caprylic through palmitic the method will measure 5-10- μ g quantities on the column within $\pm 5\%$. For the saturated and unsaturated C_{18} acids, 15-30 μ g are required to achieve an accuracy of $\pm 5\%$.

The cultures used were strains of *Pseudomonas* from the following sources: Culture A, *Pseudomonas fragi*, NRRL B-25, from Dr. W. C. Haynes, NURDD, USDA, Peoria, Illinois; Culture B, *Pseudomonas* sp. # 15, from Dr. John Ayres, Iowa State University, Ames, Iowa; Culture C, *Pseudomonas* sp. # 35, same source as B; Culture D, *Pseudomonas fluorescens*, ATCC 11251, carried in our stock culture collection for several years.

All of these cultures were polar flagellated, non-sporeforming rods, and oxidative in their glucose metabolism, and formed visible growth in peptone broth within 3 days at 1°C.

RESULTS

The previous investigation (Alford and Elliott, 1960) showed that the total amount of lipase produced per cell was greatly reduced when incubation temperature was raised from 20 to 28°C. The data in Fig. 1 suggest that a qualitative effect on the lipase also may occur when the production temperature is increased. There was a small but consistent increase in the percentage of unsaturated fatty acids liberated by the enzyme produced at 28°C, with a slight decrease in the total saturated acids, particularly stearic acid.

As expected, the earlier work showed a quantitative effect of pH on production of lipase. Table 1

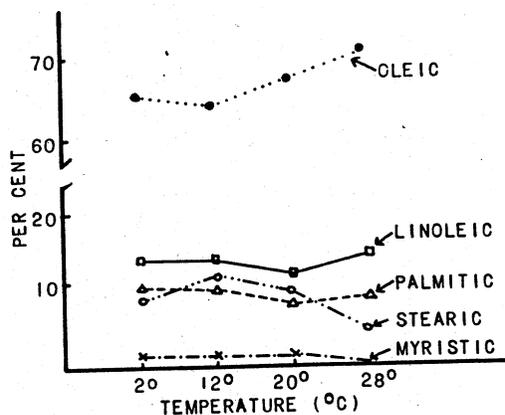


Fig. 1. Effect of temperature of lipase production on its specificity.

Table 1. Effect of pH of production medium on percentage composition of fatty acids released from lard at 2 growth temperatures.

Fatty acid	12°C		28°C	
	pH		pH	
	6.1	6.7	6.1	6.7
Myristic	1	1	tr.	tr.
Palmitic	9	10	9	9
Stearic	11	12	4	5
Oleic	65	64	69	71
Linoleic	14	13	18	15

indicates that the pH at which the lipase was produced did not affect the specificity of the enzyme. These data also support the evidence in Fig. 1 that elevated temperatures of enzyme production may affect the type of fatty acid liberated.

To determine how rate of acid formation changed during lipolysis, titratable acidity was determined at intervals, using an active culture supernatant as the source of the enzyme. The results are in Table 2. It is apparent that the rate of acid liberation is curvilinear after the first hour or two. After 24 hours, the rate drops about 50-90% below the 1-hr rate. In the case of the lipolysis of corn oil, there was an actual disappearance of acid after 6 days, indicating a loss in some of the acids. The

Table 2. Effect of length of incubation on rate of lipolytic activity of *Pseudomonas fragi*.

	1 hr		5 hr		24 hr		6 days	
	T.A. ^a	Δ /hr ^b	T.A.	Δ /hr	T.A.	Δ /hr	T.A.	Δ /hr
Lard	1.6	1.6	5.8	1.0	10.5	0.3	15.5	0.04
Corn oil	2.0	2.0	6.1	1.0	12.2	0.3	9.2	-0.02
Coconut oil	2.2	2.2	7.6	1.3	12.0	0.2	15.5	0.03

^a Ml of 0.02N NaOH per 10-ml portion.

^b Change per hour from previous figure.

slightly higher rate of lipolysis of corn and coconut oils than of lard during the first hours of incubation was consistent among different assays, even though the total acid liberated varied.

To determine whether these changes in rate were coupled with changes in the types of fatty acids liberated, aliquots were removed at various intervals and the percentage composition determined. Table 3 shows these data for the fatty acids liberated from lard by *Pseudomonas fragi*. For the first two hours, during which about 5%

of the total fatty acids were liberated, the percentage of palmitic acid released was considerably smaller than the percentage in the original fat. Conversely, the unsaturated fatty acids, which constitute only 55% of the fatty acids in the lard, accounted for 79% of the total acids released. The same pattern is apparent after 24 hours. After 6 days, however, when about 60% of the fatty acids had been released, the percentage of palmitic had risen considerably.

Table 4 shows the percentages of fatty acids

Table 3. Effect of length of incubation on the types of fatty acids liberated from lard by *Pseudomonas fragi*.

Fatty acid	Composition of lard mol %	2 hr		24 hr		7 days	
		µg *	mol %	µg	mol %	µg	mol %
Lauric	<1	<50	<1	<50	<1	100	<1
Myristic	1	<50	<1	50	<1	1,000	1
Palmitic	29	900	11	1,800	10	20,000	22
Stearic	14	1,200	13	2,100	13	9,600	10
Palmitoleic	2	<50	<1	200	1	2,300	2
Oleic	44	5,900	62	13,200	66	49,300	49
Linoleic	9	1,400	14	2,400	12	15,200	16

* Micrograms of fatty acid released per 10-ml portion.

Table 4. Per cent of various fatty acids released from coconut oil, corn oil, and tallow by *Pseudomonas fragi*.

	Coconut oil			Corn oil			Tallow	
	Orig.	2 hr	24 hr	Orig.	2 hr	24 hr	Orig.	2 hr
Caprylic	12	7	9					
Capric	9	11	10					
Lauric	45	37	38	1	1	1	4	1
Myristic	16	28	20	1	1	1		
Palmitic	9	5	11	12	16	17	31	34
Stearic	2	2	2	2	2	2	22	15
Palmitoleic	<1	0	0				1	1
Oleic	5	4	6	28	23	25	39	42
Linoleic	2	5	4	55	57	56	3	8

Table 5. Percentage of different fatty acids released by four strains of *Pseudomonas* * from three substrates.

	Lard				Corn oil				Coconut oil			
	A	B	C	D	A	B	C	D	A	B	C	D
Caprylic									<1	5	8	14
Capric									6	9	11	11
Lauric					1				47	35	42	30
Myristic					<1	<1	<1	<1	19	30	16	21
Palmitic	10	9	6	7	12	15	15	14	13	6	9	11
Stearic	14	12	13	13	2	2	1	1	2	2	3	2
Palmitoleic	1	<1	1	1								
Oleic	63	65	63	67	26	25	23	26	7	6	6	7
Linoleic	13	13	14	12	57	58	59	58	6	7	5	4

* A, *Ps. fragi*, NRRL B-25; B, *Ps. sp.* # 15; C, *Ps. sp.* # 35; *Ps. fluorescens*, ATCC 11251.

released from coconut oil, corn oil, and tallow. Again, for some of the acids, there is a variation between the percentages present in the fat and the percentages liberated in the early stages of lipolysis.

To determine whether variations occurred in the types of fatty acids released by different strains of psychrophilic bacteria, four cultures were compared on three substrates. The results are in Table 5.

On lard and corn oil, no real differences were apparent. On coconut oil, some differences did occur. Similar values for these organisms were obtained in other trials, particularly the low caprylic acid value for Culture A and the high value for Culture D. Harper (1957) showed a similar variation in specificity for lipases from different animal sources.

DISCUSSION

The previous work showed that decreasing the temperature at which an enzyme was produced caused a slight drop in the optimum pH for lipase activity. The data presented here indicate that this change in optimum pH was not the result of a change in specificity for any particular fatty acid. Although our primary concern was the effect of low temperatures on enzyme activity, it is interesting to note a slight increase in the amount of oleic and linoleic acids liberated at 28°C, with a corresponding drop in stearic acid. The lack of corresponding effect on palmitic acid may be related to its position in the triglyceride, as discussed later.

As expected in any enzymatic reaction, the rate of fatty acid liberation by bacterial lipase decreases with time. Even so, the decrease appears to be "across the board"; that is, the ratios of the fatty acids liberated remain constant, at least until about one-third of the fatty acids have been liberated.

The differences between percentages of fatty acid liberated and percentages in the original fat may be explained in two ways. There could be specificity of the lipase for the α -position on the triglyceride molecule, irrespective of the fatty acid attached to it. If the fatty acids were not randomly arranged in the fat, a larger percentage of acids esterified at the α -position would appear in the free fatty acids than were present in the whole triglyceride. The other possibility is simply that the lipase might be specific for

certain of the fatty acids. Since it is accepted by many workers that fatty acids are not randomly distributed in fats (Mattson and Lutton, 1958; Quimby *et al.*, 1953), the first possibility appears more plausible. Mattson and Lutton (1958) reported a concentration of saturated fatty acids in the β -position in lard, but did not distinguish between stearic and palmitic acids. If one assumes a similarity in mode of action of the lipases from bacteria and from the pancreas, the data presented here give additional evidence of this concentration. The data go further, however, in indicating that a much larger proportion of the palmitic than of the stearic acid is so esterified. Mattson and Beck (1955) reported, after initial hydrolysis of the fatty acids from the α -position by pancreatic lipase, some shifting of the acids from the β - to the α -position. Because of the long incubation period in our studies, such isomerization of the glycerides would be expected. Therefore, continuing lipolysis should increase the amount of palmitic acid liberated. The percentages found at 7 days substantiate this point. The predominance of only palmitic acid at the β -position, rather than of both saturated fatty acids, supports the conclusion of Quimby *et al.* (1953), who, from solvent crystallization procedures, concluded that lard was composed of 2-palmityl glycerides.

Nevertheless, there is still room for doubt that the activity of bacterial lipases (or possibly any lipase) is entirely independent of the structure of the fatty acid. There is considerable evidence on fats and oils that indicates the fatty acids have a limited random distribution. Lack of randomness, however, does not preclude the possibility that the lipase has some degree of specificity, particularly in the early stages of lipolysis. It is quite possible that a combination of positional specificity and fatty acid specificity might combine to accentuate or to obscure the real degree of randomness. The apparent variation in specificity among different strains of bacteria tends to support this, although it would be presumptuous to attach real significance to these strain differences without more detailed evidence of the effect of pH, temperature, etc., on each individual strain.

Another important variable in studies on

natural triglycerides is the triglyceride itself. Relatively wide differences have been reported in percentage composition of any particular fat or oil. It might reasonably be expected that differences in structure among different batches of the same substrate could materially affect the results obtained. Studies are now under way utilizing mixtures of pure triglycerides and randomly rearranged lard to obtain more conclusive data on the effect of chain length, as well as position, on fatty acid specificity.

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