

Lipolytic Activity of Microorganisms at Low and Intermediate Temperatures. III. Activity of Microbial Lipases at Temperatures Below 0°C

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(Manuscript received May 3, 1961)

SUMMARY

The lipolytic activity at -7 , -18 , and -29°C of strains of *Pseudomonas fragi*, *Staphylococcus aureus*, *Geotrichum candidum*, *Candida lipolytica*, *Penicillium roqueforti*, and an unidentified *Penicillium* sp. in emulsions of corn oil, coconut oil, and lard was determined. The action was measured by titratable acidity and by quantitative determination of the fatty acids by chromatography. The lipases from these microorganisms showed considerable activity within 2-4 days at -7°C and within a week at -18°C . Activity at -29°C was evident within 3 weeks by some of the cultures, particularly on corn oil. The rate of lipolysis in frozen substrates was directly related to their degree of unsaturation. However, there also were differences among genera. The lipase of *G. candidum* had considerably more specificity for oleic and linoleic acids than any of the others. *P. fragi* and *C. lipolytica* were least able to attack the β -esterified palmitic acid of lard. The ability of *S. aureus* to attack this position readily at 35°C was nullified by lowering the temperature to -18°C . A similar effect was observed on the mold lipase.

It is generally agreed that microbial counts of frozen foods decline slowly during storage even though a few bacteria and molds can grow at temperatures down to -5 to -10°C (Borgstrom, 1955). Nevertheless, changes in quality occur in frozen foods and are often quite pronounced after relatively short storage times in the range of -18 to -5°C . This deterioration is generally attributed to enzymes native to the product.

Balls and Tucker (1938) observed that pancreatic lipase was active at temperatures as low as -30°C , and Balls and Lineweaver (1938) found that chymotrypsin and pepsin were capable of bringing about proteolysis at similar temperatures. Sizer and Josephson (1942) studied lipase, trypsin, and invertase and found them active as low as -18°C . Wagenknecht *et al.* (1952) concluded that the loss of color in unblanched peas stored at -18°C was caused at least in part by an increased acid number (lipolysis), which favored the conversion of chlorophyll to pheophytin. Kuhl (1940) reported that wheat-germ lipase and the oxi-

dase and catalase activity of wheat bran were inhibited below 0°C . Peterson and Gundersen (1960a, b) reported that proteinases from *Pseudomonas fluorescens* were elaborated and active at $0-5^{\circ}\text{C}$ in defrosted chicken pies; activity in frozen substances was not reported. Studies on the blanching process for vegetables (Lee, 1958) have been concerned primarily with native enzymes, although high initial bacterial counts on peas and beans have some relationship to flavor and color deterioration in storage (Hucker *et al.*, 1952).

Previous reports from this laboratory (Alford and Elliott, 1960; Alford *et al.*, 1961) have shown that the lipases produced by different species of *Pseudomonas* are similar in their activity at temperatures ranging from 0 to 30°C . The present work was undertaken to include a study of lipases from selected bacteria, yeast, and molds, with particular emphasis on the effect of sub-freezing temperatures on their activity and specificity.

Table 1. Titratable acidities produced by lipases from different microorganisms at 35°C.

	Corn oil			Lard			Coconut oil		
	45 min	3 hr	24 hr	45 min	3 hr	24 hr	45 min	3 hr	24 hr
<i>P. fragi</i>	5.4 ^a	9.6	14.8	5.8	11.8	19.2	7.6	14.8	18.7
<i>S. aureus</i>	0.7	2.1	10.1	0.6	2.0	10.6	1.9	5.0	21.5
<i>G. candidum</i>	3.8	9.1	20.0	2.4	5.0	14.6	1.8	3.0	5.5
<i>C. lipolytica</i>	1.9	4.0	7.8	1.9	4.3	10.7	3.1	6.7	10.3
<i>P. roqueforti</i>	3.7	6.0	7.6	2.3	3.0	2.0	5.3	9.0	11.3
<i>Penicillium</i> sp.	5.5	12.4	11.6	5.6	12.8	33.2	7.2	12.8	26.3

^a ml of 0.02N acid produced.

EXPERIMENTAL METHODS

Sources and cultivation of microorganisms. The species used were obtained from the following sources: *Pseudomonas fragi*, NRRL B-27, from Dr. W. C. Haynes, NURDD, USDA, Peoria, Illinois; *Staphylococcus aureus*, D-87, from Food and Drug Administration, Washington, D. C.; *Geotrichum candidum*, from Dr. A. R. Colmer, Louisiana State University, Baton Rouge, Louisiana; *Candida (Mycotorula) lipolytica*, NRRL Y-1094, from Dr. C. W. Hesseltine, NURDD, USDA, Peoria, Illinois; *Penicillium roqueforti*, NRRL 849, from Dr. Hesseltine; and the *Penicillium* sp., isolated from the surface of aged ham.

Cultures of *P. fragi*, *S. aureus*, *G. candidum*, and the unidentified *Penicillium* were grown on 1% peptone broth buffered at pH 7.0 with 0.05M phosphate. *C. lipolytica* was grown on the medium recommended by Peters and Nelson (1948), and the *P. roqueforti* on the medium suggested by

Morris and Jezeski (1953) except that the butterfat was omitted. All cultures, except *S. aureus*, were incubated at 20°C; the *S. aureus* cultures were incubated at 32°C. Incubation periods were: *P. fragi* and *S. aureus*, 3-4 days; *G. candidum* and *C. lipolytica*, 4-6 days; and the molds, 6-10 days.

Enzyme preparation. The cells or mycelia were removed by filtration or centrifugation for 10 min at 2500 × G, and the filtrates and supernates were used as the enzyme source.

Substrate preparation. The lard, corn oil, and coconut oil emulsions were prepared as previously described (Alford and Elliott, 1960) except that homogenization was carried out at 4000-4500 lb with a recycling period of 15 min, and the emulsions were not heated after preparation. Phosphate buffer at pH 7.0 was added to the emulsions to be used for the lipases from *P. fluorescens*, *S. aureus*, and the two molds so that the final concentration after addition of enzyme would be 0.05M buffer and 2% fat. A similar procedure was used for *G. candidum* and *C. lipolytica* except that citrate buffer at pH 5.0 was used. Previous work (Alford and Elliott, 1960; Morris and Jezeski, 1953; Peters and Nelson, 1948) as well as additional observations in our laboratory indicated that assays at these pH values would give near-optimum activity.

Assay. To tubes containing 2 ml of enzyme at 3-5°C were added 8 ml of buffered substrate (also at 3-5°C), and the contents were immediately frozen in an alcohol-dry ice bath. The tube contents were below -30°C within two minutes of

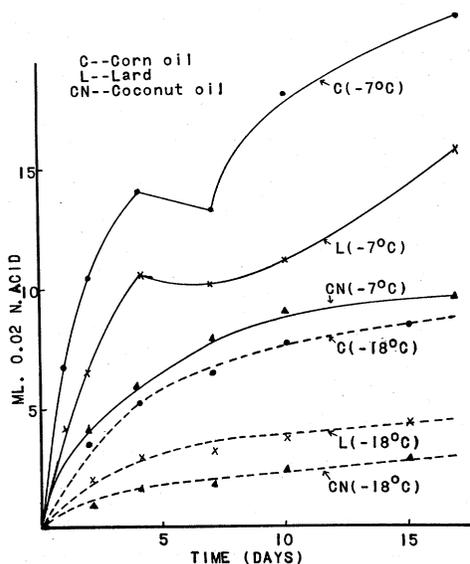


Fig. 1. Activity of *Pseudomonas fragi* on emulsified fats at low temperatures.

Table 2. Activity of lipases from different microorganisms at -29°C.

	Lard		Corn		Coconut	
	3 wk	6 wk	3 wk	6 wk	3 wk	6 wk
<i>P. fragi</i>	0.8 ^a	1.3	3.0	3.8	0.7	1.0
<i>S. aureus</i>		0.5		0.9		0.4
<i>C. lipolytica</i>	1.2	1.3	1.0	1.5		0.9
<i>Penicillium</i> sp.		0.9		3.1		4.2
						1.7

^a ml of 0.02N NaOH produced.

Table 3. Effect of temperature on percentages of fatty acids released from corn oil by different microorganisms.

Temperature (°C)	C ₁₆	C ₁₈	C ₁₈ -	C ₁₈ =	C ₁₆	C ₁₈	C ₁₈ -	C ₁₈ =
<i>Composition of original fat</i>								
	13	2	28	56				
<i>Pseudomonas fragi</i>								
35°	14	1	27	57	28	6	20	45
-7°	12	tr	27	60	19	4	24	52
-18°	7	tr	26	66	14	tr	25	59
-29°	tr	tr	35	63				
<i>Staphylococcus aureus</i>								
35°	14	1	31	53	20	2	27	50
-7°	9	tr	32	57	8	2	34	54
-18°	8	2	34	54				
<i>Geotrichum candidum</i>								
35°	3	0	40	57	14	1	31	53
-3°	20	2	27	50	9	tr	32	57
-7°	tr	tr	4	95	8	2	34	54
-18°	tr	tr	35	64				
<i>Candida lipolytica</i>								
35°	19	tr	26	55	20	tr	24	55
-7°	15	1	25	58	16	2	28	52
-18°	8	1	28	63	9	2	29	59
<i>Penicillium roqueforti</i>								
35°	19	tr	26	55	20	tr	24	55
-7°	15	1	25	58	16	2	28	52
-18°	8	1	28	63	9	2	29	59
<i>Penicillium sp.</i>								

being mixed. Replicate samples were stored at -7°C, -18°C, and -29°C. Samples were removed periodically for determination of titratable acidity by titration with 0.02N NaOH, and fatty-acid composition by gas chromatography. Samples for chromatography were usually taken when 15-35 mg of free fatty acids were present. The procedures were as reported previously (Alford and Elliott, 1960; Alford *et al.*, 1961; Hornstein *et al.*, 1960). Although activity in the control flasks was essentially negative, the zero-time blank values ranged from 1.0 to 1.6 ml of 0.02N NaOH. All values shown in the data are net values.

RESULTS AND DISCUSSION

Table 1 shows the titratable acidities produced by the different microorganisms at 35°C. Coconut oil is the most readily attacked of the three substrates by the lipases from all cultures except *G. candidum*. The low activity of *P. roqueforti* on lard is not readily explained.

Fig. 1 and Table 2 show the rate and amount of acid production by *P. fragi* lipase below 0°C on corn oil, lard, and coconut oil. Unlike the results at 35°C, the rate of lipolysis in frozen substrates was directly related to their degree of unsaturation. If one considered the average molecular weights of the fatty acids in the fats, these differences

in rate would be even greater since there are more molecules of fatty acid available in the 200 mg of coconut oil than in the others. This might also be a factor in the greater coconut oil activity at 35°C.

The apparent lag in lipolysis between the 4th and 7th days on corn oil and lard may be related to substrate utilization. As was

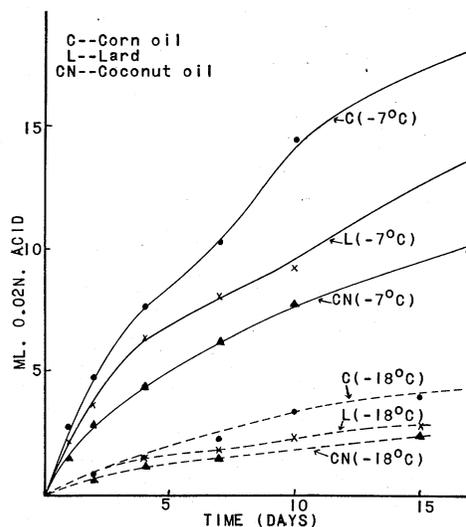


Fig. 2. Activity of *Staphylococcus aureus* on emulsified fats at low temperatures.

postulated previously (Alford *et al.*, 1961), the lipase may have a position specificity, or at least affinity, for the α -position on the triglyceride. If one assumes such a specificity, then most of the fatty acid available in the α -position will have been released when a titratable acidity of 10-15 ml of 0.02N acid is obtained. The subsequent lag may be related to the time required for the shifting of fatty acid molecules from the β -position to the α -position (isomerization). This is supported by the increase in palmitic acid released by *P. fragi* after several days at 35°C (Alford *et al.*, 1961).

Similar curves are presented in Figs. 2-6 for the other microorganisms. Essentially the same ratio of activity on the three substrates is observed for all cultures. The relatively high titratable acidity produced by *P. roqueforti* lipase on coconut oil at 35°C, coupled with the similarity of rate of hydrolysis at -7°C of coconut oil to that of lard and corn oil (Fig. 5), suggests an affinity of this lipase for the lower fatty acids. This might be expected when one considers the known activity of *P. roqueforti* on the lower fatty acids of butterfat in blue cheese manufacture (Morris and Jezeski, 1953).

Balls *et al.* (1937) reported that pancreatic lipase had very little activity on the higher triglycerides (above C₈) at 0°C. The

data presented here indicate a similar relationship of rate of attack to degree of unsaturation for microbial lipases. There is a definite shift toward increasing percentages of the unsaturated acids as the temperature decreases. More conclusive evidence is shown in Tables 3, 4, and 5, which show the types of fatty acids released from the natural triglycerides. This apparent increase in af-

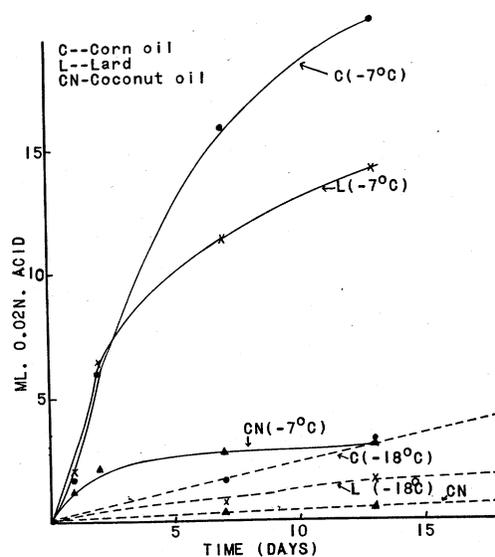


Fig. 3. Activity of *Geotrichum candidum* on emulsified fats at low temperatures.

Table 4. Effect of temperature on percentages of fatty acids released from lard by different microorganisms.

Temperature (°C)	C ₁₆	C ₁₈	C ₁₆ —	C ₁₈ —	C ₁₈ ≡	C ₁₆	C ₁₈	C ₁₆ —	C ₁₈ —	C ₁₈ ≡
<i>Composition of original fat</i>										
	26	14	5	47	8					
<i>Pseudomonas fragi</i>						<i>Staphylococcus aureus</i>				
35°	6	9	1	68	16	26	12	5	41	14
-7°	2	2	1	73	21	10	3	4	63	20
-18°	tr	1	tr	76	20	tr	3	1	67	26
<i>Geotrichum candidum</i>						<i>Candida lipolytica</i>				
35°	5	tr	2	73	19	3	7	tr	83	6
-7°	tr	tr	3	72	25	1	2	tr	76	18
-18°	tr	tr	4	66	28	1	3	tr	72	21
<i>Penicillium roqueforti</i>						<i>Penicillium sp.</i>				
35°	20	17	1	56	6	14	20	1	50	13
-7°	10	2	5	67	14	6	3	2	71	16
-18°	5	3	6	57	28	1	1	3	68	26

Table 5. Effect of temperature on the percentages of fatty acids released from coconut oil by different microorganisms.

Temperature (°C)	C ₈	C ₁₀	C ₁₂	C ₁₄	C ₁₆	C ₁₈	C ₁₈ -C ₁₈ =	C ₈	C ₁₀	C ₁₂	C ₁₄	C ₁₆	C ₁₈	C ₁₈ -C ₁₈ =		
<i>Composition of original fat</i>																
	8	8	43	17	11	2	8	2								
<i>Pseudomonas fragi</i>								<i>Staphylococcus aureus</i>								
35°	7	12	46	19	8	2	5	3	6	8	45	16	11	3	4	6
-7°	1	4	27	12	13	1	24	18	1	2	33	10	18	6	20	10
-18°	tr	4	36	14	12	6	22	6	tr	4	34	12	20	7	18	6
<i>Geotrichum candidum</i>								<i>Candida lipolytica</i>								
35°	tr	3	39	19	11	3	14	11	12	7	36	16	12	3	13	1
-7°	tr	tr	4	tr	14	0	51	29	2	4	34	15	18	4	18	4
-18°									1	5	37	15	10	8	14	11
<i>Penicillium roqueforti</i>								<i>Penicillium sp.</i>								
35°	tr	5	45	26	14	3	5	tr	tr	4	40	26	15	4	2	8
-7°	2	4	34	8	12	1	22	14	2	2	36	15	12	2	19	14
-18°									1	4	35	15	15	7	18	6

finity for unsaturated fatty acids as the temperature decreases may actually be an effect on the physical structure or degree of crystallinity of the fat. In addition to the effect of temperature on the activity of the enzymes, fundamental differences in specificity among the different genera of microorganisms are evident. This is in contrast to the slight variations in activity among different psychrophilic strains of *Pseudomonas* reported previously (Alford *et al.*, 1961).

Factors other than generic differences enter into this specificity, however. Decreasing temperature generally caused a decrease in percentage of saturated fatty acids released, but the effect was not the same on individual fatty acids by the same enzyme from different substrates. For example, the extensive activity of *S. aureus* on palmitic acid in lard is almost nullified by lowering the temperature. Its activity on palmitic acid in corn oil is reduced only about half, while in coconut oil there is an actual increase in percentage of this acid liberated as the temperature decreased. Mattson and Beck (1956) reported that pancreatic lipase is specific for the α -position of triglycerides. Employing this specificity, Mattson and Lutton (1958) gave further evidence that the palmitic acid of lard is primarily ester-

fied at the β -position. Alford and Blankenship (1961) indicated that the lipase from *S. aureus* has an affinity for palmitic acid at 35°C and that it could attack the β -position of triglycerides to a limited extent. Table 3 indicates that this ability to attack the β -position in lard is seriously retarded by lowering the temperature.

As indicated above, *G. candidum* was more

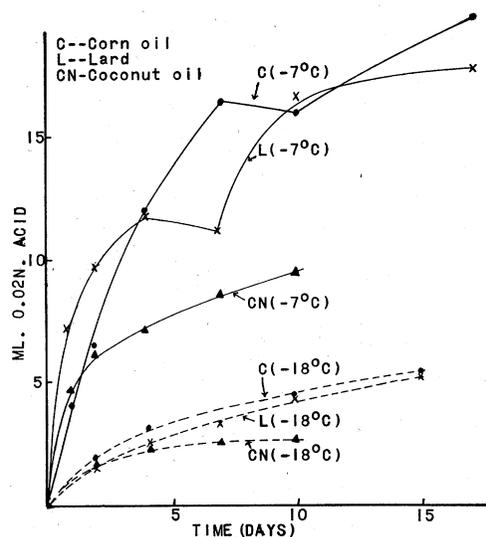


Fig. 4. Activity of *Candida lipolytica* on emulsified fats at low temperatures.

active on corn oil and lard than on coconut oil. This was particularly apparent at the lower temperatures (Fig. 3). When the types of fatty acids released were determined, a decided preference for the unsaturated acids was observed. In Table 3, the oleic and linoleic acids compose 99% of the acids hydrolyzed by *G. candidum* from corn oil at the lower temperatures. These acids account for over 90% of the acids from lard (Table 4) and 80% from the coconut oil (Table 5) even though these unsaturated acids are only 10% of the total acids in this oil.

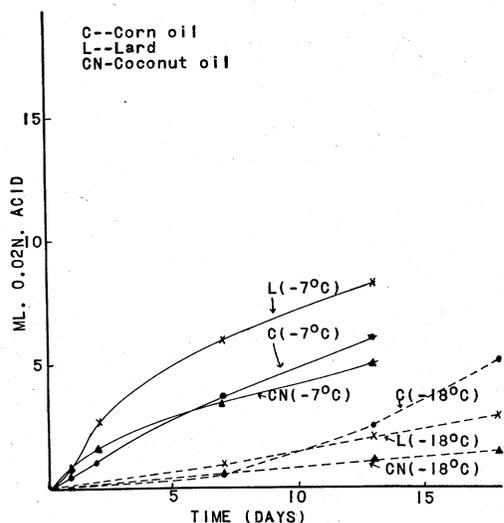


Fig. 5. Activity of *Penicillium roqueforti* on emulsified fats at low temperatures.

The other yeast-like fungus, *C. lipolytica*, showed a similar affinity for unsaturated acids from lard, but to a much less extent when corn or coconut oil was the substrate. This indicates a degree of position specificity similar to that suggested for *P. fragi*.

The unidentified *Penicillium* produces the most active lipase, but it is similar to *P. roqueforti* in the pattern of fatty acids released from the various substrates at different temperatures.

Other less pronounced differences in the activity of these lipases might be inferred from a closer examination of these data. However, factors which may affect the quantitative if not the qualitative aspects of their

activity must be considered. Different lots of culture supernatant from the same microorganisms may vary in the concentration of lipase present; the average size of the globules of the emulsified substrate may vary slightly between batches as well as among the fats; finally, the method of extraction and esterification may cause discrepancies of 5-10% between duplicates. These factors combine to make exact quantitative comparisons between different experiments difficult.

Nevertheless, these data show basic differences in microbial lipases, and that these enzymes are active in frozen substrates. They could bring about changes in quality if present in sufficient concentration. It is apparent that the type of fatty acid, as well as its position on the triglyceride, is important in determining the activity of microbial lipases. The delineations between enzymes from different sources are not sharp, and specificities overlap considerably. To the enzyme chemist interested primarily in pure systems exhibiting a high degree of specificity, the study of such systems has little to offer. However, to the food microbiologist, or technologist interested in the subtle differences in the flavor of certain foods or the incipient off-flavors associated with the beginning of spoilage, these enzymes offer intriguing possibilities for further study.

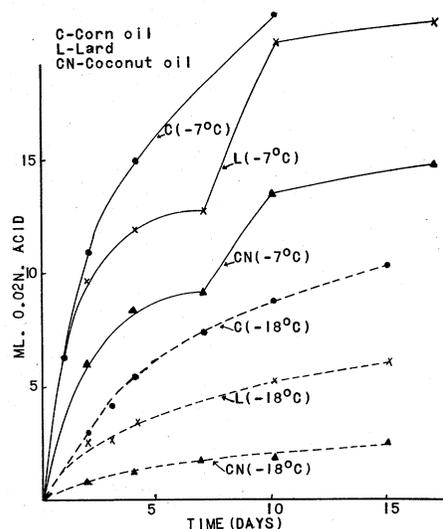


Fig. 6. Activity of *Penicillium* sp. on emulsified fats at low temperatures.

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