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Lipolytic Activity by Psychrophilic Bacteria

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In our microbiological work at Beltsville we have been interested for some time in the activities of microorganisms at those refrigerator temperatures that generally pertain in American packing house practices. These conditions include possible activity by enzymes released from the microbial cell at temperatures below the minimum for actual growth. The practical applications of such studies can readily be appreciated by considering the use of meat which is frozen after some microbial growth has occurred and is held for periods of even six months to a year at -18°C. We know from many other studies that the most serious deteriorative changes in frozen meat are changes in the fat, usually oxidative. In the case of cured meat which is aged at higher temperatures, the liberation of fatty acids plays an important part in the development of characteristic flavor. The temperature at which such meat is aged also influences the type and degree of flavor development so that temperature relationships are quite important.

We decided, therefore, in studying the microorganisms growing at low and intermediate temperatures, to devote our efforts to investigating their effects on fats. Several interesting observations have resulted which I will attempt to relay to you today. For greater detail I recommend a study of the references.

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Materials and Methods

In the course of the studies reported, the following microorganisms have been employed:

Pseudomonas fluorescens ATCC 1151

Pseudomonas fragi NRRL B-25

Pseudomonas sp. #15
Pseudomonas sp. #35
} From Dr. John Ayres, Iowa State University

Staphylococcus aureus D-87 from Food and Drug Administration

Geotrichum candidum from Dr. A. R. Colmer, Louisiana State University

Candida lipolytica NRRL Y-1094

Penicillium roqueforti NRRL 849

Penicillium sp. Meat Laboratory isolate

All of the species of *Pseudomonas* were capable of producing good viable growth in peptone broth at 1°C in three days.

Lipase was produced by bacterial cells grown in 1% peptone buffered with 0.05M phosphate to pH 7.0. Cells were removed by centrifugation at 2500 X G for 10 minutes. Lipases from the fungi were produced in various media at pH 5.0 or 7.0 and mycelial mats removed by filtration.

Fat substrates were prepared by mixing the fat (20%), emulsifier (Astec 4135, 2%), and water in a blendor and then recycling through a Manton-Gaulin homogenizer at 4000-4500 pounds for 15 to 20 minutes. The resulting emulsion can be sterilized by autoclaving.

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Assays for enzyme activity were performed by adding a definite quantity of cell-free enzyme to a flask containing 5 ml of the 20% fat emulsion, 10 ml of a 0.25M, pH 7.0, phosphate buffer, and water to make 50 ml. After incubation, an aliquot was transferred to a Mojonnier fat extract flask and extracted with successive quantities of petroleum ether. Extracts were titrated with 0.02N alcoholic NaOH, using phenolphthalien as an indicator.

Identification and estimation of fatty acids liberated by the enzymes were made according to the gas chromatographic method described by Hornstein, Alford, Elliott, and Crowe of this Laboratory.

Some Results of the Study

At the beginning of the study, experiments were performed to determine the best medium for lipase production. We found, as others had previously, that a carbohydrate-rich medium retarded lipase production and that we got our greatest yields of lipase when bacteria were grown on the fat-free, nutritionally restricted, 1% buffered peptone mentioned above.

Another problem at the beginning, was the determination of the optimum pH for enzyme production in the peptone medium. Slide 1 shows the initial and final pH and titratable acidity after 3 and 4 days of incubation at 20°C. Slide 2 shows similar data for incubation at 5°C.

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Optimum temperature for lipase production posed a similar problem and Slide 3 shows results of some experiments designed to clear up this question. You will note that, regardless of the temperature of lipase production, 40°C. seems to be the temperature at which the greatest lipolytic activity was obtained and that 20°C. was about the optimum temperature for lipase production.

Now to turn to our basic problem of fat degradation in freezer storage. Slide 4 shows the titratable acidity produced by Pseudomonas fragi lipase in emulsions of lard, corn oil, and coconut oil at several sub-freezing temperatures. You will note that there is an increase in hydrolysis of fat with time in all cases, but that this is much more marked in the case of lard and corn oil, which contain more unsaturated fatty acids. Of particular interest is the break in the curve for lard and corn oil at 7° in about four days. We think this is explained by the specificity of the Pseudomonas lipase for fatty acid position in the glyceride molecule.

Evidence of this is seen in Slide 5 which compares the activities of Pseudomonas and Staphylococcal lipases. Note that the Pseudomonas lipase releases large quantities of oleic acid from lard which has this acid in the α-position, while only a small part of the stearic and palmitic have been released from the β-position. The Staphylococcal lipases, on the other hand, released the fatty acids about in proportion to their distribution, regardless of molecular arrangement. In the case of corn oil and coconut oil, where the acids are more randomly distributed on the glyceride molecule, no similar difference between the lipases of Pseudomonas fragi and Staphylococcus aureus is seen. Slides 6 and 7.

The positional specificity of the Pseudomonas lipase is perhaps better proven by the results obtained with a synthetic unsymmetrical triglyceride, 2-oleoyl-palmito-stearin. Slide 8.

Here, where the oleic is no longer in the α-position, as it was in lard, very little oleic acid is liberated. Palmitic and stearic, which now occupy the α-position, are liberated in approximately equal quantities. The Staphylococcal lipase continues to show much less tendency toward a positional affinity, although some is indicated. The difference in quantities of acids liberated might also have been explained on the basis of a fatty acid specificity of the lipase, rather than a positional one. This can hardly be the case for the Pseudomonas lipase, however, when one compares the results with lard and with the synthetic triglyceride.

Before pursuing the interesting bypath of the specificity of microbial lipases for the ends of the glyceride molecule, we were speaking of the effects of low temperature. I am including in the handouts, which have been distributed, tables showing the effects of the cell free lipases of several microorganisms on different fatty substrates at temperatures below freezing contrasted with results at 35°.

In Table 1, we see results of lard which could be considered comparable with what might happen when pork is frozen. As you know, Pseudomonas fragi is very similar to many of the Pseudomonads commonly found on meat. We still see the positional specificity of the Pseudomonas lipase at all temperatures, while the random attack of the Staphylococcal lipase seems greatly inhibited at lower temperatures.

In all cases there seems to be a gradual change with temperature in the distribution of fatty acids released, rather than a sharp break. This may account for some of the differences in the flavor produced by different storage temperatures in such products as dry sausages and country hams.

Conclusions

From the work completed, we would like to emphasize these findings:

1) Lipase is best produced by bacteria in a nutritionally restricted medium and in the absence of fat.
2) Some bacterial lipases have positional specificity for the glyceride molecule. Lipases produced by Pseudomonas display this specificity but those from Staphylococci do not.
3) The microbial lipases tested were active at temperatures well below the growth range of the organisms which produced them. At -29°C, the lowest temperature we have tested, all strains hydrolyzed lard, corn oil, and coconut oil in 6 weeks.

for Bard

1. which kind of lipolytic b. in meat
2. Influence on the flavor

References

1. Determination of free fatty acids in fat. I. Hornstein, J. A. Alford and P. F. Crowe. Anal. Chem. 32, 540-42 (1960).

2. Lipolytic activity of microorganisms at low and intermediate temperatures. I. Action of Pseudomonas fluorescens on lard. J. A. Alford and L. E. Elliott. Food Research 25, 296-303 (1960).

3. Lipolytic activity of microorganisms at low and intermediate temperatures. II. Fatty acids released as determined by gas chromatography. J. A. Alford, L. E. Elliott, I. Hornstein, and P. F. Crowe. J. Food Sci. 26, 234-38 (1961).

4. Lipolytic activity of microorganisms at low and intermediate temperatures. III. Activity of microbial lipases at temperatures below 0°C. J. A. Alford and D. A. Pierce. J. Food Sci. In press.

Table 1. 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}^{-}	$C_{18}^{=}$
	Composition of original fat									
	26	14	5	47	8					
	Pseudomonas fragi					Staphylococcus aureus				
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
	Geotrichum candidum					Mycotorula lipolytica				
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
	Penicillium roqueforti					Penicillium sp.				
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 2. 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}=
	Composition of original fat							
	13	2	28	56				
	Pseudomonas fragi				Staphylococcus aureus			
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				
	Geotrichum candidum				Mycotorula lipolytica			
35°	3	0	40	57	14	1	31	53
- 3°					20	2	27	50
- 7°	tr	tr	4	95	9	tr	32	57
-18°	tr	tr	35	64	8	2	34	54
	Pennicillium roqueforti				Penicillium sp.			
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

Table 3. 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}=
	Composition of original fat															
	8	8	43	17	11	2	8	2								
	Pseudomonas fragi								Staphylococcus aureus							
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
	Geotrichum candidum								Mycotorula lipolytica							
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
	Penicillium roqueforti								Penicillium sp.							
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