APPLIED MICROBIOLOGY, May, 1965
Vol. 13, No. 3

Printed in U.S.A.

Action of Lipases of *Staphylococcus aureus* on Milk Fat[1]

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Received for publication 3 December 1964

ABSTRACT

VADEHRA, D. V. (Michigan State University, East Lansing), AND L. G. HARMON. Action of lipases of *Staphylococcus aureus* on milk fat. Appl. Microbiol. **13**:335-339. 1965.—The activity of the lipase(s) of two strains of coagulase-positive *Staphylococcus aureus* was determined in milk fat incubated at 15, 22, and 30 C for 8 days. Total fat hydrolysis was measured by acid degree values (ADV). Neutral lipids were separated into component groups on a Florisil column. Free fatty acids were determined by temperature-programmed gas-liquid chromatography. The ADV were 25 to 50% greater at 22 than at 15 C and 4 to 7 times greater at 30 than at 22 C. The lipases liberated as much as 0.48 g of fatty acids per gram of fat during 8 days at 30 C. The enzyme showed a predilection for the palmitic acid-glycerol bond. Addition of fatty acids C_{14} to C_{18} inclusive to inoculated sterile skim milk caused inhibition of *S. aureus* as follows: (i) complete at 0.05 and 0.10% concentration of C_{10} and (ii) partial at 0.05 and complete at 0.10% concentration of C_8. The samples showing inhibition were negative for peptonization, coagulase, and change in *p*H. Addition of oleic and stearic acid to sterile skim milk inoculated with *S. aureus* resulted in an increase in nonprotein nitrogen, and the C_4 to C_{12} acids caused a decrease in protease activity.

According to Alford, Pierce, and Suggs (1964), *Staphylococcus aureus* elaborates lipases which are strongly active against several natural and synthetic lipids. The effect of the lipolytic enzyme system of this organism on milk fat has not been investigated. The nature of this lipolytic activity is important, because the fatty acids liberated affect the growth of *S. aureus* and other organisms in foods.

The occurrence of *S. aureus* in various dairy products, particularly cheese, has been reported by several workers, including Foltz et al. (1960), Takahashi and Johns (1959), and Walker, Harmon, and Stine (1961). Previous work by Walker and Harmon (*unpublished data*) indicated that *S. aureus* caused rancidity in whole milk, and that skim milk was a better substrate than whole milk for the sustained growth of the organism. Since the fat content is the only variable component in the two substrates, experiments were conducted to delineate the nature and the effect of changes occurring in the lipid portion of milk containing 10% milk fat when inoculated with *S. aureus*. An experiment was also designed to determine the effect of various individual fatty acids on the growth of *S. aureus*.

MATERIALS AND METHODS

The activity of the lipase(s) of coagulase-positive *S. aureus* B-120 and S-1 was determined. Strain B-120, responsible for a food poisoning outbreak, was secured from the Northern Regional Research Laboratory at Peoria, Ill. Strain S-1 was isolated from milk from a cow with subclinical mastitis. The cultures were grown for 18 hr in sterile milk containing 10% milk fat and inoculated into 500 ml of the same medium, giving a final concentration of about 10^6 cells per ml. The inoculated milks were incubated at 15, 22, and 30 C for 8 days. One flask was inoculated with a culture grown in Trypticase Soy Broth (BBL) and incubated at 30 C.

Total fat hydrolysis was measured by acid degree values (ADV) as proposed by Thomas, Nielsen, and Olson (1955). Samples (50 ml) were withdrawn aseptically from each flask immediately after inoculation and mixing, and at 24-hr intervals thereafter. The samples were lyophilized and stored at −25 C. The fat was extracted from the lyophilized samples with 50 ml of ethyl alcohol, followed by 50 ml of ethyl ether, and finally taken up in 50 ml of hexane. The solvent was evaporated at approximately 60 C by use of a Rinco evaporator and a laboratory aspirator to create a vacuum of about 61 cm. The extracted lipid was dried on a steam bath under a constant stream of nitrogen. A 1-ml amount of the milk fat was dissolved in 5 ml of a solvent composed of hexane-*n*-propanol (4:1) and titrated with 0.025 N methanolic KOH; 1% alcoholic phenolphthalein was used as an indicator. The results were expressed as milliters of 1 N methanolic KOH required to titrate 100 g of fat.

The nature of the changes in the lipid fraction of milk caused by the lipase(s) of *S. aureus* was determined. The fat was extracted from samples of

[1] Michigan Agricultural Experiment Station Journal Article No. 3513.

milk handled as described previously, except that the samples were not lyophilized. A 1-g amount of the fat was dissolved in 20 ml of *n*-pentane and added to 8 g of cationic IR 400 Amberlite resin previously treated as described by Hornstein et al. (1960). The mixture was stirred for 6 min, and the supernatant fluid was removed. The resin was then washed two additional times with 25 ml of pentane, and all of the washings were collected. The solvent was evaporated and the lipid was dried as described above. The neutral lipids were separated into the various component groups on a Florisil (Floridin Co., Tallahassee, Fla.) column by use of the method of Carroll (1961).

The free fatty acids adsorbed to the resin were esterified with methanolic HCl by the method of Hornstein et al. (1960). A 20-μliter amount of the esters was analyzed by gas-liquid chromatography with the temperature programmed to obtain maximal separation between the peaks representing the individual fatty acids. The areas under the peaks on the chromatograms were calculated quantitatively by use, as a standard, of a known mixture of fatty acids esterified and treated in the same manner as the unknown samples.

During the preparation of samples for gas-liquid chromatography, extensive losses of volatile acids indicated the need for another method of measurement. The volatile acids were determined by the rapid distillation method of Kosikowski and Dahlberg (1946).

The effect of free fatty acids on the growth of *S. aureus* S-1 was determined. The saturated fatty acids C_4 through C_{18} and oleic acid were added at 0.01, 0.05, and 0.10% concentration to reconstituted skim milk, and the mixtures were homogenized at 500 psi. The milk was sterilized, inoculated with *S. aureus*, and incubated at 30 C for 8 days. The *p*H, population, and coagulase reaction were determined at 0, 1, 3, 5, and 8 days. The populations were measured by use of plate count agar according to *Standard Methods for the Examination of Dairy Products* (American Public Health Association, 1960).

Portions of the above samples were used to measure the effect of fatty acids on the proteolytic ability of *S. aureus*. After 8 days of incubation, the trichloroacetic acid-soluble nonprotein nitrogen was determined by the method of Lowry et al. (1951).

RESULTS

ADV. The amount of lipolysis occurring in milk containing 10% fat inoculated with *S. aureus* and incubated at various temperatures was measured by the ADV of the lipid extract (Table 1). Both time and temperature had a significant influence on the amount of lipolysis. The ADV, which increased as the incubation time progressed, was only 25 to 50% greater at 22 than at 15 C; at 30 C, the ADV was 4 to 7 times greater than at 22 C. These results indicate a definite preference by the enzymes for the 30 C temperature. Strain B-120 was more lipolytic than strain S-1, and the cells activated in milk were more lipolytic than those activated in broth.

Analysis of lipids. The amounts of the various fractions of the lipids extracted from milk inoculated with strain S-1 and incubated at various temperatures are shown in Table 2. There was a progressive increase in lipolytic enzyme activity, evidenced by the increase in free fatty acids, as the incubation temperature increased from 15 to 30 C and as the time increased from 0 to 8 days. Also, the amount of tri- and diglycerides decreased in approximate proportion to the increase in free fatty acids as the temperature increased and time progressed.

Analysis for free fatty acids. The amount of individual fatty acids liberated when milk was in-

TABLE 1. *Acid degree values of fat extracted from milk inoculated with Staphylococcus aureus S-1 and B-120 and incubated as indicated*

Inoculum	Incubation temp	Days of incubation								
		0*	1	2	3	4	5	6	7	8
	C									
S-1 grown in milk	15	2.40	4.00	—†	—	6.51	—	—	—	10.50
	22	2.51	5.10	—	—	10.20	—	—	—	14.20
	30	2.35	18.20	—	—	46.42	—	—	—	78.52
B-20 grown in milk	15	3.25	4.86	6.61	7.31	7.31	9.58	10.42	11.81	12.50
	22	3.37	5.81	7.42	9.72	12.78	14.53	15.54	16.94	18.34
	30	3.60	25.97	51.39	78.36	(lost)	102.78	112.22	116.94	118.19
B-20 grown in Trypticase Soy Broth	30	3.60	11.25	33.61	48.89	54.17	58.33	73.61	90.47	90.58

* Noninoculated control.
† Not determined.

TABLE 2. *Analysis of lipids extracted from milk inoculated with Staphylococcus aureus S-1 and incubated as indicated*

Compound	Noninoculated control (0 days)	15 C			22 C			30 C		
		1 day	4 days	8 days	1 day	4 days	8 days	1 day	4 days	8 days
Free fatty acids	50*	120	165	255	170	230	330	255	380	480
Monoglycerides	0	5	0	0	5	5	5	10	40	5
Diglycerides	0	40	20	10	50	5	5	50	10	10
Triglycerides†	910	770	700	640	700	615	540	615	490	445
Cholesterol	40	60	60	60	30	50	40	50	40	40
Percentage recovered	100.0	99.5	94.5	96.5	95.5	90.5	92.0	98.0	96.0	98.0

* Results are expressed as milligrams per gram of fat.
† Includes cholesterol esters.

TABLE 3. *Amount of free fatty acids liberated from milk inoculated with Staphylococcus aureus S-1 and incubated under the conditions indicated*

Acid	Noninoculated control (0 days)	15 C			22 C			30 C		
		1 day	4 days	8 days	1 day	4 days	8 days	1 day	4 days	8 days
Capric	0.335*	0.640	0.871	3.317	0.785	1.776	4.142	1.856	4.644	6.166
Lauric	0.517	0.815	1.134	1.992	1.024	2.261	5.515	2.587	6.053	8.222
Myristic	0.391	2.244	3.831	7.615	3.162	6.489	14.684	7.944	19.297	24.379
Palmitic	2.997	4.463	7.802	13.913	7.534	16.403	44.803	21.128	49.672	53.835
Stearic	1.495	2.067	2.504	3.934	1.982	4.578	13.140	7.997	17.489	26.896
Oleic	2.233	4.454	6.857	9.545	4.896	9.326	22.766	12.178	27.929	42.966

* Results expressed as milligrams per gram of fat.

TABLE 4. *Volatile fatty acids recovered from 10 g of milk (10% milk fat) inoculated with Staphylococcus aureus S-1*

Incubation temp	Days of incubation			
	0	1	4	8
C				
15	0.5*	1.33	1.92	2.15
22	0.5	1.74	2.185	2.97
30	0.5	3.50	5.47	7.28

* Results are expressed as milliliters of 0.1 N KOH required to titrate fat from 10 g of milk containing 10% fat.

oculated with *S. aureus* S-1 and incubated under various conditions is recorded in Table 3. The amount of volatile fatty acids recovered from 10 g of inoculated milk (1 g of fat) is shown in Table 4. These results also indicate a progressive increase in lipolysis as the temperature increased from 15 to 30 C and the time increased from 0 to 8 days.

Effect of free fatty acids on growth of S. aureus. The trends in population of *S. aureus* in samples of skim milk containing various concentrations of some of the fatty acids used in this work are shown in Fig. 1. None of the acids were inhibitory at 0.01% concentration. Capric acid completely inhibited *S. aureus* at 0.05 and 0.1% concentration. Also, caprylic acid was completely inhibitory at 0.1% concentration and temporarily inhibitory at 0.05% concentration. The other saturated fatty acids (C_4 to C_{18}) and oleic acid added in concentrations of 0.01, 0.05, and 0.10% had no effect on the growth of *S. aureus* S-1 in skim milk.

Effect of fatty acids on the protease and coagulase produced by S. aureus. The amount of tyrosine liberated from inoculated skim milk containing fatty acids and incubated for 8 days at 30 C was determined to ascertain whether the acids had an influence on the proteolytic enzyme system of *S. aureus* (Table 5). The short-chain fatty acids, C_6 to C_{12} inclusive, caused partial inhibition of proteolysis of skim milk when added to the milk in concentrations of 0.05 and 0.10%. Butyric acid caused some inhibition at 0.10%, but not at the lower concentrations. The long-chain fatty acids (C_{14} to C_{18}) either had no effect or were slightly stimulatory to the protease(s).

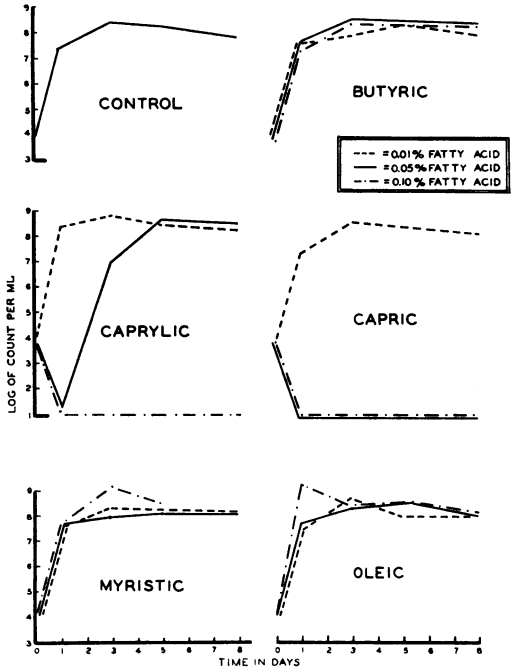

FIG. 1. *Changes in population of Staphylococcus aureus when grown in skim milk containing 0.01, 0.05, and 0.10% of the fatty acids indicated.*

TABLE 5. *Amount of tyrosine liberated when skim milk containing added fatty acids was inoculated with Staphylococcus aureus S-1 and incubated for 8 days at 30 C**

Fatty acid added	Concn of fatty acid (%)		
	0.01	0.05	0.10
Butyric	0.990†	1.080	0.750
Caproic	1.170	0.960	0.360
Caprylic	1.125	0.375	0.285
Capric	1.050	0.270	0.270
Lauric	1.550	0.745	0.795
Myristic	0.885	Lost	0.885
Palmitic	1.296	1.296	1.344
Stearic	0.915	1.185	1.185
Oleic	0.960	1.065	1.170

* Two samples which did not contain fatty acids were used as controls. Noninoculated skim milk and skim milk inoculated with *S. aureus* yielded 0.270 and 0.910 mg of tyrosine per ml of milk, respectively.

† Results are expressed as milligrams of tyrosine liberated per milliliter of milk.

At each sampling interval, the coagulase test was performed on the inoculated samples of skim milk containing the fatty acids. All of the samples were coagulase-positive, indicating substantial growth of *S. aureus*, except (i) those which contained 0.05 and 0.10% C_8, C_{10}, and C_{12} fatty acids and were incubated for 5 days and (ii) those which contained 0.05 and 0.10% C_8 and C_{10} fatty acids and were incubated for 8 days. A direct relationship existed between *p*H and inhibition, with the highest *p*H prevailing in those samples in which growth of *S. aureus* was inhibited.

DISCUSSION

The lipolytic enzyme system of *S. aureus* is very active on milk fat, as indicated by the increases in ADV and free fatty acids during incubation of inoculated samples (Table 1, 2, 3, and 4).

The greater lipolytic ability of inocula grown in milk, as compared to that grown in broth (Table 1), suggests the presence of an induced enzyme. However, inocula grown in broth also had substantial lipolytic activity, indicating the presence of more than one extracellular lipase, one of which may be adaptive.

The lipid portion of milk contains approximately 85% fatty acids which are bonded to glycerol. Calculations based on the total amount of fatty acids present indicate that about 56.4% of the milk fat was hydrolyzed by *S. aureus* S-1 during the 8-day incubation period at 30 C. Milk fat contains an average of about 32% oleic acid and 20% palmitic acid. During incubation, substantially more palmitic acid was liberated than any other fatty acid (Table 3), indicating a preference for the palmitic acid-glycerol bond by the lipase(s) of *S. aureus*.

The volatile acidity values (Table 4) include acetic, propionic, butyric, caproic, caprylic, and a substantial portion of the C_{10}, C_{12}, and C_{14} acids. Kosikowski and Dahlberg (1946) have estimated that approximately 79, 46, and 16% of the C_{10}, C_{12}, and C_{14} acids, respectively, are included in the determination for volatile acids.

The possibility exists that lipids from autolyzed cells of *S. aureus* may have contributed minor amounts of fatty acids to the values reported in Tables 2, 3, and 4, particularly toward the end of the incubation period and at the higher temperatures. Preliminary investigations show that *S. aureus* cells contain about 4% lipid on a dry weight basis, and, therefore, the amount of free fatty acids attributable to autolyzed cells of *S. aureus* is very small.

The inhibitory effect of the C_8, C_{10}, and C_{12} acids on the growth of *S. aureus* is apparently bacteriostatic rather than bactericidal. Costilow and Speck (1951) also reported that these fatty acids in concentrations of 0.05 to 0.10% were inhibitory to *Streptococcus lactis*. The conclusion that these acids are bacteriostatic is supported by

the fact that increases in population frequently followed periods of inhibition (Fig. 1). The exact mode of action of the fatty acids in functioning as inhibitors is speculative. The fatty acids could accomplish inhibition by reducing the surface tension, coating the bacterial surfaces, or selectively attaching to permease—thus blocking the flow of nutrients or preventing the release of extracellular enzymes or both. The inhibition of *S. aureus* or inactivity of the extracellular enzymes is evidenced by a decrease in proteolysis, negative coagulase tests, and stability of *p*H.

The decrease in proteolysis in the presence of the C_4 to C_{10} fatty acids and the increase in proteolysis caused by stearic and oleic acid suggests that the influence of the acids is directly related to the enzyme system rather than to the population.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant EF-00015 from the Division of Environmental Engineering and Food Protection.

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