JOURNAL OF BACTERIOLOGY, Oct. 1968, p. 1006-1010
Vol. 96, No. 4

Lipolytic Esterases in Staphylococci

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Received for publication 3 July 1968

Staphylococci split a wide range of lipid substrates by production of an enzyme complex with two main components (i) a lipase acting optimally on fat-soluble glycerides, and (ii) an esterase acting optimally on water-soluble esters. The action is dependent upon carbon chain length, interfacial dispersion, solubility, and *p*H of substrate and end products. The esterase is less susceptible to organophosphorus inhibitors than mammalian esterases. There is no apparent correlation between lipolysis and markers of pathogenicity such as production of coagulase and toxin, but the possession of a flexible lipolytic mechanism might account for the persistence of staphylococci in the fatty secretions of mammalian skin.

The lipolytic activity of staphylococci was originally observed in 1901 by Eijkman (6). This phenomenon is now known to be caused by an enzyme active against many substrates, including water-soluble and water-insoluble glycerol esters (4, 11, 19) and also water-soluble Tween polyoxyethylene esters (8).

These properties are compatible with the production of a lipase or esterase or both. Shah and Wilson (17) consider that the enzyme is a lipase because it is not inactivated by certain esterase inhibitors. Stewart (18) found evidence that two enzymes were produced, one with the properties of a lipase hydrolyzing water-insoluble lipids, the other was an esterase hydrolyzing simpler triglycerides and water-soluble esters.

In this report, we describe the prevalence in staphylococci and properties of the lipolytic mechanism, which appear to be a complex of at least two enzymes.

MATERIALS AND METHODS

To detect prevalence of lipolytic activity in staphylococci, a general survey was carried out by testing 731 strains, consecutively isolated from hospital patients, for hydrolytic activity against tripalmitin, triolein, and tributyrin. The substrates were emulsified in 1% concentration in melted 2% nutrient agar which was then poured near solidification temperature into plastic petri plates. Staphylococci were inoculated radially and tested for hydrolytic power after 24 to 48 hr at 37 C (i) by clearing of the opaque tributyrin agar, and (ii) by adding saturated copper sulfate solution to the triolein and tripalmitin agar to detect liberated fatty acid by the blue precipitate of the copper salt (2). To relate lipolytic properties to other properties, staphylococci were also tested for production of two toxic factors (α-toxin and β-hemolysin) and two other enzymes (coagulase and β-lactamase). Standard methods were used for detecting the toxins and coagulase. β-Lactamase was detected by two techniques, a membrane plate method for the inducible enzyme (10) and an indicator method for constitutive enzyme (14).

For detailed study of biochemical aspects of lipolysis, a coagulase-positive *Staphylococcus aureus* 10300, which showed consistent activity against a range of substrates, was employed. Coagulase formation was regularly checked by the slide test (3) and confirmed by incubation in plasma broth (7).

Extraction from cells. The extraction of cell extracts was performed by a modification of the method of Kohn and Reis (12). An 18-hr nutrient agar culture of the test organism was harvested and the cells were washed in normal saline and resuspended in distilled water at 50 to 60 mg (wet weight) per ml. To facilitate disruption, chloroform (0.05 ml) was then added for each millliliter of the suspension, which was then maintained at 4 C for 48 hr and centrifuged. The resulting supernatant fluid was satisfactory as a source of the enzyme, since it remained stable for at least 1 week at 4 C. For storage of the enzyme, the solution was freeze-dried in 1-ml quantities.

Chemical estimations. A weighed freeze-dried sample of the enzyme was ashed in a muffle furnace to constant weight. This inorganic residue was then analyzed for sodium and potassium by flame photometry and for calcium by ethylene diaminetetraacetate titration with acid ammonium purpurate (murexide) as the indicator. The total nitrogen content of another freeze-dried sample was determined by the method of Lowry et al. (13).

Hydrolysis and lipolysis. The *p*H optima and Michaelis constants (K_m) of the enzyme complex were determined on a phenyl ester (phenylpropionate) and on a lipid substrate (triolein). The extent of hydrolysis was determined from *p*H 6.0 to 8.6 in the following buffers: *p*H 6.0 to 8.0, potassium-disodium phosphate,

0.1 M (Sorensen); and *p*H 8.0 to 8.6, tris(hydroxymethyl)aminomethane (Tris) maleate, 0.1 M (Gomori).

Phenyl propionate. Free phenol was removed by adsorption onto alumina from a 20% (v/v) solution of phenylpropionate in light petroleum (1).

The purified phenylpropionate was shaken with appropriate buffer at 80 mg/100 ml; this was then used for the enzyme substrate. To 3 ml of the substrate-buffer mixture, prewarmed to 37 C, was added 1 ml of the enzyme solution; this was then incubated at 37 C for 15 min. The enzymatic reaction was stopped by addition of reagents for the determination of released phenol. Esterase activity was determined by measuring released phenol by the colorimetric method of Aldridge (1), which measured free phenol with 4-amino antipyrine (9). A blank was set up for each *p*H determination with 1 ml of distilled water instead of 1 ml of enzyme. The Michaelis constant was determined at *p*H 7.3 with doubling dilutions of a 1 mg/ml solution of phenylpropionate.

Triolein was emulsified at 3% (v/v) in distilled water with 4% (v/w) polyvinyl alcohol as an emulsion stabilizer using an electric stirrer. Hydrolysis was then measured by titrating the released oleic acid with 0.01 N alcoholic potassium hydroxide and with thymolphthalein as the indicator.

A 1-ml amount of enzyme solution was added to 2 ml of emulsified triolein plus 2 ml of the appropriate buffer, and the mixture was incubated at 37 C for 20 hr. The emulsion was broken by addition of 10 ml of ether-acetone mixture (50:50, v/v), and the released oleic acid was determined by titration. Blanks were set up for each *p*H value, with 1 ml of water substituted for 1 ml of the enzyme. The Michaelis constant was determined at *p*H 8.2 with doubling diultions of 0.05 M triolein.

Manometric determinations (Warburg). All determinations were performed in bicarbonate buffer containing 0.0375 M $NaHCO_3$, 0.164 M NaCl, 0.04 M $MgCl_2$ (1). Phenol and glycerol esters were suspended in buffer at 20 mg/ml, with 4% (w/v) polyvinyl alcohol as emulsion stabilizer, because this substance did not inactivate the enzyme. In all the experiments, the Warburg flasks contained 2 ml of buffered substrate in the main compartment, with 0.5 ml of enzyme plus 0.5 ml of buffer in the side arm. The flasks were gassed with 5% CO_2 in nitrogen and run at 37 C for 30 min; readings of the gas that evolved were taken at 5-min intervals.

Heat inactivation of the enzyme. Volumes (1 ml) of the enzyme solution were heated at 40, 45, 50, 55, 60, 65, 70, 75 C for 10 min and then were rapidly cooled in ice. The enzyme activity on tributyrin, triolein, and phenyl acetate was determined manometrically with all the enzyme samples. The percentage inactivation of the heated enzyme samples was determined against unheated samples run at the same time.

Electrophoresis. Cellulose acetate electrophoresis strips were run at 300 v for 75 min in 0.1 M, *p*H 8.6, barbitone-acetate buffer. Protein was stained with 0.01% nigrosine in 5% acetic acid after fixation of the strips in 5% trichloroacetic acid for 5 min.

Esterolysis was detected by placing a strip face down onto another cellulose acetate strip which had been soaked in a solution of α-naphthol acetate (0.01%), fast red T.R. salt (0.01%), in *p*H 7.3 phosphate buffer (0.1 M). The strips were then incubated at 37 C for 1 hr in a moist chamber; the esterase appeared as a red band.

Lipase activity was determined by placing the electrophoresis strip face down onto a cellulose acetate strip which had been soaked in triolein and incubated at 37 C overnight. The electrophoresis strip was then soaked in 1% copper sulfate solution; the presence of a lipase was indicated by a blue band.

Glycerol esters. Emulsions were prepared at 20 mg/ml in bicarbonate buffer of the following: triacetin, tripropionin, tributyrin, tricaproin, tricaprylin, and triolein.

Phenyl esters. After removal of any free phenol by absorption onto alumina, as with phenol propionate (above), the following were tested: phenyl acetate, phenylpropionate, phenyl butyrate, and phenyl laurate.

Choline esters. Acetylcholine, butyrol choline, and acetyl thiocholine at 0.01 M in bicarbonate buffer were used as substrates.

Inhibitor studies. The following inhibitors were used to classify the staphylococcal esterase: diethyl-*p*-nitrophenyl phosphate (E.600), diisopropyl-*p*-nitrophenyl phosphate, diisopropyl phosphorofluoridate, *N,N*-diisopropyl phosphorodiamic fluoride (Mipafox), neostigmine methylsulfate, and gentian violet. All of the above inhibitors were made up in bicarbonate buffer before use.

In all determinations with inhibitor, buffer in the side arm was replaced by 0.5 ml of inhibitor solution. The enzyme-inhibitor mixture in the side arm was incubated at 37 C for 30 min before it was added to the substrate.

RESULTS

The general survey (Table 1) showed that 85% of staphylococci hydrolyzed tripalmitin, with a significant correlation between hydrolysis of this substrate and coagulase production in the case of nonpigmented strains. Statistically, a negative correlation with β-lactamase production, constitutively and inducibly, was also apparent (Table 2) but not with or against production of

TABLE 1. *Hydrolysis of tripalmitin by staphylococci compared with pigment and coagulase production*[a]

Pigment	Coagulase	Hydrolysis	No. hydrolysis	Total
Present	Positive	523	109	632
	Negative	19	0	19
Absent	Positive	61	2	63
	Negative	17	0	17
Total no. of strains		620 (84.8%)	111	731

[a] $\chi^2 = 15.5872$; $0.002 > P > 0.001$.

α-toxin or β-hemolysin. All strains, irrespective of other properties, hydrolyzed tributyrin and triolein, which were therefore used as standard controls for comparison with other substrates in the tests carried out with strain 10300.

The enzyme complex extracted from cells of this strain hydrolyzed all the water-soluble esters and the short-chain glycerol esters in aqueous solution. The longer chain fat-soluble glycerol esters, tricaproin, tricaprylin, and triolein, were hydrolyzed only after emulsification. In terms of substrate specificity, therefore, two components were present, one with the actions of an esterase and one with an action of a lipase (Table 3). By our method, these were extracted as one complex of which about 40% was protein (Table 4). Electrophoresis at *p*H 8.6 separated this protein into the two constituent enzymes.

The complex showed maximal activity, expressed as quantity hydrolyzed, against tributyrin; glycerol esters with a carbon content greater or less than this were less susceptible. With the exception of phenyl acetate, the phenyl esters were less susceptible to hydrolysis than their homologous glycerol esters (Table 5). Activity was maximal against phenyl acetate, decreasing with increase in chain length. Choline esters were not hydrolyzed. The glycerol esters were more effectively hydrolyzed when emulsified in polyvinyl alcohol and water, more or less in proportion to their chain length. Emulsification did not alter the rate of hydrolysis of the water-soluble phenyl esters.

The organophosphorus compounds were without effect on the two enzymes, except for diethyl-*p*-nitrophenyl phosphate which inhibited the hydrolysis of tributyrin and the phenyl esters by about 25%. Neostigmine was inactive, and gentian violet inhibited only in proportion to its general inhibition of growth.

The results reported here relate to the enzyme obtained from one strain of *S. aureus* which we believe, in this respect, to be representative of the lipolytic enzymes found in this genus. Most staphylococci produce lipolytic enzymes capable of hydrolyzing simple fats and esters (18), but there is considerable limitation of activity upon glycerides with a chain length greater than C 15.

TABLE 4. *Composition of lipolytic enzyme complex extracted from S. aureus*

Component	Constituents (mg/ml of extract)	Percentage of dry weight
Weight (freeze-dried)	3.850	
Ash	1.800	46.76
Protein	1.520	39.48
Na^+	0.250	6.49
K^+	0.505	1.31
Ca^{++}	0.020	0.52
Unidentified	0.210	5.45

DISCUSSION

The results show that lipolytic power is a constant property of staphylococci, more so than coagulase production, pigment, or toxin; lipolysis should, therefore, be quoted as an essential

TABLE 2. *Correlation matrix of prevalence of biochemical marker properties in staphylococci*[a]

Marker	Marker				
	Tripalmitin lipase	α-Toxin	β-Hemolysin	β-Lactamase (membrane)	β-Lactamase (indicator)
Tripalmitin lipase	1				
α-Toxin	0.038	1			
β-Hemolysin	0.029	0.040	1		
β-Lactamase (membrane)	−0.201[b]	−0.032	0.031	1	
β-Lactamase (indicator)	−0.117[b]	0.001	0.021	0.708[b]	1

[a] Number of strains tested, 731.
[b] $P < 0.01$.

TABLE 3. *Properties of lipolytic enzyme complex extracted from S. aureus*

Fraction	Substrate	Optimal *p*H	Michaelis constant (M)	Temp of 50% inactivation (C)	Emulsification (% increase in hydrolytic activity)	Electrophoretic charge
Esterase	Phenylpropionate	7.3	9.8×10^{-4}	59	0	Cationic
Lipase	Triolene	8.2	9.5×10^{-4}	64	83	Neutral

TABLE 5. *Hydrolytic activity on glycerol and phenyl ester substrates of lipolytic enzyme complex from S. aureus*

Fatty acid	C chain	Hydrolytic activity (as % of tributyrin hydrolysis)	
		Glycerol ester	Phenyl ester
Acetic.....	1	25.6	26.0
Propionic...	2	55.0	17.0
Butyric.....	3	100.0	12.0
Caproic.....	5	35.0	
Caprylic....	7	27.8	
Lauric.....	11		9.6
Oleic.....	17	4.5	

property in classification. The nature and extent of this lipolysis depend, however, upon the substrate used for its detection. The so-called lipase is, in fact, a complex of at least two enzymes, one with preferred specificities for water-soluble aromatic esters and one with fat-soluble aliphatic esters. In accordance with present nomenclature (5), it would, therefore, be more precise to describe the two components as an esterase with the properties of carboxylic ester hydrolase and aryl ester hydrolase and a lipase or glycerol ester hydrolase. Chemically, however, many fats are esters irrespective of their solubility; since the products of reaction in the case of each component indicate a cleavage at the ester linkage (18), it would be more accurate to regard the enzyme protein as a complex of esterases with specificities determined by physical factors such as solubility, *p*H, and carbon chain structure.

This interpretation would account also for limitation of activity upon the longer chain glycerides which are effectively hydrolyzed only when the reaction site is extended at an oil-water interface by emulsification. It is, in fact, a general characteristic of glycerides that they are hydrolyzed better when water is available for solvation of the enzyme protein and the hydrophilic products of reaction, as in alimentary digestion. Fat-soluble products will naturally remain in the lipid phase and, by reducing dispersion, stop the reaction. This explains the poor rate of hydrolysis observed with the longer chain fats which disperse in water only with difficulty.

The inhibition of esterase by organophosphorus compounds in high dilution (1, 5) was not found in our study. One such compound, diethyl-*p*-nitrophenol phosphate, showed inhibitory activity but not of a high order. Susceptibility to the organophosphorus inhibitors has been studied, however, mainly with esterases produced by mammalian cells. It is likely that enzymes from bacteria are fundamentally different because choline esters are unlikely to be encountered as substrates.

The ability to hydrolyze simple water-soluble esters and fats with a carbon chain length below 15 is a constant property of staphylococci, whereas hydrolysis of higher fats, like tripalmitin, is a more variable property. Since cutaneous fats and secretions differ in composition according to species, race, and diet, it is likely that staphylococci with wide substrate affinities enjoy an advantage in colonizing the skin.

It is not our purpose here to discuss further the biological significance of esterases in staphylococci, except to state that the biochemical finding might well explain the tenacity with which these organisms remain in lipoid secretions in their cutaneous habitat (16) and might, therefore, account ecologically for the prevalence of the organism in most human communities.

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