Modification of bactericidal fatty acids by an enzyme of *Staphylococcus aureus*

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Summary. Certain strains of *Staphylococcus aureus* produce an enzyme capable of inactivating the bactericidal fatty acids produced in staphylococcal abscesses by esterification to various alcohols. The enzyme, called FAME (fatty acid modifying enzyme), has a pH optimum between 5.5 and 6.0 and a temperature optimum of about 40°C. Enzyme activity is not affected by edetic acid or by the presence or absence of sodium and potassium ions. Although FAME can utilise methanol, ethanol, 1-propanol, 2-propanol, 1-butanol or cholesterol as substrates, cholesterol appears to be the preferred substrate. FAME esterifies without being an esterase operating in reverse. Strains capable of producing the enzyme can synthesise it in trypticase soy broth and in a chemically defined medium, but not necessarily in equal amounts. FAME production is correlated with the ability of a strain to grow and survive within the tissues.

Introduction

While studying the fate of *Staphylococcus aureus* in abscesses, it was found that the elimination of the cocci from these lesions was mediated by two kinds of staphylocidal lipids.^{1–3} One of these consists of a pool of long-chain, unsaturated, free fatty acids and the other is an unidentified lipid (UBL) which has several characteristics that distinguish it from the more abundant fatty acids.^{3,4}

The bactericidal activity of abscess homogenates can be increased by incubating such preparations with either live *S. aureus* or culture filtrates. We have called this phenomenon “activation”.¹ Activation results from the release of fatty acids from glycerides by the action of staphylococcal lipase thus contributing to the total fatty acid pool, but it does not involve the production of more UBL.

Recently we found that freshly prepared culture filtrates also contain an enzyme capable of destroying the bactericidal activity of both lipids under proper conditions. It is called “fatty acid modifying enzyme” (FAME). This report describes the properties of FAME and discusses its possible role in the staphylococcal host-parasite interaction.

Materials and methods

Staphylococcal strains

S. aureus strains 18Z, 18Z-G, 18Z-H, P78, P78-22, PG114, PG114-1, TG, 689 and 303 have been described previously.² Strains M36, M56 and M68 were other isolates taken from our culture collection. All had been evaluated for their ability either to survive within intraperitoneal abscesses or to multiply in kidneys. Because FAME production was first detected in strain 18Z, this strain was used for enzyme production in this study.

Abscess homogenates and bactericidal assay

Abscesses were generated in mice by the intraperitoneal inoculation of 10⁹ cfu of *S. aureus* strain 18Z. After 7 days, the abscesses were excised and homogenised, and the bactericidal activity was assayed as previously described.¹

Lipid extracts and fractionation of lipids

Lipids were extracted from abscess homogenates by the method of Bligh and Dyer⁵ and the fatty acid fraction was recovered as previously described.³

Preparation of crude FAME

S. aureus strain 18Z was grown in trypticase soy broth (TSB) at 37°C for 18 h with continuous agitation.

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The culture was centrifuged at 10 000 *g* for 20 min and the supernatant fluid was sterilised by passage through a 0.22- μ m membrane filter. The culture filtrate was concentrated 10-fold on a PM30 membrane (Amicon Corp.) and the concentrate was dialysed overnight against several changes of distilled water at 4°C. After dialysis, the preparation was lyophilised and stored at 4°C until used.

Fatty acid analysis

Fatty acid methyl esters were produced with diazomethane.⁶ The esters were separated on a column of Alltech CS-10 10% on chromosorb W-AW in a Packard Model 428 gas chromatograph fitted with an automatic integrator.

FAME assay

The fatty acid substrate, [9,10-³H(N)]-oleic acid (New England Nuclear), was diluted with unlabelled oleic acid to provide 2.5×10^5 cpm/mg of fatty acid. The radiolabelled oleic acid was dissolved in hexane and aliquots of the stock solution containing 200 μ g of fatty acid were dispensed into lipid-free vials. The hexane was evaporated under a stream of nitrogen at 40°C and the fatty acid was again dissolved by adding 0.1 ml of ethanol to each vial.

FAME was dissolved in 0.1 M phosphate buffer, pH 6.0, and serial three-fold dilutions were prepared in the same diluent. Of each enzyme dilution, 0.5 ml was added to a vial containing the oleic acid solution and the final volume was brought up to 1 ml with phosphate buffer. The mixture was incubated in a water bath, with shaking, at 37°C for 20 min.

After incubation, the lipids were removed by extracting the mixture twice with 2.5-ml volumes of diethyl ether:methanol (6:1 v:v) and once with 2.5 ml of diethyl ether. The ether phases (upper phases) were combined and evaporated to dryness under nitrogen. The extracted lipids were dissolved in 70 μ l of chloroform and the entire amount was spotted on silica gel G-coated glass plates. The vials were rinsed

successively with 70 μ l of chloroform and 70 μ l of acetone and these rinses were added to the same spot.

The fatty acids were separated from the ester by developing the plates in hexane:diethyl ether:acetic acid (80:20:1) and the lipid spots were visualised with iodine vapour. The spots were scraped and collected in vials containing cocktail (complete counting cocktail 3a 70, Research Products International, Mt Pleasant, IL) and counted in a scintillation counter. The percentage of fatty acid esterified was calculated by dividing the counts of the ester spot (minus the negative control) by the total counts in the sample.

Chemicals and glassware

All glassware was cleaned with chloroform:methanol (2:1 v:v) before use.

Non-radiolabelled lipids and cholesterol were purchased from Sigma Chemical Co, St Louis, MO, USA.

Results

Preliminary studies

When the fatty acid fraction isolated from abscess homogenates was incubated with freshly prepared *S. aureus* culture filtrate (CF), the bactericidal activity of the lipid fraction was destroyed (table I). Heated CF, or CF stored at -20°C for 7 months, no longer had this ability. Similarly, CF could abolish the bactericidal activity of pure oleic acid, one of the most abundant fatty acids in the fatty acid pool in abscesses.

Gas chromatography of the fatty-acid fraction incubated with CF revealed the presence of a series of new peaks, each trailing the peak of a known fatty acid. These new peaks did not appear in the absence of CF or in preparations incubated with heated CF. Furthermore, with progressive incubation, the known fatty acid peaks diminished while the new peaks increased in size suggesting that the fatty acids were being converted to derivatives with increased retention times. These findings were confirmed with pure

Table I. Effect of *S. aureus* CF on the bactericidal activity of fatty acids found in abscesses

Culture filtrate preparation	Bactericidal activity (μ g of lipid/LD ₅₀) of	
	Free fatty acid fraction from abscess homogenate	Oleic acid
Control (no CF)	1.8	2.3
Unheated CF	> 50	> 25
Heated CF		
60°C, 1 h	5.1	...
80°C, 1 h	1.7	...
100°C, 1 h	2.2	1.1
Stored CF (-20°C, 7 months)	1.8	...

Table II. Composition of fatty acids (FA) incubated with *S. aureus* CF

CF preparation	Percentage composition of FA preparation			
	Oleic acid	Oleic acid derivative	Linoleic acid	Linoleic acid derivative
Control (no CF)	100	0	100	0
CF	24.2	75.8	8.8	91.2
Boiled CF	100	0	98.1	0
TSB (no <i>S. aureus</i>)	100	0	...	...
Relative retention time	1.00	0.90	1.00	0.91

samples of oleic acid and linoleic acid as substrates (table II).

The fatty acid derivatives could be separated from the parent fatty acids by chromatography on Silica G plates; this permitted the recovery of the derivative and its analysis by mass spectroscopy. When oleic acid was used as the starting material, the derivative proved to be ethyl oleate. Considering the composition of the reaction mixture, it was obvious that the source of the ethyl group was the ethanol used in preparing the fatty acid suspensions. These results indicated that FAME was an enzyme capable of esterifying free fatty acids with ethanol.

When the esterification of oleic acid by crude FAME was examined, it was found that the extent of esterification was linear over the enzyme concentration range 0–300 µg/ml (fig. 1). Under the conditions selected for the assay, one unit of FAME activity was defined as that amount of enzyme needed to esterify 10 nmol of oleic acid/min. With a 20-min incubation period, this was equivalent to the esterification of 28% of the fatty acid in the reaction mixture. FAME either heated to 100°C for 60 min or treated with protease K (1 mg/ml at 37°C for 1 h) had no esterifying activity.

pH Optimum

A constant amount of FAME was dissolved in either 0.1 M sodium phosphate buffer (for pH range

Fig. 1. Esterification of oleic acid to ethanol by crude FAME. Reaction mixture: FAME in 0.9 ml of 0.1 M phosphate buffer (pH 6.0), with 200 µg of oleic acid in 0.1 ml of ethanol, incubated at 37°C for 20 min. Vertical bars represent the SD of the mean of 3–8 experiments.

Fig. 2. The effect of pH on FAME activity. Reaction mixture: 200 µg of crude FAME in 0.9 ml of either 0.1 M sodium phosphate buffer (pH 5.0–8.0) or 0.1 M citric acid/sodium citrate buffer (pH 3.5–6.0), 200 µg of oleic acid in 0.1 ml ethanol, incubated at 37°C for 20 min.

5.0–8.0) or 0.1 M citric acid/sodium citrate buffer (for pH range 3.5–6.0). The optimum activity occurred between pH 5.5 and pH 6.0 and little activity was seen at pH 4.0 or pH 8.0 (fig. 2).

Incubation temperature

Samples of a constant reaction mixture were incubated in a water bath, with shaking, for 20 min at temperatures from 0° to 60°C. The extent of esterification was proportional to the incubation temperature over the range 0–30°C and was maximal at about 40°C. Little activity was noted at 60°C (fig. 3).

The effect of monovalent and divalent cations

The requirement for divalent cations was evaluated by adding 5, 10 or 20 mM edetic acid to the standard reaction mixture. The effect of sodium and potassium ions on FAME activity was examined by dissolving FAME in 5 mega-ohm distilled water and substituting 10 mM HEPES-Tris buffer (pH 6.0) for the usual phosphate buffer. NaCl or KCl, at concentrations of 0.25, 0.5 or 1.0%, were then added to samples of the reaction mixture. FAME activity was not affected by the addition of edetic acid, potassium ions or sodium ions.

Fig. 3. Effect of incubation temperature on FAME activity. A mixture of 150 μ g of crude FAME in 0.9 ml of 0.1 M phosphate buffer (pH 6.0) and 200 μ g of oleic acid in 0.1 ml of ethanol was incubated for 20 min at various temperatures in a water bath with shaking.

Substrates for esterification to oleic acid

Initially, qualitative data were obtained to determine if alcohols other than ethanol might be suitable substrates for the esterification reaction. These included methanol, 1-propanol, 2-propanol, 1-butanol and isoamyl alcohol. Certain staphylococcal metabolites, which might accumulate in abscesses, were also studied. These included: 2,3-butanediol; 2,3-butanedione; 3-hydroxybutanoic acid and 3-hydroxy-2-butanone. Glycerol, cholesterol and glucose were examined also as host products that might be available to participate in the esterification reaction in lesions. In these studies, equal molar concentrations of the aforementioned compounds were substituted for the ethanol and the amount of FAME was held constant. Otherwise, conditions were the same as in the standard assay except that the incubation period was extended to 4 h. Methanol, ethanol, 1-propanol, 2-propanol and 1-butanol were esterified to oleic acid by FAME, but isoamyl alcohol was not esterified under these conditions (table III). None of the staphylococcal metabolites tested were esterified and, of the host-related products, only cholesterol could be esterified to oleic acid.

To compare the relative efficiency of the different alcohols in the esterification reaction, a fixed amount of oleic acid was mixed with various amounts of the alcohol to generate different alcohol:oleic acid ratios. A constant amount of enzyme was added and the extent of esterification under standard conditions was measured. The concentration of oleic acid was held at 7×10^{-4} M and the concentration of the alcohols was varied from 2.6×10^{-4} to 8.6×10^{-1} M. Because of poor solubility of some alcohols, the buffer, fatty acid and alcohol mixtures were sonicated before the addition of the enzyme. From these data, the molar ratio of alcohol:oleic acid necessary to achieve 30% esterification under the test conditions was calculated (table IV).

From among the series of primary alcohols, as the chain length increased from one carbon to four carbon

Table III. Evaluation of various compounds for their ability to be esterified to oleic acid by *S. aureus* FAME*

Compound	Esterification
Methanol	+
Ethanol	+
1-Propanol	+
2-Propanol	+
1-Butanol	+
3-Methyl-1-butanol	—
Glucose	—
Glycerol	—
Cholesterol	+
3-Hydroxybutanoic acid	—
3-Hydroxy-2-butanone	—
2,3-Butanediol	—
2,3-Butanedione	—

* The reaction mixture consisted of 150 μ g of crude FAME in 0.9 ml of 0.1 M phosphate buffer (pH 6.0) and 7.0×10^{-4} M oleic acid. The concentration of the other substrates was 1.7×10^{-3} M. Incubation was at 37°C for 4 h.

Table IV. Relative efficiency of different alcohols in the esterification of oleic acid by *S. aureus* FAME

Alcohol	Alcohol:Oleic acid ratio*
Methanol	848
Ethanol	732
1-Propanol	141
1-Butanol	36.6
Cholesterol	0.89

* Ratio to achieve 30% esterification of the fatty acid under the test conditions: 150 μ g crude FAME and 7.1×10^{-4} M oleic acid. Incubation was at 37°C for 4 h.

atoms, the efficiency increased, since less alcohol was required per mol of oleic acid to give the same degree of esterification. However, cholesterol proved to be an even better substrate for this reaction than 1-butanol. This suggested that from among the alcohols which could be esterified to oleic acid by FAME, cholesterol was the preferred substrate.

Production of FAME by staphylococcal strains

The ability of various *S. aureus* strains to produce FAME in either TSB or in a chemically defined amino acid medium⁷ was evaluated. The strains selected for study had all been examined for their ability to survive within intraperitoneal abscesses or for their ability to multiply within kidneys.² From each strain, culture filtrates were prepared and assayed for FAME as previously described. The concentration of organisms was determined either by optical density measurements or by plate counts.

Strains 18Z, PG114, TG, P78, M36, M56 and M68 produced substantial amounts of the enzyme whereas strains 18Z-G, 18Z-H, PG114-1, P78-22, 689 and 303 produced none. Furthermore, all those strains capable

Table V. Relationship between FAME production and invasiveness of *S. aureus* strains

Strain no.	FAME production (units/10 ⁹ cfu)		Invasiveness†
	defined medium*	TSB	
P78	0.47	0.10	+
PG114	0.24	0.65	+
18Z	0.76	0.10	+
TG	0.24	0.10	+
M36	...	0.90	+
M56	...	0.48	+
M68	...	0.73	+
PG114-1	...	0.0	—
18Z-G	...	0.0	—
18Z-H	0.0	0.0	—
P78-22	0.0	0.0	—
689	0.0	0.0	—
303	0.0	0.0	—

* An amino acid, salts, vitamin medium.⁷

† Invasiveness is defined as the ability either to survive within intraperitoneal abscesses or to multiply in the kidneys.

of producing FAME were able to survive or multiply in host tissues, whereas those strains unable to synthesise the enzyme could not (table V).

Discussion

Previous work described an infection model that involved the destruction of *S. aureus* in intraperitoneal abscesses by two kinds of bactericidal lipids.¹⁻³ The detection of an enzyme capable of inactivating these lipids suggested that this enzyme might be involved in the host-parasite interaction and, therefore, deserved further study.

The original finding that FAME could esterify fatty acids to ethanol explained the appearance of new peaks during gas chromatography. When fatty-acid samples were treated with FAME, ethyl esters were produced. The remaining free fatty acids would then be converted to the methyl esters for GC analysis. The additional carbon unit present in the ethyl ester retarded elution from the column and resulted in the appearance of a second peak for each fatty acid. Esterification also explained the loss of bactericidal activity, since previous studies had shown that fatty acid esters were unable to kill staphylococci.³

Subsequent data indicate that FAME esterifies free fatty acids to various short chain primary alcohols and cholesterol but not to certain other mono-, di- or polyhydric compounds. FAME has a temperature optimum of about 40°C and a pH optimum of 5.5–6.0.

Although the pH of normal tissue is above the optimum, there is evidence that the pH within abscesses is decidedly lower. It has been reported that abscesses induced with turpentine and staphylococci have a pH between 6.2 and 7.0, whereas abscesses produced with turpentine alone have a pH 7.1–7.2.⁸

We have found that homogenates prepared from intraperitoneal staphylococcal abscesses have a pH of 5.9–6.1. This suggests that the conditions within staphylococcal abscesses may be close to the pH optimum for the enzyme.

We elected to use ethanol as a substrate when assaying FAME activity, but this choice was strictly a matter of convenience because stable cholesterol suspensions are more difficult to prepare. However, cholesterol seems to be the preferred substrate and cholesterol is available in the core of abscesses in the form of membrane fragments resulting from the disintegration of leucocytes. Thus, it appears likely that abscesses provide not only a suitable pH, but also an appropriate substrate to permit the esterification and neutralisation of bactericidal fatty acids.

FAME has yet to be purified and characterised in detail. However, there are various types of enzymes that catalyse reactions involving esters of fatty acids.⁹ One possibility is that FAME is an acyl-transferase [EC 2.3.1]. However, because of the requirements of this enzyme for ATP, magnesium ions and coenzyme A carrier,⁹ it seems unlikely that FAME would fall into this category. FAME was found to be insensitive to edetic acid and was unaffected by prolonged dialysis against distilled water.

A second possibility is that FAME is a representative of the sterolester hydrolases [EC 3.1.1.13], a group of enzymes which usually catalyse the hydrolysis of sterol-fatty acid esters, but which may also catalyse the reverse reaction at a lower pH. Cholesterol ester hydrolases have been described in *Saccharomyces cerevisiae*¹⁰ and *Pseudomonas fluorescens*.¹¹ These enzymes have not been studied extensively, but, for the *P. fluorescens* enzyme, the optimum pH for hydrolysis of cholesterol esters was pH 7.3. It has also been reported that the Wood 46 strain of *S. aureus* produces a cholesterol ester hydrolase.¹²⁻¹⁴ Workers studying this enzyme observed hydrolytic activity at pH 7.0–7.5, but made no mention of any possible reverse reaction.

While it is conceivable that FAME is related to a cholesterol esterase, we have been unable to detect esterase activity over the range pH 4–9, either in the presence or absence of calcium. Thus, at present FAME appears to be a unique type of enzyme.

The ability of FAME to esterify fatty acids suggests that the enzyme may play an important role in the host-parasite interaction, as the esterification of the bactericidal fatty acids found in abscesses results in their inactivation.³ Amongst the 13 *S. aureus* strains tested, only those able to elaborate FAME were able to survive within host tissues (table V). Those strains lacking FAME were rapidly destroyed when introduced into the tissues,² but they did appear able to colonise mucosal surfaces.

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