Antibacterial Spectra and Minimum Inhibition Concentration of Clupeine and Salmine*1

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(Accepted February 20, 1984)

Two types of fish protamines (clupeine sulfate and salmine sulfate) were tested for their antibacterial action on a number of bacterial strains. All the Gram-positive organisms studied were more or less sensitive to these protamines, while there was little or no effect on Gram-negatives. The action was almost same in the two protamines. According to MIC values, Bacillus coagulans and Bacillus megaterium were the most sensitive organisms. The protamines were bactericidal against five out of six bacillus organisms studied and the organism Bacillus licheniformis was capable to resist the bactericidal action of the protamines.

Protamines are peptides which are very basic in nature and found in association with DNA of spermatozoan nuclei of number of species of fish, birds, and mammals.1,2 For many years, it has been known about the antimicrobial characteristics of fish protamines, but in fact, serious attention has never been paid regarding this aspect. In some early reports, antimicrobial action of protamines on number of bacterial strains was described. More recently, the bactericidal effect of salmon sperm protamine on Bacillus subtilis cells has been reported.9 Another report9 described the bactericolytic effect of protamine on some strains and assumed that the primary site of action of the protamine was the cell wall.

The available reports on antibacterial action of protamines, however, are inconclusive and apparently fragmentary, involving only a few strains of organisms and describing antibacterial spectrum of the protamines rather unprecisely. The present paper reports on antibacterial spectra and minimum inhibition concentration of clupeine and salmine.

Materials and Methods

Strains

The following bacterial strains were included in this study: Pseudomonas fluorescens ATCC 13525; Escherichia coli ATCC 25922; Serratia marcescens ATCC 13880; Proteus morganii ATCC 25830; Salmonella enteritidis ATCC 13311; Staphylococcus aureus ATCC 25923; Vibrio parahaemolyticus (clinical isolate); Enterobacter aerogenes IFO 13534; Bacillus subtilis subtilis IFO 3026; B. subtilis niger IFO 13721; B. subtilis mesentericus IFO 3034; B. megaterium IFO 12108; B. licheniformis IFO 12200; B. coagulans IFO 12583; Lactobacillus plantarum IFO 12519; L. casei IFO 3533; and Streptococcus faecalis IFO 12580. The strains of ATCC and vibrio were obtained from Nissui Seiyaku Co. Ltd., Central laboratory, Tokyo, and the rest of them were from Institute of Fermentation, Osaka.

Protamines

Two types of commercially available protamines were included in this study. Clupeine sulfate (from herring roe) and salmine sulfate (from salmon roe) were obtained from Wako Pure Chemical Industries Ltd. Protamine solutions were prepared by dissolving them in distilled water, sterilized with Millex-GS filter units (Millipore Corp., U. S. A.), and used immediately after preparation.

Media

The following media were used in this study: Media for Gram-negative organisms includes- (a) tryptcase soy agar (Difco, U.S.A.), (b) nutrient agar medium composed of 1.0% polypeptone, 0.5% meat extract, 0.2% NaCl (3.0% for vibrio), and 1.5% agar, pH 7.0. Media for bacillus or-
organisms- 0.5% polypeptone, 0.3% meat extract, 0.2% MgSO₄·7H₂O, pH 7.0. Media for lactobacillus and streptococcus- tomato juice agar (Eiken Chemical Co. Ltd., Tokyo). The liquid versions of the above media were used for broth culture.

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**Sensitivity Test and Determination of Minimum Inhibition Concentration (MIC)**

Agar dilution method: Two-fold dilutions of protamines in water were mixed with agar medium and incorporated into the petri dishes, then the solid surface was inoculated (spot application) with diluted 18h broth culture which contained approximately 10⁷ cells per ml. The plates were then incubated for 20 h at 37°C except that of *Pseudomonas fluorescens* which was incubated at 25°C. The grade of growth inhibition was judged by naked eye comparing with that of control plate. The end point for MIC reading was taken as the lowest concentration of protamine giving complete inhibition of growth.

Agar diffusion method: Protamine discs were prepared by incorporating different concentrations of protamine solutions in 8 mm diameter sterile paper discs and allowed to dry overnight at 37°C. Double layer agar plates were used in this method. The protamine discs were firmly applied on the surface of the upper layer which contained about 10⁷ cells per ml. The plates were incubated at 37°C for 20 h. The inhibition zones around discs were measured as the grades of growth inhibition.

**Determination of Bactericidal Action of the Protamines**

Aliquots of broth media of 4.375 ml each were distributed in 14mm x 160 mm test tubes. Each series of tubes for each strain was completed by adding 0.5 ml of inoculum of suspension of 10⁸ cells per ml prepared from 18 h broth culture and 0.125 ml of adequately diluted protamine solutions to give different concentration in each series of tubes. The tubes were then incubated at 37°C for 20 h and the total viable count of the survivors in each concentration of protamine were made by standard plate count method as colony forming units.

**Results and Discussion**

**Sensitivity Test**

With the aim of screening the protamine sensitive organisms, both clupeine and salmine were tested at a concentration of 500 µg per ml medium in agar dilution method and 500 µg per disc in agar diffusion method. The results are given in Table 1. Out of all the Gram-negative organisms included in this study only enterobacter was slightly sensitive and the rest of them were resistant to both

<table>
<thead>
<tr>
<th>Strain</th>
<th>Agar dilution method 500 µg/ml media</th>
<th>Paper disc method 500 µg/disc</th>
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<tbody>
<tr>
<td></td>
<td>Clupeine</td>
<td>Salmine</td>
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<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>-</td>
<td>-</td>
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<tr>
<td><em>Serratia marcesens</em></td>
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<tr>
<td><em>Proteus morganii</em></td>
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<td><em>Escherichia coli</em></td>
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<td><em>Salmonella enteritidis</em></td>
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<tr>
<td><em>Vibrio paraahemolyticus</em></td>
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<td>-</td>
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<tr>
<td><em>Enterobacter aerogenes</em></td>
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<td>+</td>
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<td><em>Staphylococcus aureus</em></td>
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<td>+</td>
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<tr>
<td><em>Bacillus coagulans</em></td>
<td>++</td>
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<tr>
<td><em>B. megaterium</em></td>
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<tr>
<td><em>B. licheniformis</em></td>
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<td><em>B. subtilis subtilis</em></td>
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<td><em>B. subtilis niger</em></td>
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<td><em>S. subtilis mesentericus</em></td>
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<td><em>Lactobacillus plantarum</em></td>
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<td><em>Lactobacillus casei</em></td>
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<tr>
<td><em>Streptococcus faecalis</em></td>
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</tbody>
</table>

- = resistant; + = growth inhibition

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clupeine and salmine. In contrast all the Gram-positive organisms were more or less sensitive to the protamines.

The above results indicated that the Gram-positive organisms are more vulnerable to protamines than Gram-negative ones, being in agreement with the previous reports\(^3,5\) which revealed that the salmon sperm protamine was more bacteriostatic against Gram-positive bacteria. According to the grade of growth inhibition exerted by the protamines it can be assumed that the antibacterial action was almost same in clupeine and salmine.

Out of the two methods involved in this study, the agar dilution method was more suitable than the agar diffusion method. In agar diffusion method the inhibition zones were much smaller than expected and the largest zone diameter recorded was only 8 mm. Another method, the broth dilution method should have been more convenient for this study. It was observed, however, that due to some unknown reasons the addition of protamine solution to the broth medium resulted in the turbidity of the medium and consequently made the interpretation of the photometric measurements impossible. Therefore the broth dilution method was excluded from this study.

### Minimum Inhibition Concentration (MIC)

The MIC of both clupeine and salmine in agar dilution method are given in Table 2. Bacillus coagulans and Bacillus megaterium were found most sensitive organisms with MIC 75 \(\mu\)g protamine per ml medium and the Enterobacter aerogenes was least sensitive. The MIC of lactobacillus organisms ranged from 100 \(\mu\)g–150 \(\mu\)g protamine per ml medium, but previously it was reported that the growth inhibition of Lactobacillus plantarum and L. casei was occured at 5 \(\mu\)g and 10 \(\mu\)g clupeine per ml respectively.\(^7\) This discrepancy can be explained by differences in many aspects including the media, methods, preparation of protamines, and the bacterial strains involved.

The results of the paper disc method are given in Fig. 1. Only the bacillus organisms were included in this method. Six different concentrations of both clupeine and salmine ranging from 100 \(\mu\)g to...
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Fig. 2. Lethal effect of clupeine sulfate on bacillus organisms.

- - - : B. subtilis ruber, - - - - : B. subtilis nigere
- - - - - : B. subtilis mesentericus, - - - : B. megaterium, - - - : B. licheniformis, - - - - : B. coagulans.

600 µg per disc were used for each organisms. For all the organisms measureable inhibition zones were found at 200 µg per disc. The results of this method also indicated that the antibacterial action of clupeine is slightly higher than that of salmine and this observation supports the results of agar dilution methods.

Lethal Effect of Protamine

Only clupeine was tested for lethal effect. This protamine had lethal effect on five out of six bacillus organisms. From Fig. 2 it can be noticed that at a concentration of 50–100 µg per ml the survivability of the most of the organisms started to decrease significantly and it reached close to zero at 200–300 µg per ml medium. Survivability of B. coagulans and B. megaterium decreased to 0% at the concentration of as low as 100 µg per ml.

However, clupeine sulfate had very little effect on B. licheniformis as far as the lethal effect is concerned. The reason for the less lethal effect of clupeine sulfate to B. licheniformis is not emphasized yet, but it should further be studied in detail.

The above results indicated that clupeine is bactericidal rather than bacteriostatic at least for some particular organisms. The previous reports also supports this observation. It is to be mentioned here that most of the known polypeptide antibiotics are bactericidal and more active on Gram-positive organisms.

In consequence, even the action is moderate, protamines have shown some similarity to the other polypeptide antibiotics as far as the antibacterial characteristics is concerned. It was of particular interest in this study that all the bacillus organisms included in this study were sensitive to the protamines.

References