Effects of pH, Temperature, Metal Ions and Organic Matters on the Bactericidal Action of Clupeine Sulfate*1

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Various factors influencing the bactericidal action of clupeine sulfate on Bacillus organisms were studied. Clupeine sulfate was found more active in neutral and alkaline pH regions. The ionic strength of the buffer greatly influenced the bactericidal action, 0.5 M phosphate buffer almost inhibited the bactericidal action of clupeine sulfate. Temperature had only a little effect on the bactericidal action. Although the increased salt concentration in the media largely reduced the bactericidal action of clupeine, the ionic strength of the metal ions was far more important than that of molarity of the salts. The ionic strengths of more than 0.15-0.20 of metal ions drastically reduced the bactericidal action. Mg and Ca ions were more inhibitory than that of Na and K ions. Organic substances in the media had very little or no effect on the bactericidal action of the protamine under study.

Few studies are available on the bactericidal action of protamine under different nutritional and environmental conditions. Among the available reports, however, the studies carried out by NEGRONI and FISCHER1,2) are the most elaborate, and others are the works regarding some factors which can influence the antibacterial action of protamine.3-5) BLOOM et al.6) reported that the antibacterial action of protamine on B. subtilis cells were reversed by nucleic acids and ribonucleic acids. A recent report also described the factors influencing the bacteriolytic action of protamine on B. subtilis cells.7) Although a number of papers dealt with the study of factors influencing the antibacterial action of protamine, they were neither specific nor consistent and were rather contradictory in some cases. The present paper reports the effects of pH, temperature, metal ions and organic matters on bactericidal action of clupeine sulfate. In view of the marked bactericidal action of protamines against Bacillus species,13) only these organisms were included in this study.

Materials and Methods

Strains

Bacillus subtilis ruber IFO 3026 was the principal subject matter of this study. The other organisms included were B. coagulans IFO 12583 and B. licheniformis IFO 12200. All the strains were obtained from Institute of Fermentation, Osaka and continuously maintained in our laboratory.

Protamine

Clupeine sulfate (from herring roe) was obtained from Wako Pure Chemical Industries Ltd. Clupeine solution was prepared by dissolving it either in distilled water or 0.01 M phosphate buffer and sterilized with Millex-GS filter units (Millipore Corp. U.S.A.). If not stated otherwise in the text the protamine solutions were used immediately after preparation.

Media

Phosphate buffer was used as basal medium for the study of bactericidal action under different conditions. Nutrient broth consisting of 0.5% peptone, 0.3% meat extract and 0.2% MgSO₄·7H₂O; Penassy Broth Antibiotic Medium NO. 3 (Difco); Nutrient agar and Tryptose Blood Agar Base (Difco) were routinely used for growth and preservation of the strains.

Initial Suspensions of Bacteria

Initial suspensions of bacteria were prepared from 18 h broth culture either in nutrient or Penassy Broths. The culture was centrifuged and

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the pellet obtained was resuspended in sterilized phosphate buffer and photometrically quantified at 340 nm. A standard plate count was made simultaneously with each experiment.

**Bactericidal Action of Clupeine Sulfate**

The basic principle of the method involved in the study of bactericidal action of protamine was as follows:—the basal medium was distributed in 0.8 ml aliquots in series of tubes of 14 mm x 160 mm. Each aliquot from the series was completed by adding 0.1 ml of adequately diluted protamine solution and 0.1 ml of quantified bacterial suspension. The samples were then incubated at 37°C (if not stated otherwise in the text) in a thermostat water-bath for desired length of period. After incubation viable organisms were estimated by standard plate count as colony forming units and the percent of surviving organisms were calculated. All experiments were accompanied with control samples without protamine. Any modification of the above technique, when made, are discussed in the text.

**Results and Discussion**

**Effect of pH**

The relationship between pH and bactericidal action of clupeine is presented in Table 1. Bactericidal action of clupeine was found to be much higher at neutral and alkaline pH. This result contradicted with a previous report suggesting the higher antibacterial action of protamine in slightly acidic and neutral media.4) Another interesting observation found in the present study was that in acetate buffer (pH 4.0 and 5.0) bacterial ino-
culum in the control tubes were partially killed but at pH 4.0 where most of the organisms killed in the control tubes, a significant number of organisms survived in the protamine tubes. This result suggests that addition of protamine in the acidic media somehow protected some of the organisms from the lethal effect of lower pH (Fig. 1). Considering the overall effect of pH on the bactericidal action of clupeine, it is reasonably suggested that the polycationic nature of protamine due to their high arginine content may partially be altered in the acidic medium, consequently resulting in the decreased ability to combine with the bacterial cell surface. On the other hand, it is quite natural to think that cationic nature of protein is not subject to be changed considerably in the alkaline media because isoelectric point of protamine is reported to be about 12.0.16)

Bactericidal action of protamine also varied significantly at different ionic strength of buffer (Table 2). In 0.01 M phosphate buffer at pH 7.0 the percent survival of organisms was only 0.14 but this figure increased up to 62.5 in 0.5 M buffer at the same pH. From this result it can be assumed that increased concentration of anionic phosphate molecules prevented protamine to reach the reactive cell surface. The same observation on E. coli K-12 has been reported for another arginine rich basic protein histone.8)

**Effect of Temperature**

The data presented in Table 3, indicated that although bactericidal effect of clupeine sulfate is slightly higher at higher temperatures, it is not likely that temperature can influence the bactericidal action significantly. Killing at different temperatures as a function of time are given in Table 4. Killing took place at slightly rapid rate at higher temperature, but at all the temperatures studied most of the organisms were killed within 5 minutes and after that reduction in number was quite insignificant. The technique employed in this study was not sufficiently accurate to establish actual rate of killing in the early course of the reaction, therefore, quantitative relationship between rate of killing and temperature was not established.

**Effect of Metal Ions**

Influence of various metal ions with different ionic strengths on bactericidal action of clupeine sulfate are given in Table 5. Salt concentration of the medium greatly influenced the bactericidal action and the ionic strength of the metal ions was far more important than that of molarity of the salts. Although the effect of molarity on bactericidal action varied depending on the salt used, ionic strength higher than 0.15-0.20 in general led to increased survivability of Bacillus subtilis cells. Mg and Ca ions were more inhibitory than that of Na and K ions. Previous studies have also supported the present finding, which suggested that presence of Al, Mg and Ca ions decreased the efficiency of antibacterial action of protamine.9)

Considering the multilayered cell wall of Gram-positive organism which is composed of peptidoglycan and teichoic acid and stabilized as polyanionic polysaccaride,10,11) it is reasonable to assume that the metal ions may compete with polycationic protamine for binding with reactive cell surface. Recent evidence has suggested that polyanionic polysaccharides from bacterial cell wall can antagonize the bactericidal and bacteriolytic action of protamine.7)

**Effect of Media Containing Organic Matters**

Effect of some common bacterial culture media
on bactericidal action of protamine are presented in Table 6. From these results it can easily be understood that the presence of these organic matters in the media had very little or no influence on strong bactericidal action of protamine. Moreover in some of the media bactericidal action was even stronger than that of in plain phosphate buffer. Only in peptone the bactericidal action was slightly decreased but that seemed to be quite insignificant and ignoreable. The earlier reports of ineffectiveness of protamines in broth media was greatly contradicted with the results of the present study. This can be explained by differences in preparation of protamines, organisms involved and the experimental condition employed. Considering the biochemical nature of protamine it is not unlikely that it may combine with organic substances like nucleic acids, some proteins and anionic polysaccharides and this phenomenon can antagonize the bactericidal action. Some previous evidences supports this view.

Throughout the present study phosphate buffer was used as the basal medium to avoid the undesireable interference by other materials. Although previously a number of studies have been made on antibacterial action of protamine, the mechanism by which protamine kill some particular organisms is not yet clearly known. There are only some speculations that protamine acts like cationic bactericidal detergents and poly-peptide antibiotics, which adsorb on the cell surface, alter the osmotic integrity, and disturb respiration. It is almost clear from the above study that bactericidal action of protamine is more or less substrate dependent, and especially the pH and ionic strength of the media are the most important.

Table 5. Effect of metal ions on bactericidal action of clupeine sulfate

<table>
<thead>
<tr>
<th>Metal ions</th>
<th>Molarity</th>
<th>0.005</th>
<th>0.01</th>
<th>0.05</th>
<th>0.1</th>
<th>0.15</th>
<th>0.2</th>
<th>0.3</th>
<th>0.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>—</td>
<td>—</td>
<td>0.09</td>
<td>0.09</td>
<td>0.12</td>
<td>0.96</td>
<td>5.00</td>
<td>31.40</td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>—</td>
<td>—</td>
<td>0.13</td>
<td>0.15</td>
<td>0.19</td>
<td>1.21</td>
<td>6.28</td>
<td>34.20</td>
<td></td>
</tr>
<tr>
<td>MgSO</td>
<td>0.07</td>
<td>0.19</td>
<td>2.70</td>
<td>35.50</td>
<td>—</td>
<td>80.20</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.16</td>
<td>0.86</td>
<td>4.05</td>
<td>37.50</td>
<td>—</td>
<td>84.00</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.25</td>
<td>1.90</td>
<td>5.58</td>
<td>30.30</td>
<td>—</td>
<td>70.50</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

Initial cell number: 2.0 x 10⁶ cells/ml
Test organism: B. subtilis ruber
Clupeine sulfate: 200 μg/ml in M/100 phosphate buffer (pH 7.0)
Incubation time: 60 min at 37°C.

Table 6. Effect of different culture media on bactericidal action of clupeine sulfate

<table>
<thead>
<tr>
<th>Glucose (0.1 M)</th>
<th>Peptone (1.0%)</th>
<th>Meat extract (0.5%)</th>
<th>Penassay broth</th>
<th>Tryptic soy broth</th>
<th>Heart infusion broth</th>
<th>Phosphate buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.60</td>
<td>0.93</td>
<td>0.08</td>
<td>0.0</td>
<td>0.03</td>
<td>0.06</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Initial cell number: 2.0 x 10⁸ cells/ml
Test organism: B. subtilis ruber
Incubation time: 2 h at 37°C

References
12) O. R. Braekkan and G. J. Boege: Reports on Technological Research Concerning Norwegian
Factors Affecting Bactericidal Action of Clupeine


