Antibacterial Characteristics of Fish Protamines-IX
Effect of clupeine sulfate on the metabolic
activities in *Bacillus subtilis*

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Abstract

Effect of clupeine sulfate (a commercially available protamine from herring milt) on the major metabolic processes in *Bacillus subtilis* was investigated. Clupeine sulfate severely inhibited the *in vivo* synthesis of macromolecules including peptidoglycan, DNA, RNA and protein at both lethal and sublethal concentrations. Clupeine sulfate also decreased the cellular ATP level and inhibited the respiratory activity of the bacterial cell. The above findings indicated that clupeine interfered with the metabolic activities of the cytoplasmic membrane of *Bacillus subtilis*, but inhibition of the synthesis of a specific macromolecule could not be attributed to the antibacterial action of clupeine.

Introduction

Nuclear basic proteins from fish spermatozoan known as protamines are known to possess antibacterial action particularly against Gram-positive organisms and *Bacillus* spores[1-6]. But until now, the mechanism of action of protamine has not been completely elucidated. In our previous studies, we described that fish protamine bind to the bacterial cell wall, change the cellular morphology and release the cytoplasmic materials from the intact cells[7]. It was also reported that, protamine interact with the phospholipid of the bacterial cell membrane[8], increase the membrane-ATPase activity and induce changes in the membrane structure[9].

Interaction between antibacterial agent and the cell membrane and release of cytoplasmic materials are not always the decisive factors in the loss of viability of the cells, unless the extent of damage is such that it is irreversible[10]. Irreversible damage of the membrane is usually accompanied by the destruction of vital metabolic function of the membrane.

In the present paper, we describe the effect of clupeine sulfate (a commercially available protamine from herring milt) on the metabolic processes associated with the cytoplasmic membrane of *Bacillus subtilis*.

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Materials and Methods

Organism and growth condition. *Bacillus subtilis* IFO 3026 was used through-out this study and they were grown in TSB (Trypticase soy broth, Difco) or Heart infusion broth (Difco) at 37°C. Growth condition was modified when necessary and described in the text.

Chemicals. Clupeine sulfate (a protamine from herring milt) was obtained from Wako Ltd. Radio chemicals: [D-^{14}C] N-acetylglucosamine (3.4 m Ci/m mole), [U-^{14}c] thymidine (56.0 m Ci/m mole), [2-^{14}C] uracil (40.0 m Ci/m mole), and [U-^{14}C] phenylalanine (522 m Ci/m mole) were purchased from Amersham (England). [^{32}P] orthophosphate, (carrier free, original specific activity 3 Ci/m mole P) was the product of New England Nuclear (FRG). All other chemicals used were of reagent grade.

In vivo synthesis of peptidoglycan. Incorporation of [^{14}C] N-acetyl-glucosamine into trichloroacetic acid (TCA) precipitable material was studied according to the method described by Uchida and Zahner [11]. *Bacillus subtilis* cells were inoculated into a synthetic medium consisting of 0.3% KH_2PO_4, 0.7% K_2 HPO_4, 0.05% sodium citrate, 0.1% $(NH_4)_2SO_4$, 0.01% $MgSO_4$ and 0.2% glucose. After 20 hr incubation at 37°C, a fresh medium was inoculated with this suspension and culture continued until exponential growth was achieved (optical density 0.2 at 600 nm). [^{14}C] N-acetylglucosamine (0.1 μ Ci/ml) was added to this growing culture (30 ml) and incubatd at 37°C for 15 min. The culture was then split into three equal portions, one culture served as control and different concentrations of clupeine were added to the remaining two portions. Culture samples (0.1 ml) at intervals were added to 10% TCA (5.0 ml), whirled, placed on ice for 30 min and finally heated at 90-95°C for 20 min. On cooling, each was filtered on a membrane filter (0.45 μm), washed successively with 5% TCA (3×5 ml) and ethanol (2×10 ml), air dried and counted in 5 ml of toluene (containing 0.6% butyl-PBD and 5% nepthalene) in a liquid scintillation spectrometer (Beckman LS-230).

DNA and RNA synthesis. Incorporations of [^{14}C] thymidine and [^{14}C] uracil into TCA precipitable material with or without clupeine were measured as described above with the following inclusions: culture sample (1.0 ml) was added to 2% sodium lauryl sulfate (1.0 ml), whirled and placed in ice for 30 min, 5% TCA added to each and the samples left for a further 30 min before filtering etc. as above.

Protein synthesis. Incorporation of [^{14}C] phenylalanine into TCA precipitable material was measured by following the same procedure described for peptidoglycan synthesis.

Stability of cellular DNA. Effect of clupeine on the intracellular DNA prelabelled with [^{14}C] thymidine was examined as described by Das et al[12]. Exponentially growing *Bacillus subtilis* culture was supplemented with 0.1 μ Ci/ml of [^{14}C] thymidine. Isotope labelled cells were treated with clupeine or saline (control) and then extracted with TCA. Radioactivity retained in the precipitate and filtrate was determined by radiocount.

Determination of cellular ATP level. The [^{32}P] orthophosphate exchange method of Cashel[13] as described by Ogilvie et al.[14] was used for the incorporation of [^{32}P] orthophosphate into cellular ATP. Aliquots of exponentially growing cultures were added with clupeine and [^{32}P] orthophosphate, samples without

clupeine were served as control.   After the incubation, cells were extracted with formic acid and supernatants spotted on polyethyleneimine-cellulose (PEI-cellulose) TLC plates (Merck) and eluted with 1.5 M KH_2PO_4 (pH 3.4).   [^{32}P] ATP spots were located by autoradiography, removed and radiocounted.   The R_f value of the ATP (0.8) was identical to that quoted by Cashel[13].

Determination of oxygen uptake by Warburg respirometry.   To a series of Warburg flasks, 1.0 ml of washed suspension of *Bacillus subtilis* (18 hr culture) in 0.1 M phosphate buffer (pH 7.2) containing 1.0 mg dry weight cells/ml, 1.0 ml of 0.06 M glucose and 0.5 ml of clupeine solution containing 500 μg protamine were added. Oxygen consumption was recorded every 10 min in the water-bath at 37°C.   The data obtained were plotted as microliter of oxygen consumed.

Results

The in vivo peptidoglycan synthesis

Incorporations of [^{14}C] N-acetylglucosamine in the cell wall of a growing culture of *Bacillus subtilis* with or without clupeine were measured in the TCA precipitable material.   Results given in Fig. 1 show that almost immediate inhibition of radioactive incorporation was occurred after the addition of clupeine. Effects of 100 μg/ml and 200 μg/ml clupeine were almost same and at both the concentrations peptidoglycan synthesis practically stopped within 5 min after the addition of protamine.   These concentrations were bacteriostatic and bactericidal concentrations of clupeine against *Bacillus subtilis*.

In vivo synthesis of DNA, RNA and protein

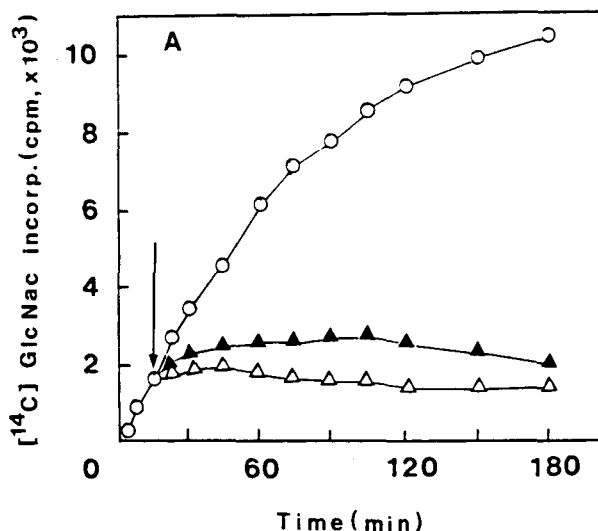Incorporations of [^{14}C] thymidine, [^{14}C] uracil and [^{14}C] phenylalanine into
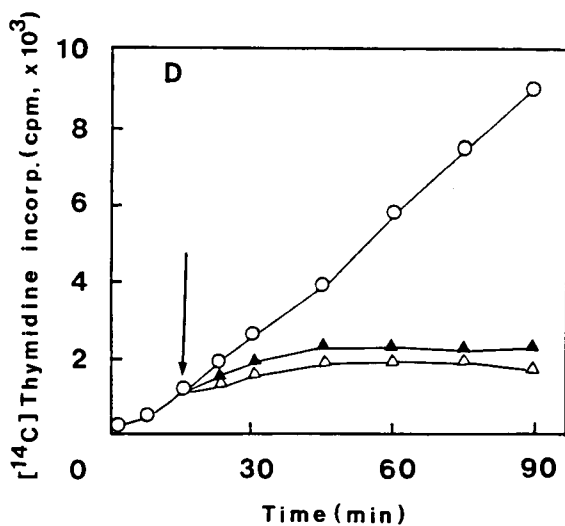
Fig. 1.   Effect of clupeine on the peptidoglycan synthesis in *B. subtilis*.
○: Control; ▲: clupeine 100 μg/ml; △: clupeine 200 μg/ml.   Arrow indicates the addition of clupeine.

Fig. 2. Effect of clupeine on the DNA synthesis in *B. subtilis*.
○ : Control ; ▲ : clupeine 100 μg/ml ; △ : clupeine 200 μg/ml. Arrow indicates the addition of clupeine.

the respective macromolecules were measured. The effects of clupeine on the synthesis of these macromolecules are presented in Figs. 2, 3 and 4. The incorporation of labelled materials were inhibited simultaneously within 5-10 min by 100-200 μg clupeine/ml.

In order to confirm the inhibition of macromolecular synthesis by clupeine rather than degradation of cellular macromolecules, the stability of cellular DNA was investigated. As shown in Fig. 5, clupeine did not induce degradation of DNA prelabelled with [^{14}C] thymidine. At 45 min exposure to clupeine, the amount of
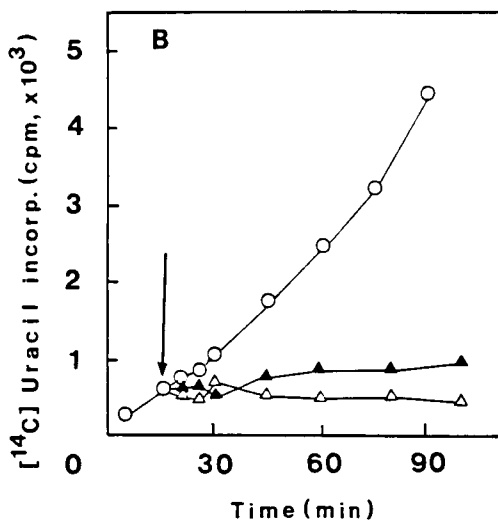

Fig. 3. Effect of clupeine on the RNA synthesis in *B. subtilis*.
○ : Control ; ▲ : clupeine 100 μg/ml ;
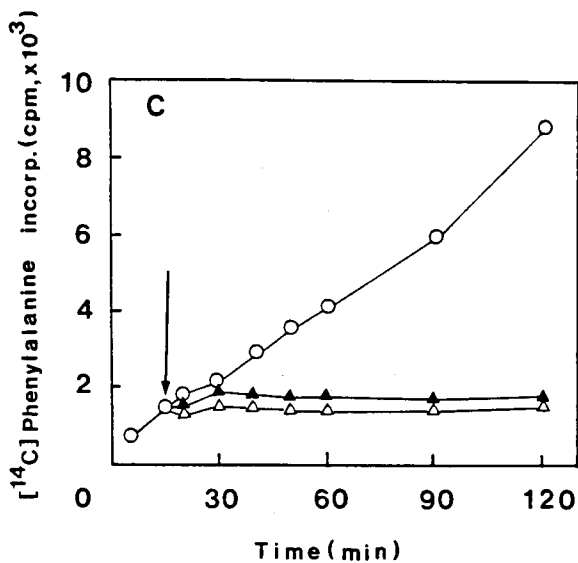△ : clupeine 200 μg/ml. Arrow indicates the addition of clupeine.

Fig. 4. Effect of clupeine on the protein synthesis in *B. subtilis*.
○ : Control ; ▲ : protein 100 μg/ml ; △ : protamine 200 μg/ml. Arrow indicates the
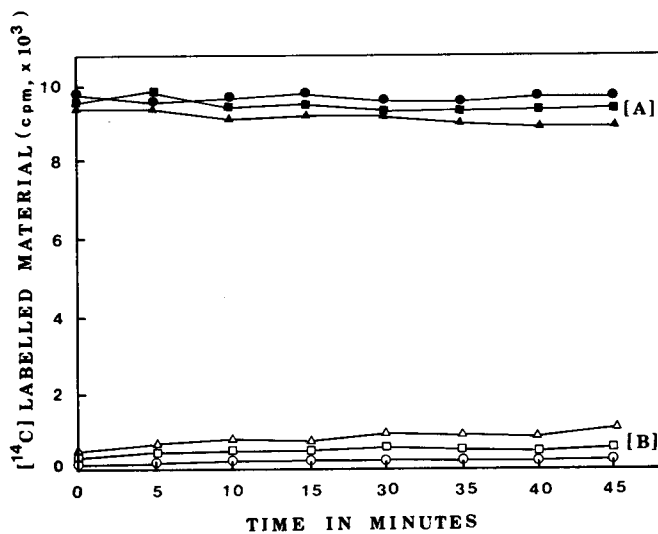addition of clupeine.

Fig. 5. Effect of clupeine on the stability of cellular DNA.
A) Thymidine in TCA-precipitable fraction of cells. ● : Control ; ■ : clupeine 100
μg/ml ; ▲ : clupeine 200 μg/ml.
B) Thymidine in TCA-soluble fraction of cells. ○ : Control ; □ : clupeine 100 μg/
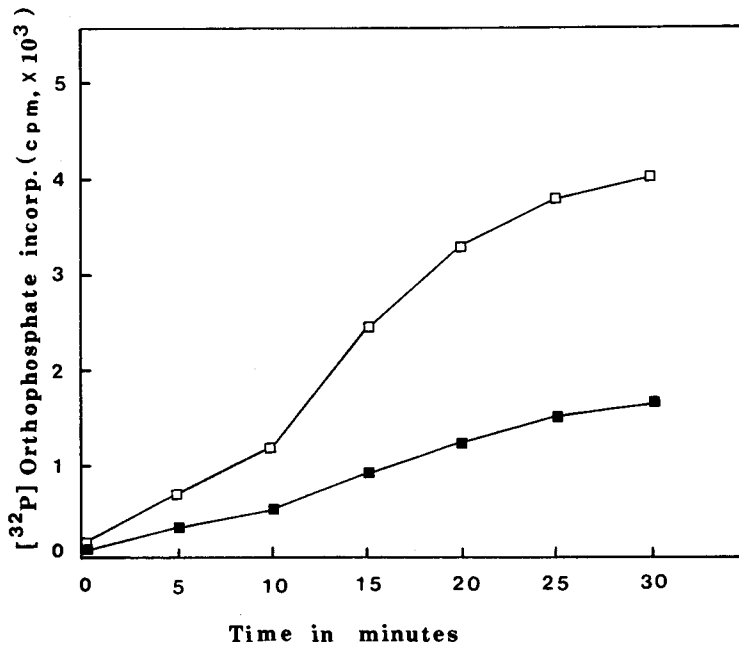ml ; △ : clupeine 200 μg/ml.

Fig. 6.   Effect of clupeine on cellular ATP levels in *B. subtilis*.   □ : Control ; ■ : clupeine 200 μg/ml.
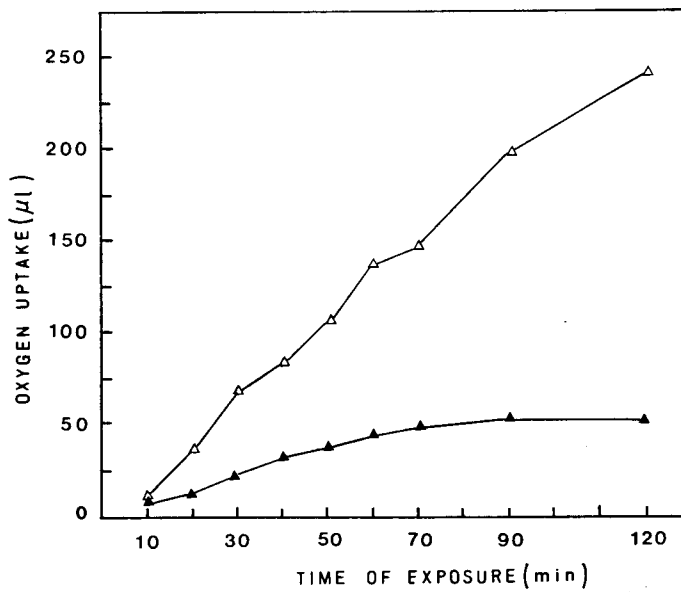

Fig. 7.   Effect clupeine on the respiratory activity of *B. subtilis*.   △ : Control ; ▲ : clupeine 200 μg/ml.

radioactivity remaining in TCA precipitable fraction was almost same as that of control. This result suggested that the apparent decrease of ^{14}C incorporation by clupeine was not due to degradation of DNA but due to inhibition of synthesis.

Effect of clupeine on cellular ATP level

Incorporation of [^{32}P] orthophosphate in the cellular ATP was measured in presence or absence of clupeine. Fig. 6 shows that at a concentration of 200 μg clupeine/ml cellular ATP level was considerably decreased to about 40% of the control level within 30 min.

Effect of clupeine on respiratory activity

Oxygen consumption by *Bacillus subtilis* cells in presence or absence of clupeine was determined by a Warburg apparatus. As shown in Fig. 7, immediate inhibition of oxygen consumption by bacterial cells treated with clupeine was observed starting within 10-15 min of incubation. The bacterial suspension without clupeine continued to consume oxygen in an almost linear fashion with respect to time. The respiratory activity of the treated bacteria continued at a reduced rate for 90 min and then stopped.

Discussion

In a previous report, we described that clupeine interacts with the cell membrane particularly with the phospholipid component of the membrane[8]. From the experimental results of the present study it is evident that clupeine deeply interferes with the metabolic processes associated with the cell membrane of *Bacillus subtilis*.

Addition of clupeine to a growing culture of *Bacillus subtilis* rapidly inhibited the incorporation of ^{14}C substrates in the macromolecules. Inhibition of cell wall (peptidoglycan) biosynthesis would certainly be sufficient to eliminate further bacterial growth. But, our previous studies showed that clupeine rapidly lysed the protoplasts which are devoid of peptidoglycan[8]. From this observation it can be assumed that synthesis of this polymer cannot be a prerequisite for the antibacterial action of clupeine. In addition, simultaneous inhibition of DNA, RNA and protein synthesis by clupeine indicated that inhibition of any specific macromolecule synthesis cannot be attributed to the bactericidal effect of clupeine. More than one reason could be considered for the inhibition of macromolecule synthesis, first of all, interaction between clupeine and cell membrane components may result in the change of membranous environment for the normal metabolic activities of the membrane. Moreover, loss of cytoplasmic constituents[7] vital for the synthesis process may also partially contribute to the inhibition of macromolecular synthesis. The possibility of a degradation of cellular macromolecules by clupeine was also investigated, but it was found that clupeine did not cause degradation of cellular DNA, therefore, it can be assumed that degradation of intracellular material by clupeine was not the cause of inhibition of macromolecular synthesis.

So far, no report has been published on the inhibition of bacterial macromolecular synthesis by protamine, but inhibition of nucleic acid and protein synthesis in eucaryotic cell has been reported earlier[16,17]. Decrease of cellular ATP content and inhibition of respiratory activity of *Bacillus subtilis* by clupeine

suggested that clupeine may interfere with the oxidative phosphorylation and energy processes dependent on it. Known uncouplers of oxidative phosphorylation like 2,4-dinitrophenol[18] has been reported to reduce the ATP content of the *Staphylococcus aureus* cells[19]. Popinigis et al.[20] suggested that protamine sulfate (from salmon) affects mitochondrial metabolic processes as an inhibitor of energy transfer. It has also been proposed that protamine reacts with the mitochondrial membrane and inhibits proton back diffusion into the mitochondria which was followed by inhibition of respiration in isolated mitochondria[21]. In bacteria lacking mitochondria condition may be different and much ambiguous than the eucaryotic cells. Harold[15] pointed out that respiratory chain linked to the ATP generation is apparently obligatorily associated with cytoplasmic membrane structure of bacteria. It is well established that dehydrogenases and the electron carriers of respiration are found in the membrane after disintegration of the cell. The above evidences indicate that clupeine-membrane interaction could interfere with the energy system of *Bacillus subtilis* which eventually would result in the shortage of energy supply for macromolecular synthesis and other energy requiring processes. It is to be noted that action of clupeine on the cell membrane may permit passage into the cell of ions that are normally excluded, and under some conditions this may cause uncoupling of oxidative phosphorylation.

In the present study, it was observed that both bactericidal concentration (200 μg/ml) and a sublethal or bacteriostatic concentration (100 μg/ml) of clupeine inhibited the incorporation of ^{14}C substrates in the macromolecules. This observation suggested that inhibition of macromolecular synthesis do not necessarily require a concentration of clupeine which induce bactericidal effect and supports the view that inhibition of macromolecular synthesis may not be the primary cause of bectericidal action of clupeine.

The data presented in this paper and in our previous papers[7-9] suggest that this nuclear basic protein primarily interact with the cytoplasmic membrane of the *Bacillus subtilis* and subsequently destroy the metabolic function of the membrane as the secondary effect. Therefore, inhibition of the synthesis of a particular cellular macromolecule could not be assigned for the cause of antibacterial action of clupeine, but the overall effect of clupeine on the bacterial membrane is responsible for the antibacterial action of this basic protein.

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