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CEPHALOSPORIN β-LACTAM GROUP OF ANTIBIOTIC DRUG RESPONSE STUDY AGAINST DIFFERENT TIME FRAME USING HPLC TECHNIQUE

Manish Sharma[1], Nitesh Chandra Mishra[2] and Kiran Singh[2*]

[1]Department of Microbiology, Triveni Dental College& Research Centre, Bilaspur,C.G., India.
[2]Department of Biological Science and Engineering,
Maulana Azad National Institute of Technology (MANIT), Bhopal, M.P., India
*Corresponding authors: Email: researchnm.kiran@gmail.com,
Tel:+91-9893338285, +91-9165461492,

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ABSTRACT:

The study aims at specific characteristic of antimicrobial agents as microbicidal and microbiostatic. Some antibiotics are more selective than others with respect to the bacterial species, which they inhibit. In narrow spectrum, antibiotics may be targeted at a particular pathogen for e.g. a particular bacterial species. In contrast broad-spectrum antibiotics inhibit a relatively wide range of bacterial species, including both gram- positive and gram-negative types. The mode of action of β-lactam antibiotics involves the inhibition of peptidoglycan biosyntheses. During the inhibition process these β-lactam antibiotics binds to specific proteins located on the cytoplasmic membrane or in the periplasmic space between the inner and the outer membrane of the gram negative bacteria. These specific proteins are popularly known as penicillin binding proteins (PBP's). These proteins are evolved in transpeptidase, transglycocylase, D-D-carboxypeptidases and endopeptidases activities. This study tried to reveal the expression of different known or unknown proteins being expressed during the process of resistance.

Keywords: Cephalosporins, Ceftriaxone sodium, Cefazolin sodium,EDTA,HPLC and*Bacillus subtilis*

[I] INTRODUCTION

In contrast to the optimal growth conditions in the laboratory environment, cell spends most of their lifespan in stress full environmental conditions in their natural ecosystem, the soil. To cope with this stress, this bacterium has developed a very complex adaptation network inside which the induction of general and unspecific stress proteins seems to be very important component [35]. The cell wall of this bacterium contains highly electronegative polymer PG corresponding its 40 layers to one layer of *Escherichia coli*, that is 1nm thick and glycerol – based teichoic acid to produce a net negative charge with high metal binding capacity. During metabolism, a membrane induced proton motive force continuously pumps protons into the wall – fabric. As a result, a competition between protons and metal ions for anionic wall sites

occur and less metal is bound in living cells than in non-living cells or those in which the plasma membrane has been uncoupled. However, when *Bacillus subtilis* cells are grown in the presence of phosphate, its high density electronegative sites within its wall gives it affinity for metals and its ability to form metal precipitates [54]. Therefore, there is some selectivity for cations, and the wall on *Bacillus subtilis* becomes an especially good sink for protons (H^+). Although this work is based on one microorganism it seems logical to presume that the results can be applied in a general way to all bacteria which shells themselves in anionic cell wall polymers. *Bacillus subtilis* known to undergo sporulation in response to environmental stress and nutrient limitations[40]. The complex structure spore coat is composed of a dozen or more polypeptides that are arranged in an electron dense outer layer and a lamellar inner layer. These layers protect the spore from certain bactericidal agents, and play a role in the responsiveness of the endospore to germination.

Genomic study can provide very little information with respect to deciphering how microorganisms adapt to constantly changing environment. While the presence of certain genes, characterized by their sequence identity to a bio-chemically characterized database homologue, suggests an organism may have the ability to adapt to certain ecological niches and utilize various substrates. The knowledge of protein expression under these conditions is essential to fully understand how the organism responds to a given challenge. The global analysis of protein expression from a genome under a given set of conditions is best summarized, by the term 'proteomics'. The techniques or proteomics (2-DE and protein characterization by HPLC, MS) are widely used for microbiological research changes in outer membrane proteins have been investigated using proteomic approach [45]. To get the identity of a particular protein (eg. Mol. Wt.) Liquid chromatography (LC) based systems is more conclusive as it leaves the protein sample in solution instead of being bound in the matrix. On subjecting the microorganism like *Bacillus subtilis* to an antibiotic stress, some changes in the cellular and membranous proteins are expected. To identify and characterize the proteins (cellular or membranous) which are expressed as a result of the antibiotic (CT, CZ) stress, HPLC analysis will be done. Various combinations of size exclusion Chromatography, HPLC based reverse phase chromatography has been used for separation of complex cellular lysates[49]. The RP-HPLC separation of any peptide or protein mixture is dependent upon the strength of the hydrophobic interactions of each component in the mixture with the hydrophobic surface of the column matrix and the elution strength of the organic solvent in the mobile phase, as the concentration of the organic solvent increases, the interactions between the peptides or proteins and the column matrix are diminished, and elution of the polar species occurs first followed by the elution of non-polar species. Peptide or protein mixtures are applied an RP-HPLC column containing a chromatographic matrix with defined hydrophobic character. The adsorbed peptides or proteins are eluted in order of least to most strongly bound molecules by increasing the organic solvent concentration in the elution buffer. The only drawback of RP-HPLC lies in the fact that inspite of high recovery of proteins the molecules are donated and thus becomes difficult to recover in biologically active form as well as high mass yields due to irreversible precipitation of the column.

[II] MATERIALS AND METHODS

Bacillus subtilis NCIM 2063 strain used for the present study is a non pathogenic Gram- positive rod, and obligate aerobe (Plate 01: a; b). It is

known to form protective end spore thereby providing tolerance to extreme environmental conditions. It was obtained from National Chemical Laboratory (NCL) Pune, India. Chemical as well as reagents used in the present experiments were of reagent grade. However, antibiotics preferred for the experimental purpose for eg. Ceftriaxone (CT) sodium and Cefazolin (CZ) sodium were procured from Lupin Laboratories Ltd. M.P., India under the common generic names.

During experimentation *Bacillus subtilis* cultures were grown in Erlenmeyer flask containing presterilised growth medium. Incubation was done at 37^0C ± 0.1 in thermostatically controlled orbital shaker (Lab India, Model, 3521) under aerobic condition with plate rotation of 180 revolutions per minute.

2.1 Protein Assay:

Protein concentration in the supernatant (released) was estimated by Lowry's (1951) and ultraviolet absorption method (1951) (Warburg and Christian., 1941).

2.1.1 Lowrey's Method:

Protein estimation was done by the method of (Lowry et. al., 1951) as detailed for which following reagents were used.

2% Na_2CO_3 in 0.1 N NaOH (a) 0.5% $CuSO_4.5H_2O$ in 1% sodium or potassium tartarate (b) 50 ml of reagent (a) plus 1.ml of (b); 1N Folin – Ciocaleten phenol reagent; 100 µg/ml lysozyme solution. The standard curve for Lysozyme is obtained by plotting optical density against concentration. The graph obtained is a straight line with the aid of which the concentration of an unknown solution can be readily determined. The optical density and the absorbance is defined as the O.D. of the solution of unit concentration kept in the cell of unit length. Energy is absorbed by all components depending upon their chemical characteristics. The amount of light energy absorbed by a solution depends upon the intensity of the color of the solution. At particular wave length the absorption is maximum and these are called - maximum and absorption maximum. The absorptions is governed by 2 laws:-

1. Lambert's Law: It states that the amount of light transmitted through a solution depends on the distance of the light path.
2. Beer's Law: It states that the amount of light absorbed in 1 Unit thickness is proportional to the concentration of the absorbing particles. It can be shown as follows:-

$$\log \frac{Io}{I} = \log \frac{I}{T} = KtC = \text{optical density}$$

I = intensity of Incident light

Io = intensity of transmitted light

T = transmittance – the distance through which the light passes

C = Solution concentration

K = Extinction coefficient

Protein was estimated at different phase of growth in the presence and absence CT and CZ by the ultraviolet absorbance method[56]. Absorbance measurements were made at 280 and 260 nm. If the ratio of absorbance at 280 nm and 260 nm is not greater than 1.70, except with solutions known to contain pure protein, the equation used to calculate the concentration of protein is as follows:

Protein concentration (mg/ml^{-1}) = 1.45 A280 – 0.74 A260

The equation is used to subtract the contribution of nucleic acids to the ultraviolet absorbance at the solution [38] .

Bacillus subtilis NCIM 2063 cells were grown in Dyes minimal medium and incubated at 37^0C. The concentration of inoculum used (0.5% v/v) was suspended in 1 ml of the minimal medium. The cells were harvested at exponential growth phase and treated with CT and CZ, separately. Aliquots (3ml) were aseptically removed from the flasks at hourly intervals and placed in a 1 cm

path length cuvette of the spectrophotometer. The absorbance was taken at 280 nm and 260 nm with UV-VIS spectrophotometer (Shimadzu 1610) against a medium blank.

The protein assay was also performed by treating the cells of log phase under the influence of various physiological stress parameters such as pH (5.0, 7.0, 8.0), Temperature (25^0C, 37^0C, 50^0C), EDTA (0.25 mM, 0.5 mM and 0.75 mM) Mg^{2+} ions (5mM, 10mM, 15mM). EDTA, Mg^{2+}ions combination (1+0.25, 1+0.75,15+0.25,15+0.75). The experiments were performed with and without CT and CZ respectively. 3 ml aliquots were then read at 550 nm with UV-VIS spectrophotometer (Shimadzu 1610).

2.2 Assay of protein profile using HPLC:

Confirmation of stress proteins as evident by the presence of β-lactams , CT and CZ was done by the HPLC (Shimadzu model LC-10AT) for the study of molecular weight determination of proteins found in the sample Bovine serum albumin (67 kDa), Ovalabumin (45 kDa), Chymotrypsinogen (25 kDa), Cytochrome (12.5 kDa) and Insulin (0.57 kDa) was used in pure form. The migration of protein in the column was observed when 25 µl of protein samples were injected. The proteins show different rate of movement due to their different molecular size for every sample 4000 psi pressure was maintained and flow rate was adjusted to 1.0 ml min-1 the solid phase was protein-PAK column (Shimadzu) and mobile phase was 0.1 M phosphate buffer (pH 6.8). The proteins were measured at absorbance of 280 nm after carrying out elution process for 30 minutes. Protein –PAK column (7.8 mmx30 cm, total permeation volume of 12 ml) provide rapid separation, purification and characterization of proteins using gel filtration. These columns are packed with a rigid hydrophobic porous silica gel and are manufactured with exclusive bonding processes that improve column stability and minimize non-specific adsorption. HPLC technique offers advantages of speed and specificity and uses equipment that is versatile in its operation. Column is the essential feature of HPLC. The column allows high resolution at speedy flow rate. HPLC offers advantages in speed and ease of sample recovery. Therefore, it offers greater advantage over electrophoresis and open column chromatography. The column Chromatographic analysis helps in separation and identification of biological samples. Allowing the sample to move in a column containing partitioning material and eluting the mixture by pumping the solvent through the column do this.

[III] RESULTS

The transport / intake of antibiotics including β-lactams across the bacterial outer surface to cell interior plays an important role with respect to susceptibility of *Bacillus subtilis*. When HPLC studies were performed under such experimental conditions molecular property of bacterial cell wall reflects the size of proteins present in the supernatant at pH 5.0, 7.0 and 8.0 **(Fig. 3 .1; 3.2; & 3.3).**

Fig: 3.1: Molecular weight determination of protein released in supernatant of intact cells of *B.subtilis* in the presence of antibiotic stress at pH 5.0 as monitored by HPLC. (--- without antibiotic,-x-x- presence of CT and presence of CZ)

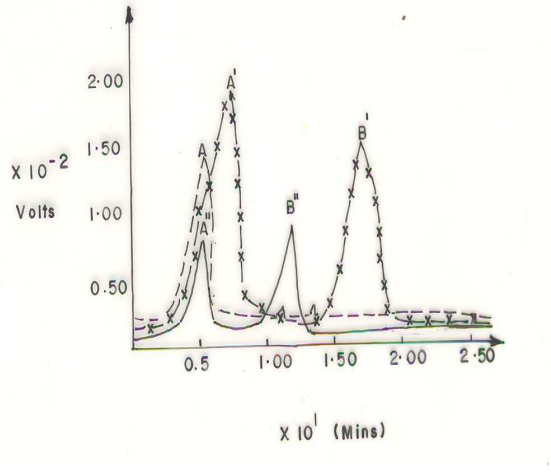


Fig: 3.2: Molecular weight determination of protein released in supernatant of intact cells of *B.subtilis* in the presence of antibiotic stress at pH 7.0 as monitored by HPLC. (---without antibiotic,-x-x- presence of CT and presence of CZ)

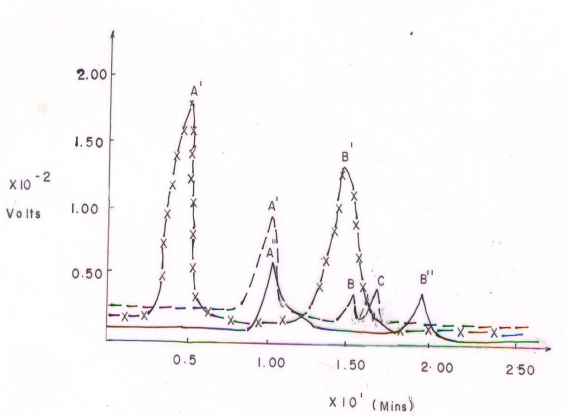
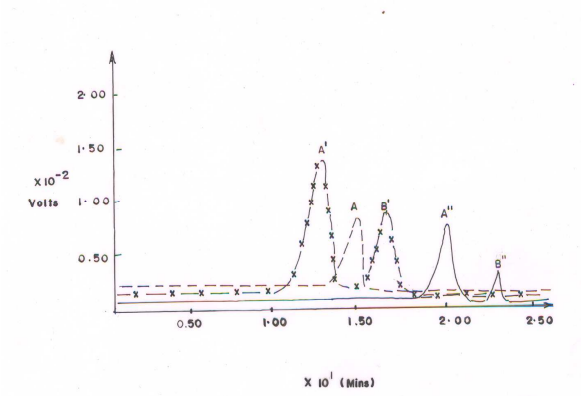


Fig: 3.3: Molecular weight determination of protein released in supernatant of intact cells of *B.subtilis* in the presence of antibiotic stress at pH8.0 as monitored by HPLC. (---without antibiotic,-x-x- presence of CT and presence of CZ)



The molecular size of protein observed at pH 5.0 in the absence of β-lactam at MIC_{50}. Only one species of protein was seen **(Fig. 3.1).** As far as, speciation of protein leakage is concerned CT, caused release of protein (A) after 5 mins which falls under category of protein smaller than 5.7 kDa, the known mol. Wt. Of Insulin indicated in standard **(Fig. 3.1).** With CT the two peaks A' and B' were visible after 6.0 and 17 mins approximately and both fall below 67 kDa, the known mol. wt. of Bovine serum albumin indicated in standard **(Fig. 3.1).** At pH 7.0 in the presence of CZ two species of proteins were visible A" and B" and all appear before 20.0 mins of flow peak C was seen after 15 mins with reasonably low resolution therefore cannot be predicted as a component of *Bacillus subtilis* associated with membranes and thus available in washed form in the supernatant. In the presence of CZ the first peak appeared after 20 mins, where as CT caused leakage of membrane proteins even after 12 mins of exposure peak A' **(Fig. 3.3).**

In case of intact cells of *Bacillus subtilis* the protein released in the medium when cells were subjected to EDTA stress was found to be more in the presence CZ (MIC_{50}). However the pattern of protein released from membrane vesicles prepared from *B.subtilis* showed maximum protein in the suspended medium in the absence of EDTA (0.25mM).

The type of proteins when determined by HPLC shows no remarkable variation in appearance of peaks after withdrawal of sample from 30 mins of incubation **(Fig. 3.4 and 3.5).**The only reason of similar pattern of appearance of protein peaks might be due to involvement of EDTA only at outer surface not at molecular level.

Fig: 3.4: Molecular weight determination of protein released in supernatant of intact cells of *B.subtilis* in the presence of antibiotic stress at pH8.0 as monitored by HPLC, in the presence of 0.25mM EDTA after 30 mins of incubation. (---- ---- without antibiotic,-x-x- presence of CT and presence of CZ)

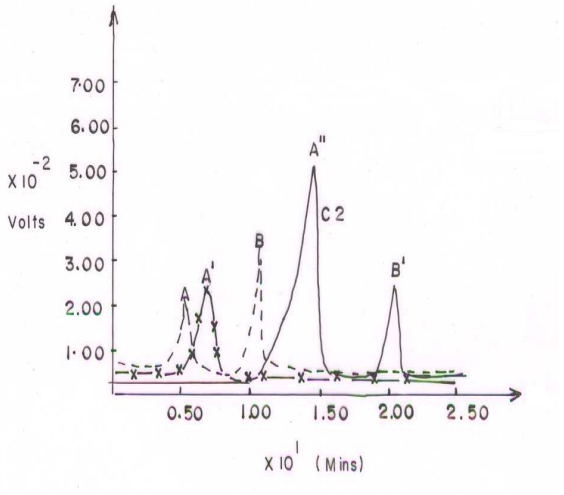
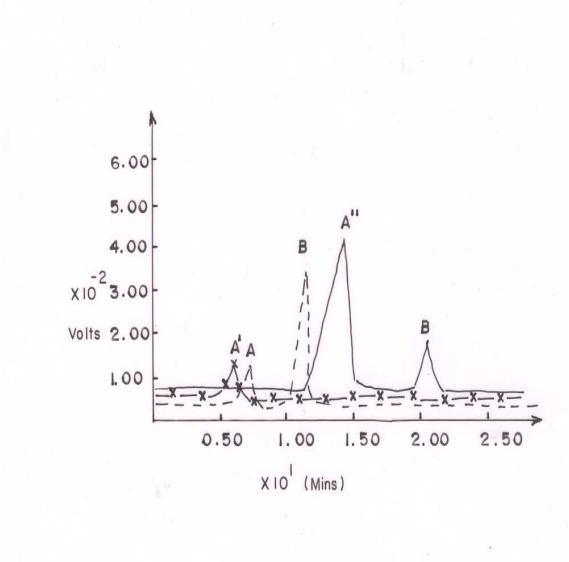


Fig: 3.5: Molecular weight determination of protein released from membrane vesicles of *B.subtilis* suspended in Tris-HCL buffer in the presence of antibiotic stress at pH8.0 as monitored by HPLC. (-------- without antibiotic,-x-x- presence of CT and presence of CZ)



[IV] DISCUSSION

No much effect on pattern of inhibition of growth by chelation (EDTA, 0.25 mM) contrary to, that the protein released from the intact cells when *Bacillus subtilis* was grown in the presence of EDTA (0.25 mM) protein leakage in the exterior of the cell was potentiated by CZ. Therefore, from the present study it could be inferred that the toxicity of CT as observed greater than CZ was not due to washing of membrane proteins but alteration of intake (uptake) pattern regulated by cellular transport mechanism. Similar to EDTA, Mg^{2+} ion alone was found to enhance protein release even at 5mM concentration up to 15 mM in the presence of CZ not in the absence, of β-lactam. However, the absence of Mg^{2+} ions in the growth medium caused similar pattern of protein release into the exterior of the cells. The resolution analysis of proteins in the presence of 1.0 mM Mg^{2+} and 0.25 mM EDTA shows appearance of protein peak after 20 mins of sample run in the column showing presence of different molecular configuration of protein greater than 67 kDa when referred with standard curve **(Fig. 3.1).**

The Gram-positive bacteria, *Bacillus subtilis* when subjected to antibiotic stress the profile of protein as studied in the presence of Ceftriaxone (CT) and Cefazolin (CZ) β-lactam antibiotic stress reflects variation in protein profile by HPLC studies. Similarly *Listeria monocytogenes* Gram-positive bacteria cells has been reported to have acid shock with HCL had significantly greater heat resistance when compared with non-acid-shocked cells[23]. Studies with Gram-negative *Escherichia coli* have shown that a shift to a lower pH induces the synthesis of at least four heat shock proteins [40].It has been shown that acid induced death is the direct result of lowered pH_i [25]. Severe acidic pH creates a situation whereby protons leak across the membrane faster than housekeeping pH homeostasis (the ability of an organism to maintain its cytoplasmic pH at a value close to neutrality, despite fluctuations in the external pH) systems can remove them. The result is an intracellular acidification to levels that damage or disrupt key biochemical processes [13]. Weak acids in their unprotonated form can diffuse into the cell and dissociate thereby lowering the intracellular pH (pH_{in}) and resulting in the inhibition of various essential metabolic and anabolic processes. In response to encounters with acids, microorganisms have evolved complex inducible acid survival strategies [4].

The gradual susceptibility of *Bacillus subtilis* to CT and MIC_{50} (1.5 ppm) in contrast to CZ MIC_{50} (18 ppm) appears to be differential selectivity of bacterial membrane to both the β-lactams even at neutral pH / the sensitivity to drugs was more pronounced at alkaline pH. The integrity of membrane proteins in the influence of CT reflects valuable amount of release of protein **(Fig. 3.2)**, after 30 mins of run shows reason for susceptibility to β-lactam. In the present study the

availability of proteins under stress was seen to be modified under various physiological conditions. The appearance of drug resistance in therapeutic purposes under treatment conditions might be responsible for induction of variable protein species under clinical procedures. The appearance of proteins in the medium cannot be confirmed by present investigation that they were genetically induced by β-lactam antibiotics.

[V] CONCLUSION

The ATPase activity when recorded under varying physiological conditions reflects modulation in enzyme activity as estimated in membrane vesicles derived from *Bacillus subtilis*. The invented vesicles showed ATPase activity under controlled conditions i.e. pH 7.0 in the absence of any stress and measured as 630 $\mu gPi.mg.protein^{-1}.min^{-1}$ Mg^{2+} ions at concentration of 1mM at pH 7.0 reduced ATPase activity and only 380 $\mu gPi.mg.protein^{-1}.min^{-1}$ was recorded. In contrast CT and CZ alone at pH 7.0 was capable of showing 160 $\mu gPi.mg.protein^{-1}.min^{-1}$ and 220 $\mu gPi.mg.protein^{-1}.min^{-1}$ ATPase activity in inverted vesicles of *B.subtilis*. The large number of cephalosoporins makes a system of classification, which is based upon chemical structure, clinical pharmacology; resistance to β-lactamase, the well-accepted system of classification by "generation" is acceptable. Although fourth generation-Cefapine is already available offering more resistance to β-lactamase but the existing third generation cephalosporin selected from the study is not been rejected by the system. The development of resistance although considered to be genetic in bacterial system, the resistance gets reported soon after lack antibacterial agent enters into clinical practice. The present study throws some light, which may help to predict future needs in study of infectious diseases as several unpredictable consequences promote the establishment of unrecognized infections. The results of HPLC helped us in reaching to the conclusion that during various stresses applied on *B.subtilis* resulted in to a significant change in protein release.

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