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Binding studies of a universal transcriptional metallo-regulator, DtxR, from *Corynebacterium diphtheriae* with divalent cations

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Abstract
Physiological role of iron metal is well documented as it is essential as a co-factor for the enzymes involved in the basic bio-chemical and metabolic reactions necessary for the microorganism like cellular respiration, as a part of the components of electron transport chain and for the enzymes involved in DNA replication. Micro-organisms have in place eloquent methods for iron metal uptake. Microorganisms employs a battery of iron, Fe^{2+}, sensing genes, which are essential for quenching, acquiring and assimilating iron metal from the micro-organisms milieu and thus, important for surviving successfully inside the host. The DtxR protein family is a widely established family of transcriptional regulators influencing transcription of other essential genes too. Transcriptional regulators from DtxR family are strictly iron metal dependant, though in-vitro, these metallo-regulators are known to bind to other divalent cations too. DtxR, metallo-regulator was first isolated from *Corynebacterium diphtheriae*, where it influences the synthesis of diptheriae toxin gene, *tox*, in the presence of iron metal. DtxR is a universal transcriptional regulator present in other micro-organisms too. The present study was designed to amplify, express, purify the DtxR protein and study it's binding characteristics with various divalent cations through bio-physical technique. Controlling iron levels inside the micro-organism is very essential as its excess leads to the oxidative stress and deficiency impedes micro-organisms survival and both the conditions are deleterious for the micro-organism.

Keywords: Metallo-regulator, hydrodynamic radius, stokes-einstein equation, iron, manganese, magnesium

1. Introduction
Iron metal is indispensable for the survival of the micro-organism, as it acts as co-factor in various essential bio-physiological and metabolic processes, like respiration, replication, photosynthesis and N_2 fixation. On the other hand, iron being capable of inducing Fenton's reaction, is toxic, because of it's ability to form active species of oxygen, thus destroying the bio-molecules like lipids, protein and DNA. Thus, iron metal uptake has to be exquisitely regulated to maintain the intracellular concentration of the metal [1]. Free form of iron metal in the form of Fe^{2+} is very limited, because of it's ability to undergo rapid oxidation to Fe^{3+} ion in the presence of oxygen. Hence, many micro-organisms secrete high affinity iron chelators, known as siderophores, in order to extract iron from the environment. The synthesis of siderophores, their internalization and storage of iron metal inside the micro-organism is accomplished by a battery of genes controlling acquisition, assimilation and storage of iron metal inside the micro-organism [2]. The whole physiology of iron metal metabolism is under the control of iron dependent metallo-regulators. These metallo-regulators are proteins influencing iron metal homeostasis, by acting as transcriptional regulators. These transcriptional regulators modulate expression of various genes involved directly or indirectly in iron metal uptake from the surrounding, according to the iron metal deficiency or iron metal abundance inside the micro-organism [3]. Both gram-positive bacteria and gram-negative bacteria are equipped with efficient and unique metallo-regulators, which though distantly related serve the same purpose of influencing iron metal uptake. In gram-positive bacteria such as *Corneybacterium*, *Streptomyces* and *Mycobacteria* metabolic iron balance is under the control of a metallo-regulator protein known as DtxR. Diptheria toxin regulator (DtxR) was first described in *Corynebacterium diphtheriae* [4, 5]. DtxR family which contains IdeR and SirR metallo-regulators, have been enumerated as global gene regulators. DtxR protein binding to iron, establishes necessary conformational changes in the protein, making it vulnerable to bind to the "Iron box" upstream of the gene, thus influencing the binding of the

RNA polymerase to the cognate promoter and hence up-regulating or down-regulating the expression of the downstream genes, depending upon it's availability inside the micro-organism [11, 13]. In gram-negative bacteria iron regulation is carried by other universal iron metal ion regulator, Fur protein, ferric iron uptake regulator. Fur like protein regulators have also been reported in the low GC content gram-positive bacteria like *Bacillus subtilis*. The *E. coli* Fur protein binds to divalent iron metal and has a molecular weight of 17 kDa. Fur protein is known to control the transcription of about 90 genes in *E. coli*. It does so by binding to the Fur box of about 19 bp palindromic sequence, present upstream of the various genes involved in iron metabolism, like siderophore (iron-quencher) synthesis genes and other iron related function genes [6]. The iron-regulators from gram-positive and gram-negative bacteria share no sequence similarity, but the structural aspects in terms of domain organization and metal binding site are very similar [7, 8]. Both the iron dependent regulators, Fur and DtxR, are documented to exist as homo-dimer, with each monomer having three domains, domain 1 is the DNA binding domain, domain 2 houses the metal binding site and is responsible for dimerization, and domain 3 binds to domain 2 through a seven-amino acid linker, having same topology as SH3 like domain. The putative Fe^{2+} metal binding site is composed of two histidine residues, two aspartate residues and one glutamate residues. These amino acids are known to form the active site of the metallo-regulator [12, 13]. Though in-vivo Fe^{2+} is the preferred metal ion for binding to the active site of the protein, but in in-vitro conditions other divalent metal cations are also known to bind to the active site of the protein [9, 10]. The following study was conducted to analyze the binding of the gram–positive metal binding protein, DtxR, to Fe^{2+} as well as to Mn^{2+} and Mg^{2+}.

2. Materials and Methods

2.1 Isolation of Genomic DNA from *Corynebacterium diphtheriae*

Corynebacterium diphtheriae strain *ACTC 700971/ NCTC 13129* was cultured for genomic DNA isolation, on blood agar (Himedia), and incubated at 37 °C for 24 hours. A single colony was inoculated from the blood agar plate into the LB broth growth media (Himedia). The following day genomic DNA was isolated using the CTAB method. The genomic DNA was looked for it's purity and quantity by taking absorbance at A_{260} and the ratio of A_{260}/A_{280}. After conforming the purity and quantity of isolated genomic DNA, PCR amplification was performed.

2.2 PCR amplification of the *dtxR* gene:

Gene specific primers were synthesized by retrieving the *dtxR* gene sequence from the KEGG database (http://www.genome.jp/kegg/kegg.html), (KEGG database sequence accession number for *dtxR* gene is DIP1414). Primers with the restriction enzyme site underlined are shown in the table 1.

Table 1: Primers for *dtxR* gene with restriction enzyme site underlined.

dtxR F	5' CGC <u>GGA TCC</u> GCG ATG AAG GAC TTA GTC GAT 3' *BamH1*
dtxR R	5' CCG <u>CTC GAG</u> CGG TTA GAG TTC TTC GAT ACG 3' *Xho1*

PCR amplification was done with the Agilent thermocycler (USA) under following conditions.

1. 98 ^{0}C for 30 sec
2. 98 ^{0}C for 10 sec
3. 66 ^{0}C for 10 sec
4. 72 ^{0}C for 30 sec

(35 cycles of 2-4)

5. 4 ^{0}C for 30 sec

The amplified PCR product was gel purified (IBI Gel Purification Kit, USA) and stored in -20 °C for further cloning reaction.

2.3 Cloning of the *dtxR* gene

E. coli DH5α strain was used for cloning of the *dtxR* gene into the pET-28a expression vector. *dtxR* gene PCR product and pET-28a expression vector were double digested with the desired restriction enzymes (*BamH1* and *Xho1*, NEB) for 4 hours. Both the double digested *dtxR* PCR product and pET-28a expression vector were gel purified (IBI Gel Purification Kit, USA). Cloning was set up with the pET-28a expression vector and double restriction enzyme digested *dtxR* gene as insert with the DNA Ligase (NEB) at 16 °C overnight. The following day the cloned product was transformed into chemically competent *E. coli DH5α* cells. The transformed cells were spread on LB agar (Himedia) plate with kanamycin (50μg/ml) (Himedia) as selectable marker and incubated at 37 °C overnight. The next day single colony was inoculated into LB broth with appropriate antibiotic and incubated at 37 °C overnight. The clone was isolated with the plasmid isolation kit (IBI Plasmid Isolation Kit, USA).

2.4 Expression and Purification of the DtxR protein

E.coli BL21(DE3) expression strain was transformed with the *dtxR* gene clone and selected with the desired antibiotic. A single cell colony was incubated in LB broth media (Himedia) at 37 °C overnight. The following day the cells were grown till an OD of 0.8, and were subjected to induction with 1mM IPTG (Himedia) and grown for 4 hours at 37 °C in an orbital shaker at 200 rpm. The cells were pelleted and were suspended in the lysis buffer (50mM NaH_2Po_4, 300mM NaCl, 300μg/ml lysozyme, 10mM Imidazole, pH=8.0), according to the weight of the cell pellet. The suspended cells were subjected to sonication to disrupt the cells and release the expressed protein. The sonicated cell lysate was centrifuged for 20 minutes at 11,000 rpm to clarify the cell lysate. The cell lysate was loaded on to a pre-calibrated Ni-NTA slurry with wash buffer, (50mM NaH_2Po_4, 300mM NaCl, 20mM Imidazole, pH=8.0). The Ni-NTA slurry and cell lysate were incubated at 4 °C for effective binding of the protein to the Ni-NTA. The cell lysate and Ni-NTA slurry was washed with varying Imidazole (Himedia) concentration to wash off the unbound protein. Final protein elution was done at 250mM Imidazole (Himedia) concentration. The eluted protein was subjected to SDS-PAGE analysis and protein quantification was done by Bradford method with BSA as standard. The eluted protein was dialysed with phosphate buffer and concentrated through Amicon centrifugal Unit (EMD Millipore) to remove imidazole, so that it should not hinder in downstream experiments.

2.5 Dynamic Light Scattering:

Dynamic Light Scattering is a non-invasive and rapid method for protein size estimation. Due to particle diffusion, there

arise intensity fluctuations which are measured as hydrodynamic radius according to the Stokes-Einstein equation. Because of the binding of the divalent cation at the active sites of the protein, the protein undergoes inherent conformational changes to satisfy the thermodynamic parameters like, enthalpy change, Entropy change and Gibbs free energy change. These conformational changes to accommodate divalent metal cation, affects the overall 3D structure of the protein, which is manifested in form of increase in relative size of the protein as compared to the un-bound protein. This increase in size is picked up as the intensity fluctuations and hence affects the hydrodynamic radius of the protein [14].

$$R_H = \frac{k_B T}{6\pi\eta D_\circ}$$

where: R_H = Hydrodynamic radius of the particle.
K_B = Boltzmann's constant.
T = Temperature.
η = viscosity of the solvent.
$D_\circ$ = Diffusion co-efficient.
A 30µM concentration of DtxR protein was titrated with 5µM to 20µM concentration of the following divalent cations, Fe^{2+}, Mn^{2+} and Mg^{2+}.

3. Results

3.1 PCR amplification of the *dtxR* gene gave a product size of 600 bp.

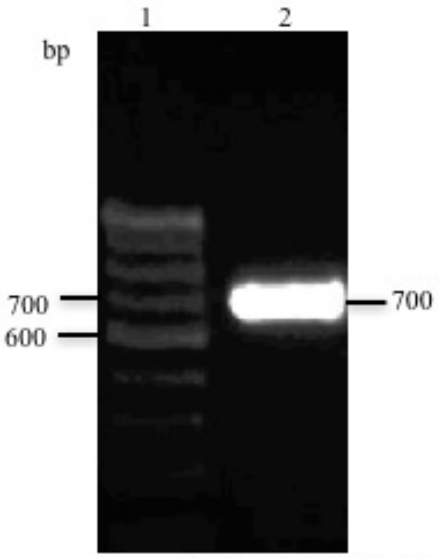

Fig 1: PCR amplification, dtxR gene.
Lane 1: DNA Marker, 100 bp, Genei.
Lane 2: PCR product, dtxR gene.

3.2 The desired clone was confirmed by double digesting *dtxR* gene clone. Double digestion with desired restriction enzymes gave pET-28a expression vector and *dtxR* gene insert as products.

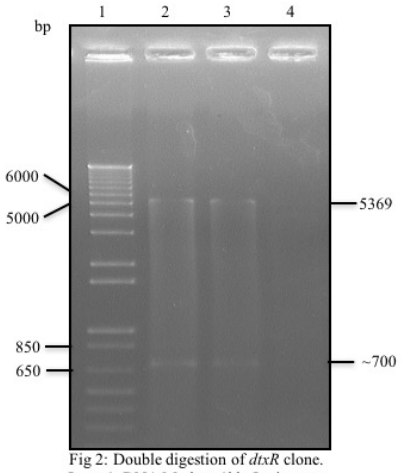

Fig 2: Double digestion of *dtxR* clone.
Lane 1: DNA Marker, 1kb, Invitrogen.
Lane 2,3,4,5: Double digested vector and *dtxR* clone.

3.3 SDS-PAGE analysis of the expressed DtxR protein shows it's size to be 25kDa.

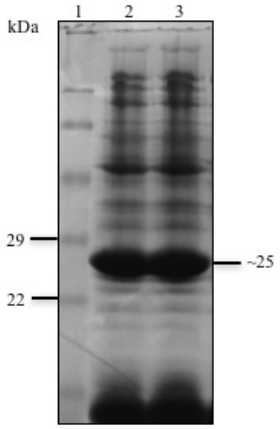

Fig 3: SDS-PAGE of DtxR protein.
Lane 1: Protein Marker, Invitrogen.
Lane 2,3: SDS-PAGE analysis of DtxR clones.

3.4 SDS-PAGE of the purified DtxR protein.

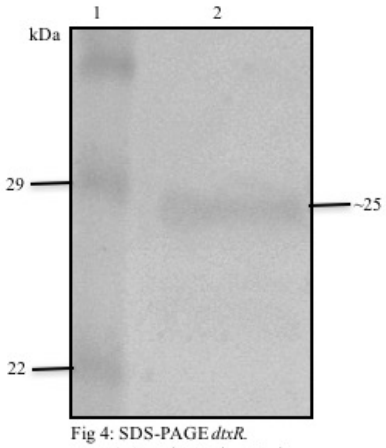

Fig 4: SDS-PAGE *dtxR*.
Lane 1: Protein Marker, Invitrogen.
Lane 2: *dtxR*, 25 kDa.

3.5 Dynamic Light Scattering results for DtxR protein:

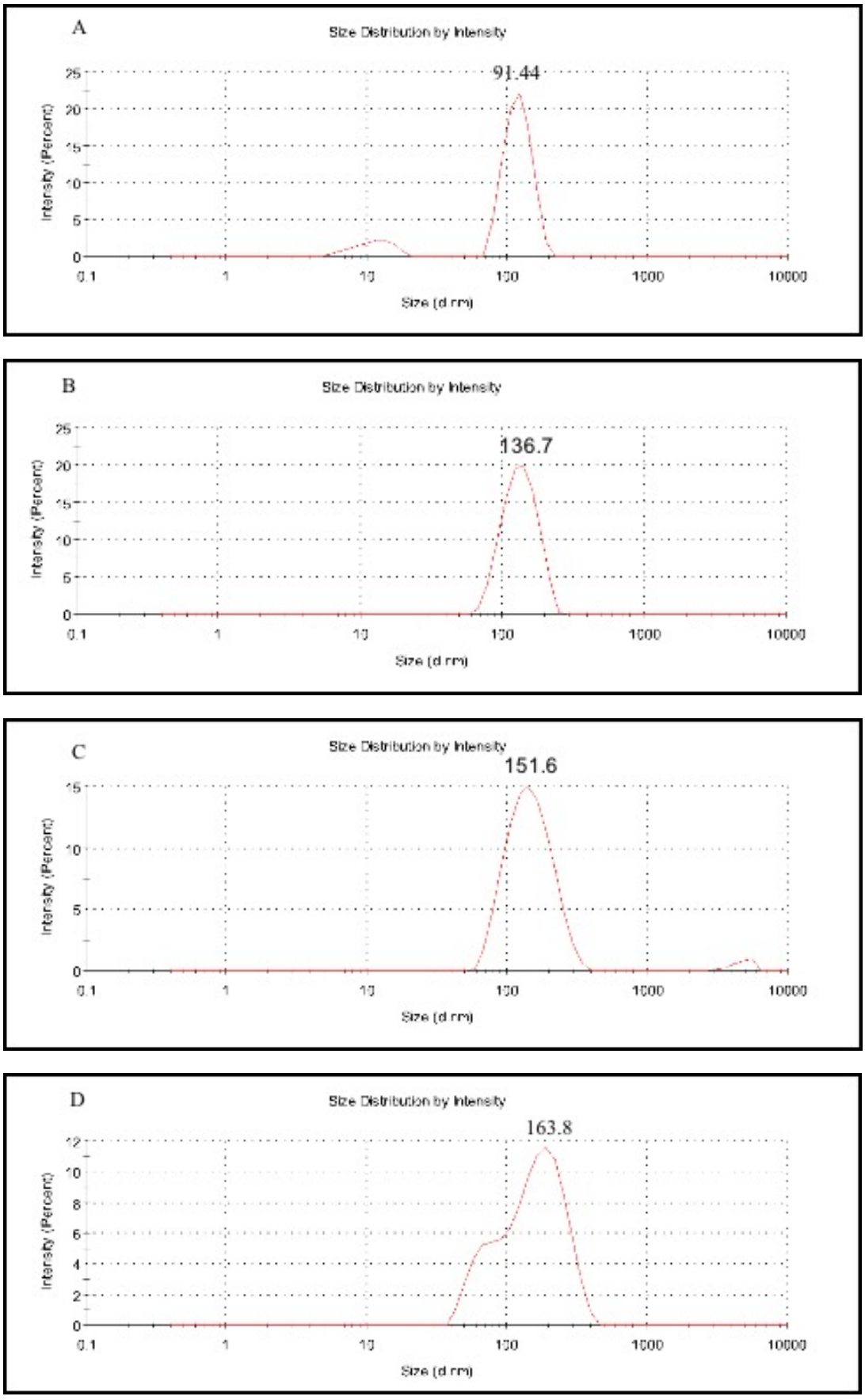

Fig 5: (A Native size of DtxR protein) (B, C, D Increase in the size of the protein after addition of Fe^{2+} ion at various ion concentration.)

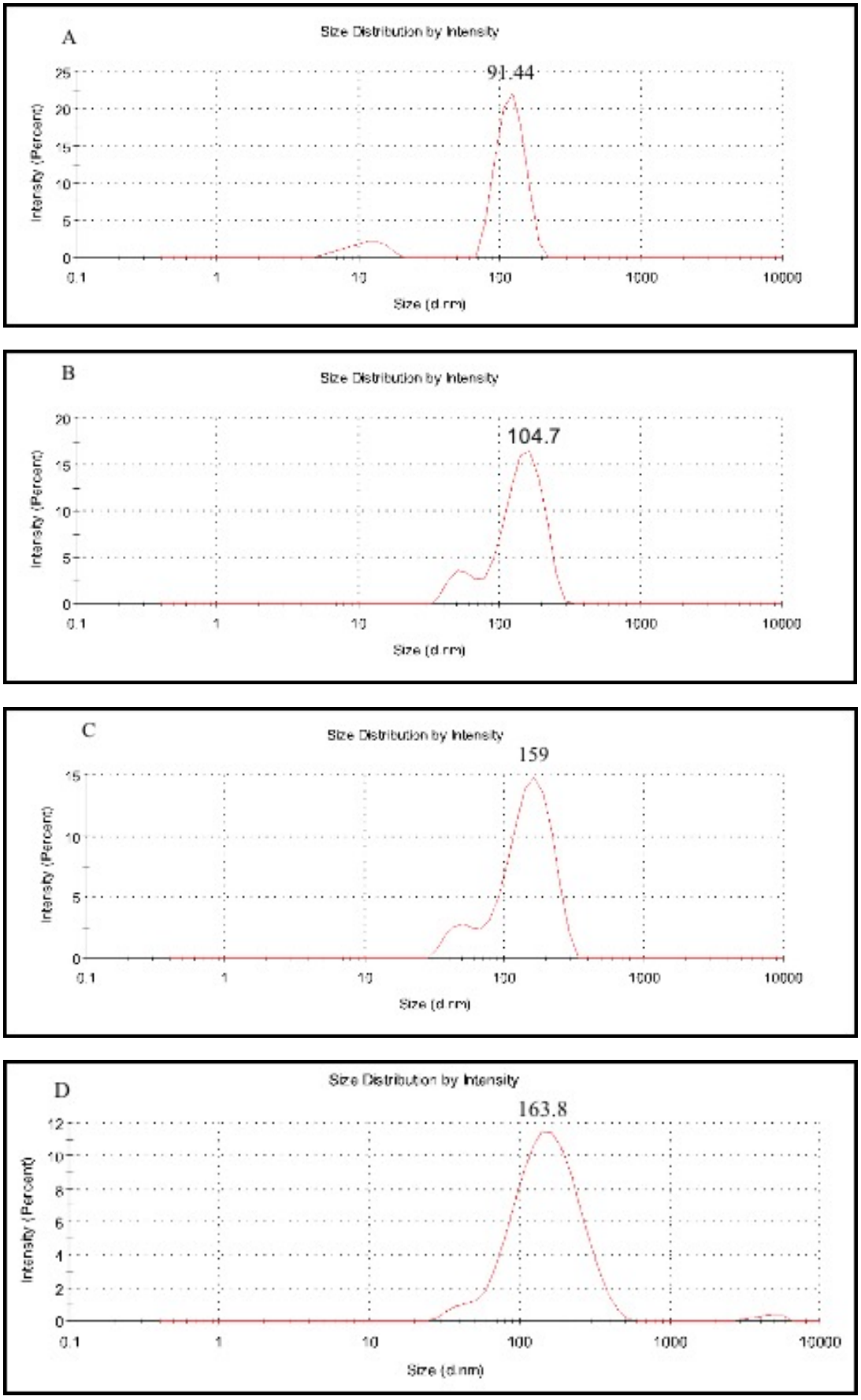

Fig 6: (A Native size of DtxR protein.) (B, C, D Relative increase in the size of the protein after addition of Mn^{2+} ion at different ion concentration.)

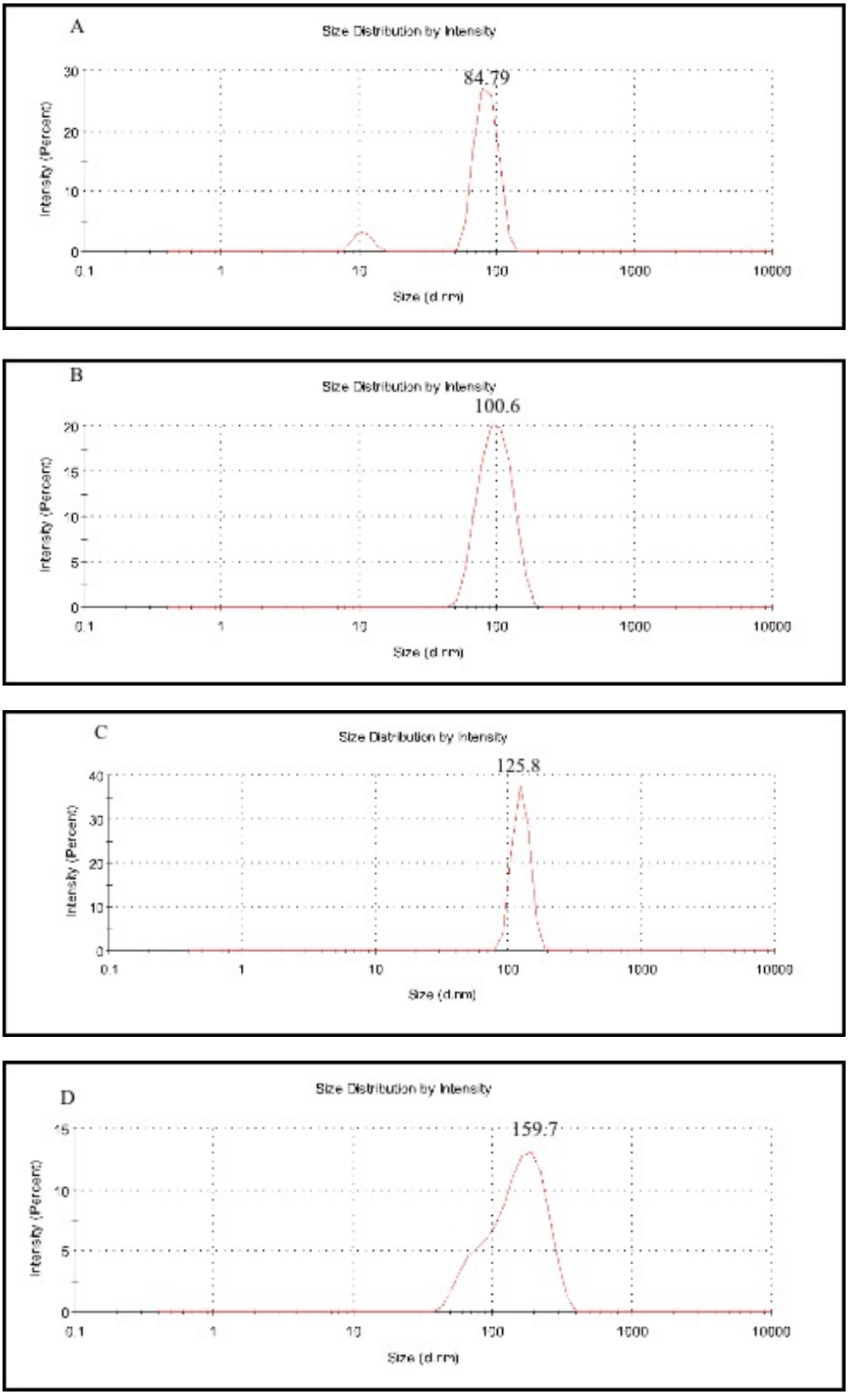

Fig 7 (A Native size of DtxR protein.) (B, C, D Relative increase in the size of the protein after addition of Mg^{2+} ion at different ion concentration.)

The relative change in the size of the DtxR protein was measured by Dynamic Light Scattering with Malvern Zetasizer instrument (Malvern Instruments Ltd., Worcestershire, UK). With the addition of the divalent cation, there is increase in the size of the protein, thus showing the binding of the divalent cation with the protein as compared with the un-bound protein. The addition of divalent cations at a concentration of 5µM, 10µM, 15µM and 20µM showed

incremental increase in the size of the protein.

4. Conclusion

Metal ion acting as a co-repressor for the efficient functioning of the protein has been documented and described in an elaborate way. Iron metal ion is associated with many vital and essential cellular pathways, like cellular respiration, DNA replication. Hence, physiological balance of iron metal inside micro-organism is necessary for it's survival. Iron dependent regulators play an important role in sensing iron metal concentration inside micro-organism. Both the scarcity and abundance of iron metal inside micro-organism triggers an elaborate pathway for it's acquisition through the expression of a battery of genes, and this is mediated by iron dependent metallo-regulators. Thus, a complete interpretation of various iron dependent regulator family protein is essential in understanding the bio-physiological pathways and it's effect on the micro-organisms survival inside the host.

5. Acknowledgements

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6. Conflict of Interest

The authors declare that they have no conflict of interests.

7. Ethical statement

Since this work does not involve any animal studies and human studies, hence ethical clearance is not reqiured.

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