Identification of Protein L-isoaspartate (O) Methyltransferase Interacting Proteins in *Salmonella* Typhimurium

Thesis

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IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Master of Veterinary Science
(Animal Biochemistry)

2015

Dedicated to....

My Beloved Family

भारतीय पशु चिकित्सा अनुसंधान संस्थान
(सम विश्वविद्यालय)
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Certificate

This is to be certified that the research work embodied in this thesis entitled "Identification of protein L-isoaspartate (O) methyltransferase interacting proteins in *Salmonella* Typhimurium" *submitted by* Dr. Prasanta Kumar Koustasa Mishra, Roll No. M-5349, *for the award of* Master of Veterinary Science *Degree in* Animal Biochemistry *at Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate himself under my supervision and guidance.*

It is further certified that Dr. Prasanta Kumar Koustasa Mishra, Roll No. 5349, *has worked for more than 21 months in the Institute and has put in more than 150 days attendance under me from the date of registration for the* Master of Veterinary Science *Degree in this Deemed University, as required under the relevant ordinance.*

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We the undersigned members of Advisory Committee of **Dr. Prasanta Kumar Koustasa Mishra, Roll No. M-5349,** *a candidate for the degree of* **Master of Veterinary Science** *with the major discipline in* **Animal Biochemistry**, *agree that the thesis entitled* "**Identification of protein L-isoaspartate (O) methyltransferase interacting proteins in *Salmonella* Typhimurium** " *may be submitted in partial fulfillment of the requirement for the degree.*

We have gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented for the award of **Master of Veterinary Science** *Degree of this Institute.*

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Prasanta ku. k mishra

Date 1/7/2015
Place: IVRI, Izatnagar

Prasanta Kumar Koustasa Mishra

ABBREVIATIONS

%	:	Per cent
µg	:	Microgram
µl	:	Microlitre
Asn	:	Asparagine
Asp	:	Aspartic acid
BCA	:	Bicinchoninic acid
BCIP	:	5-Bromo-4-chloro-3'-indolyphosphate
CAM	:	Chloramphenicol
CBB	:	Coomassie brilliant blue
CcmA	:	Cytochrome c biogenesis protein A
CL	:	Column load
CO-IP	:	Co-immunoprecipitation
Conc	:	Concentration
DNA	:	Deoxy-ribonucleic acid
DSS	:	Disuccinimidyl suberate
DTT	:	Dithiothreitol
EDTA	:	Ethylenediaminetetraacetic acid
Fig.	:	Figure
FT	:	Flow through
g	:	Gram
H_2O_2	:	Hydrogen peroxide
HEA	:	Hektoen enteric agar
IPTG	:	Isopropyl-β-D-thiogalactopyranoside
iso-Asp	:	Isoaspartate
Kan	:	Kanamycin
Kb	:	Kilobasepairs
kDa	:	Kilodalton
LB	:	Luria Bertani
LC-MS	:	Liquid chromatography mass spectrometry
M	:	Molar
M-cells	:	Microfold cells
Mec	:	Mechanosensitive channel protein

Met (SO)	:	Methionine sulfoxide
Met	:	Methionine
min	:	Minute
ml	:	Millilitre
mM	:	Millimole
Msr	:	Methionine sulfoxide reductase
NaCl	:	Sodium chloride
NapA	:	Periplasmic nitrate reductase A
NBT	:	Nitro-blue tetrazolium chloride
ng	:	Nanogram
NO	:	Nitric oxide
O_2^-	:	Superoxide anion radical
OD	:	Optical density
OH^-	:	Hydroxyl radical
$ONOO^-$	:	Peroxynitrite
PAGE	:	Polyacrylamide gel electrophoresis
PBS	:	Phosphate buffered saline
PBST	:	Phosphate buffered saline with Tween 20
pH	:	Log hydrogen ion concentration
PIMT	:	Protein-L-isoaspartate (O) methyl transferase
pmol	:	Picomoles
PVDF	:	Polyvinyl difluoride
RNS	:	Reactive nitrogen species
ROS	:	Reactive oxygen species
rplC	:	Ribosomal protein L3
rpm	:	Rotations per minute
S. T	:	*Salmonella* Typhimurium
SDS	:	Sodium Dodecyl Sulphate
SPDP	:	N-succinimidyl 3-(2-pyridyldithio) propionate
SPI	:	*Salmonella* pathogenicity island
T3SS	:	Type III secretion system
UV	:	Ultra-violet
β-ME	:	β Mercaptoethanol

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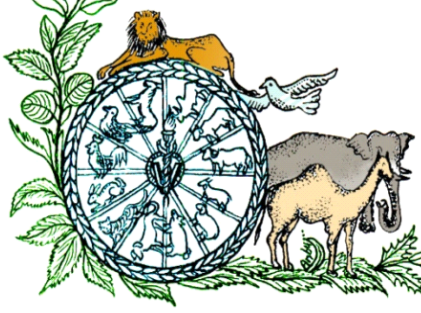


1 INTRODUCTION

Macromolecules like carbohydrate, lipid, protein, nucleic acid forms the basis of cellular structure and function. Out of these complex biochemical elements, nucleic acids and proteins play major role in the flow of information. The central dogma of information starts from DNA, gets transcribed into RNA and results into protein, the 'executor' (Crick, 1970). The ubiquitous phenomenon of wear and tear is also applied to these elements during the process of metabolic turnover of cells. Also various stress conditions induce structural as well as functional changes in these macromolecules. Whenever any living cell (like eukaryotic or prokaryotic) confronts these damages it has got two options to deal with it.

1. To replace the altered macromolecules (which is an energy consuming process)
2. To repair them

Clearly, many times cell will go for the second option to conserve its energy. Extensive research in genomics and proteomics has provided a fair insight into the damage and repair system of nucleic acids and proteins. The knowledge of such pathways has provided a new platform in the field of prevention of various infectious diseases.

DNA, the store house of information confronts various damages in various conditions. Such as UV mediated damages like formation of cyclobutane pyrimidine dimers (CPD), pyrimidine pyrimidone photoproducts (6-4PP) (Goosen and Moolenar, 2008). Apart from UV mediated damages, conformational and covalent modifications also elicited by heat, oxidative reagents, change in pH (Viscik and Clarke, 1995). DNA is also subjected to depurination or deamination through spontaneous chemical reactions (Kushner, 1987). Many

of the above mentioned damages are also common to RNA. In cell various mechanisms of DNA repair exists, most of them are mediated via enzymes. These mechanisms includes direct reversal of damages by photolyases (photoreactivation), repair of the damaged base by DNA glycosylase (base excision repair), incision of the DNA adjacent to the damage by endonucleases (UV damage endonucleases), removal of complete oligonucleotide containing the damage (nucleotide excision repair) (Goosen and Moolenar, 2008). One of the important group of damaging agents are endogenous (i. e. S-adenosyl methionine) and environmental alkylating agents (i. e. Chloromethane and other halocarbons) (Drablos *et al*., 2004). Alkylating agents cause alkylation of the majority of N- and O- atoms in DNA and RNA bases. Bacterial and mammalian AlkB proteins (Fe^{2+}) and 2–oxo-glutarate dependent dioxygenase can reverse methylation damages. The *E. coli* protein AlkB was recently shown to be an oxidative DNA methylase that repairs the cytotoxic lesions like 1-meA and 3-meC in DNA (Drablos *et al*., 2004).

1.1 Execution of the executor

Like DNA, proteins are also subjected to various forms of damages that can render them nonfunctional. According to physiological regulation of cell, various proteins undergo spontaneous conformational and covalent modifications. But the rates of these reactions can be increased by environmental/ other stresses such as heat, oxidative changes or changes in pH and osmotic conditions (Viscik and Clarke, 1995).

Protein damages can be classified generally into two different categories.

1. Conformational damage or alteration of the three dimensional structure of the protein without changing its chemical makeup.
2. Covalent modification of the amino acids (Viscik and Clarke, 1995).

Bacteria deals with these damages mostly by 3 different mechanisms

- Chaperones play an important role in *de novo* folding of newly synthesized proteins. Apart from that they also take care of conformationally distorted proteins.
- Enzymatic system exists, which can repair covalently modified amino acids. For

example isomerized prolines, oxidized methionines and iso-Asp can be repaired enzymatically.

- When these two above mentioned mechanisms unable to repair the damaged protein the cell takes necessary steps to eliminate it.

1.2 Role of chaperone in protein refolding

All the information required for protein folding resides on its amino acid sequence (Anfinsen, 1973). Despite of their correct initial folding, there is always some tendency for proteins to subsequently unfold or aggregate. It is now clear that GroEL and DnaK help to maintain the functional conformations of existing proteins. Their ability to bind unfolded polypeptides and facilitate renaturation has been demonstrated both *in vitro* and *in vivo* (Kawata *et al.,* 1994; Martin *et al.,* 1992; Skowyra *et al.,* 1990). Increased production of chaperones is common to nearly all known stress responses. Heat, ethanol, nutrient limitation, oxidizing agents, high or low pH, nalidixic acid, UV irradiation, puromycin, phage infection and alkylating agents are all factors that induce GroEL and DnaK synthesis (Neidhardt and VanBogelen, 1987) Similarly the quaternary, tertiary or secondary structure of proteins can be disturbed by many factors, including heat, cold, salts, acid or alkaline pH, high osmotic pressure, alcohol and oxidizing agents, both *in vitro* (Davies and Delsignore, 1987) and *in vivo* (Nguyen *et al.,* 1989). Apart from these, Hsp 33 and Prohibitin are two new world chaperones found to be important in protein modification process (Wholey and Jacob, 2012; Gionnato *et al*., 2015).

1.3 Covalent damages of protein needs specific enzymes for their repair

Covalent modifications of amino acids lead in altered proteins in cell which has to be repair for cellular survival. One of these kinds of change is formation of disulfide bonds in proteins under oxidizing environment. Protein disulfide isomerase, which catalyses this process, is an important member of the protein-folding pathway (Freedman, 1989). In *E. coli* the periplasmic proteins DsbA and DsbC (Wulfing and Pluckthun, 1994) catalyzes isomerization of disulfide bonds needed for activity of periplasmic and outer-membrane proteins.

Another good example will be proline isomerization and its repair. In proline, peptide bonds can exist in either the 'normal' *trans*-configuration or in the *cis*-form (Fischer *et al.,* 1983). The presence of the inappropriate *cis*-isomers can greatly retard protein folding or

refolding (Lang *et al.,* 1987) which results in compromised function and importantly impaired degradation and removal from the cell (Yaron and Naider, 1993). Peptidyl-prolyl *cis-trans* isomerases catalyse proline isomerization, have been identified in many organisms and shown to increase the rate of protein folding (Schmid *et al.,* 1993). *E. coli* has genes for at least three such isomerases as *ppiA* and *ppiB,* which encode periplasmic and cytoplasmic enzymes, respectively (Hayano *et al.,* 1991), and *ppiC,* encoding a new protein of unknown localization.

Oxidizing agents contribute a major part of covalent modification. Hydroxyl (OH^-), hydroperoxyl (HOO^-), superoxide (O_2^-) or lipid peroxyl radicals are the major oxidants generated in the biological system. These oxidizing agents mostly target amino acids like methionine, cysteine, tyrosine, histidine and tryptophan (Stadtman, 1992). Methionine sulfoxide reductase recognizes methionine sulfoxide as an abnormal amino acid and can reduce it back to methionine, using reduced thioredoxin as a substrate (Lunn and Pigiet, 1987 and Rahman *et al.,* 1992).

L-isoaspartyl protein carboxyl methyItransferase specifically binds L-iso aspartyl residues in proteins (Fu *et al.,* 1991) and methylates the side-chain carboxyl group using S-adenosyl methionine (SAM) as a methyl donor thus helps in isoaspartate repair. This particular enzyme has been found to be distributed in various bacteria (Li and Clarke, 1992a) suggesting role of this enzyme in cellular survival under various stress conditions.

1.4 MsrA and PIMT play important roles in repair of covalent damages in protein

Mutants strains in *msr* gene of *Mycobacterium smegmatis* (Douglas *et al*., 2004), *Staphylococcus aureus* (Singh and Moskovitz, 2003), *E. coli* (John *et al*., 2001), *Helicobacter pylori* (Alamuri and Maier, 2004) and *Neisseria gonorrhea* (Skarr *et al*., 2002) were found to be hypersensitive to oxidative stress. Mahawar *et al* in 2011 showed that catalase lost its structure and fuction when exposed to HOCl. The activity of this enzyme was restored by Msr with the help of GroEL. Defective response has been shown to oxidative stress in protein L-isoaspartyl repair-deficient *C. elegans* (Khare *et al.,* 2009). Repair of spontaneously deamidated HPr phosphocarrier protein was shown to be catalyzed by PIMT (Brennan *et al*., 1994). Mice deficient of *pimt* experienced epileptic seizures (Morrison *et al*., 2012).

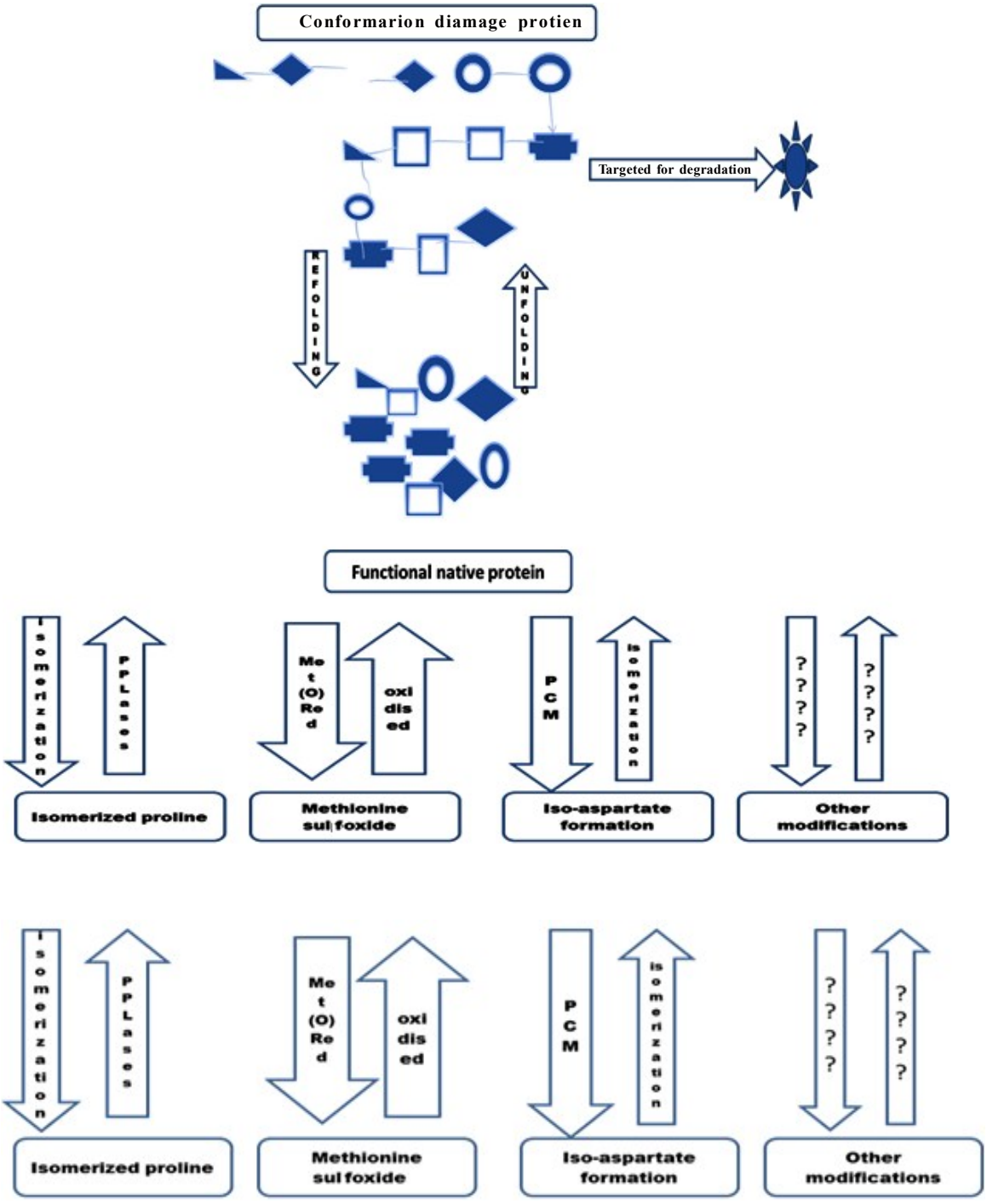


Fig. 1: Different modifications of protein and their repair processes

1.5 *Salmonella enterica* serovar Typhimurium can be a suitable model for the study of PIMT mediated repair of isoaspartate in the protein.

PIMT helps in repair of many proteins, which were found to be very essential for normal metabolism of cells. Some of them are:

- Prokaryotic HPr protein which is a component of sugar phosphate translocase system of *E. coli* (Brennan *et al*., 1994).
- PIMT mediated activity restoration of deamidated calmodulin has been reported (Johnson *et al.,* 1987).
- Proteins in the mice brain extracts like aconitase 2, enolase, creatine kinase B, synuclein are the targets of PIMT (Zhu *et al*., 2006).
- *Arabidopsis* ATP-dependent DEAD box RNA helicase activity is shown to be restored by PIMT (Nayak *et al.,* 2013)
- Histones are a group of basic proteins associated with DNA compaction and packaging by formation of nucleosome. These long lived intracellular proteins are subject to various post translational modifications inside the cell. Formation of isoaspartates makes histone H4 protein prone to proteosomal degradation. Recently methylation of H4D24 (that can be done by PIMT) was proposed to be associated with longevity of this protein (Biterge *et al*., 2014).
- The role of PIMT in age related CNS dysfunction has been demonstrated by taking PIMT +/- mice as a model (Qin *et al*., 2015). The extracts from the brain of knockout mice showed accumulation of isoaspartate containing proteins.
- In a separate study PCMT1 has been found to be a target of has-miR-195, which promotes its silencing at the post transcriptional stages of PCMT1 expression . PCMT1 was found to be involved in different signaling pathways of hepatocellular carcinoma and associated with long term sustainability of tumor cells (Amer *et al*., 2014).
- α, β - tubulin have been found to be most susceptible to ethanol mediated damage of brain tissues in alcoholics. There are various types of damages confronted by these tissues i. e. Na+/ k+ ATPase malfunction, proteasome dysregulation and Iso-Asp formation during exposure to alcohol. An increase of 9 % in Iso-Asp residue and 28 % of PIMT up regulation in alcoholics has been reported (Erdozain *et al*., 2014).

- Detoxification of ammonia is mostly mediated by converting it to urea in Kreb's cycle. Conversion of NH_3+CO_2 →Carbamoyl phosphate is the first and rate limiting step of urea cycle is catalysed by mitochondrial CPS-I. Recently CPS-I has been found to be a target of PIMT (Carter *et al*., 2015). In the same experiment they have also demonstrated the role of Iso-Asp, CPS-I and CPS-III as a biomarker of liver injury.
- Na^+/ K^+ ATPase gets repaired via PIMT mediated pathway in humans (Adav *et al*., 2014).
- In *pimt* knockout mice, formation of iso-Asp residue in creatine kinase B have been reported (Dimitrijevic *et al*., 2014).
- Asn deamidation and repair in SOD-1 of human have been observed (Bierczyńska-Krzysik *et al*., 2014).

Apart from repair of all these proteins evidences like defective survivability of *pimt mutant* of *E. coli* at 55 ˚C and stationary phase suggest that PIMT is an indispensable enzyme for bacteria at higher temperature (Li and Clarke, 1992).

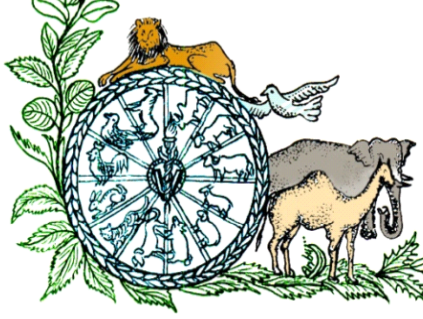
In case of *Arabidopsis* it has been seen that the activity of PIMT is more at 50 ˚C (villa *et al*., 2006). These above statements may lead to put forward one assumption that iso-Asp formation is more at higher temperature which has been experimentally shown (Sharma *et al.,* 1993).

These evidences make PIMT an interesting enzyme, playing important role in survival at higher temperature. In case of poultry, carrier for *Salmonella* Typhimurium, the normal body temperature is 42 ˚C (Cooper and Washburn., 1998). This temperature is higher than the normal body temperature of humans, who are the natural host of *Salmonella* Typhimurium. So we can postulate one hypothesis that in poultry the *Salmonella* Typhimurium must be confronting some temperature induced stress resulting in formation of Iso-Asp residues in important proteins and PIMT plays an important role to repair them. Therefore, identification of the proteins which interact and get repaired by PIMT is of prime importance. In my current research, I plan to identify target proteins which interact with PIMT in *Salmonella* Typhimurium.

Objectives

1. Isolation and identification of protein L-isoaspartate (O) methyltransferase interacting proteins in *Salmonella* Typhimurium

Review of Literature



2 REVIEW OF LITERATURE

2.1 Salmonellosis: A brief review

It is an infectious disease of humans and animals caused by organisms of two different species of *Salmonella* (OIE, 2010). These are etiological agents of diarrhea and systematic infection in humans, mostly as secondary contaminants of food originating from animals and the environment (OIE, 2010). According to WHO the most common vehicles of transmission are eggs and egg products, poultry meat and meat products. *Salmonella* is a gram-negative, facultative intracellular anaerobe belongs to family **Enterobacteriaceae**. Recent classification (Grimont and Weill, 2007) suggests that *Salmonella* consists of two major species: *S. enterica* and *S. bongori*. Out of these two classes *S. enterica* is mostly responsible for human and animal salmonellosis.

Salmonella enterica is further grouped into six subspecies according to biochemical parameters and susceptibility to lysis by bacteriophage Felix O1 (OIE, 2010). These sub species are as: subspecies I (enteric), subspecies II (salamae), subspecies III (arizonae), subspecies IIIb (diarizonae), subspecies IV (houtenae), subspecies V (indica). Strains of *Salmonella* are classified into serovars on the basis of extensive diversity of lipopolysaccharides, somatic antigen O and flagellar antigens H, in accordance with Kauffman- white scheme (OIE, 2010). In *salmonella* over 2500 serovars has been recognized (Grimont and Weill, 2007).

Salmonella enterica are typically orally acquired pathogens that causes one of the four major syndromes: i. e. typhoid (enteric fever), enterocolitis, bacteremia, chronic asymptomatic carriage (Coburn *et al.*, 2006). Out of which we are interested mostly in enterocolitis (Non typhoidal salmonellosis).

Human salmonellosis is one of the most important zoonotic disease (OIE, 2010). Each year 93.8 million cases reported worldwide, out of which the disease claims 0.15 million lives (Majowicj *et al*., 2010). But in animals like poultry and pigs, infection may be there but they do not show any clinical illness (Wray and Wray, 2000).

Pathogenesis of *Salmonella* have been classified into two categories as per the pro inflammatory stimuli generated course of infection (Coburn *et al.*, 2006).

- PAMP mediated, that is capable of stimulating innate immunity
- Virulence associated pro inflammatory behavior that results into host disease pathology.

The pathogenesis is mostly mediated via specific pathogenesis islands present in *salmonella*, mostly SPI-1 and SPI-2. These islands encode molecular apparatus called as a type III secretion system (T3SS). This system injects bacterial proteins known as effectors. *Salmonella* SPI-1 T3S results in activation of MAPK and induces NF-κB cascade and leads to inflammation by effector like SopE (Palmer *et al.,* 1998). In contrast SPI-2 is essential for intracellular parasitism, virulence and evasion of phagocyte oxidase machinery of host (Henshel *et al.,* 1995). The SPI-2 plays an important role in early and complete induction of *Salmonella* mediated enterocolitis (Coombes *et al*., 2005). Apart from SPI the PAMP of *Salmonella* like LPS and flagellin plays important role in pathogenesis of *Salmonella* (Zeng *et al*., 2003).

During infection *Salmonella* survives in macrophages and evade immune response. It overcomes various stresses inside macrophages by certain repairing systems mediated by PIMT and MsrA (enzymes) and others.

2.2 PIMT repairs some unusual isoaspartate residues in proteins

The protein L- isoaspartate (D-aspartate) O-methyltransferase (PIMT/ PCM) plays an important role in restoring the native structure of protein by catalyzing the conversion of isoaspartate to aspartate. The enzyme commission name is EC- 2.1.1.77, so it indicates that, this enzyme belongs to transferases family (Clarke, 1999). Gene coding for this enzyme have been found in almost all organisms studied (Kagan *et al*., 1997). The enzyme shows high degree of sequence conservation among various species of both prokaryotes and eukaryotes

(Kagan *et al*., 1997). The ubiquitous distribution of this enzyme among eubacteria and archea has been demonstrated (Ichikawa and Clarke, 1998). Direct analysis of methyltransferase activity in various bacterial extracts confirmed its wide distribution in the gamma subdivision of gram – ve bacteria (Li and Clarke, 1992a).

It has been reported that, except in *Archaeoglobus fulgidus*, PIMT is coded by a single gene (Ichikawa and Clarke, 1998). Though in eukaryotes introns are present but the coding regions are constant throughout all species. While it appears that bacterial and animal PIMT genes are more or less constitutively active, but in case of plants, this gene appear to be regulated in a more complex manner and involve elements of hormonal, developmental and environmental controls (Mudgett *et al*., 1993). The L–isoaspartyl methyltransferase is a monomer and is normally localized in the cytosolic fraction of cell (Clarke, 1999). Also the enzyme has been found to be associated with cell membrane of eukaryotes.

2.3 Crystal structure of PIMT

PIMT is a monomeric protein but can be sub divided into three functional sub domains (Skinner *et al.*, 2000). The central sub domain closely resembles other SAM dependent methyltransferases but differs in the topological connectivity. The sides of the enzymes are formed by serine-threonine rich β-strands which may provide hydrogen bonds for specific interaction with isoaspartyl residues.

The binding site of PIMT in *T. maritima* forms a single binding site for iso aspartate residue but in case of human, 4 binding sites has been reported (Lowenson and Clarke, 1991). PIMT is supposed to function without a covalent intermediate and without the aid of metal ions. This has been confirmed that there are no cysteines near the active site, nor are any bound ions or any obvious metal ligands. Apparently, the –'O' of the isoaspartyl α-carboxylate is a strong enough nucleophile that neither a covalent intermediate nor a metal ion is required (Skinner *et al.*, 2000).

2.4 Mechanism of iso aspartate formation

Normal L-aspartic acid and L-aspargine residues are susceptible to intramolecular reaction under physiological conditions that lead via succinimide intermediate, to the formation

of isomerized L-isoaspartate residues (Clarke, 1999). In proteins, deamidation and isoaspartyl formation generally occur at Asn/ Asp-Gly/ Ser sequences (Skinner *et al.*, 2000). In peptides this reaction occurs 10-fold more rapidly at asparagine than at aspartic acid. In proteins however, aspartates are more labile than any asparagines (Geiger and Clarke, 1987). It has been reported in *E. coli* that mischarging of an aspartyl tRNA with L-aspartate would lead to coupling of its β- COOH group rather than α-COOH group (Momand *et al.,* 1990). Usage of this charged tRNA would then result in the incorporation of L-isoaspartate in the newly synthesized protein. Apart from these, temperature and pH extremes can lead to polypeptide denaturation and disruption of conformation, which makes protein even more vulnerable to covalent damages such as deamidation and racemization (Aswad *et al*., 2000). *In vivo* L-iso-Asp accumulation is found to be pH dependent (Hicks *et al*., 2005). Neutral to alkaline pH conditions favours L– iso-Asp formation in proteins *in vitro* (Brennan and Clarke, 1995). A link between an increased temperature and increased formation of L-isoaspartate residues have been demonstrated in HPr protein (Sharma *et al*., 1993)**.** Stress developed due to methanol and long term stationary phase survival also results in formation of isoaspartate residues (Hicks *et al*., 2005).

2.5 Isoaspartate distorts the native structure of a protein

Normally the iso- term is used for those peptide bonds which are formed as the result of amide bond formed between other amino or carboxyl group of an amino acid apart from the α groups. The aspartic acid contains two – COOH groups and in Asn two- NH_2 groups (i. e. the β groups). This β group forms peptide linkage in the altered structure.

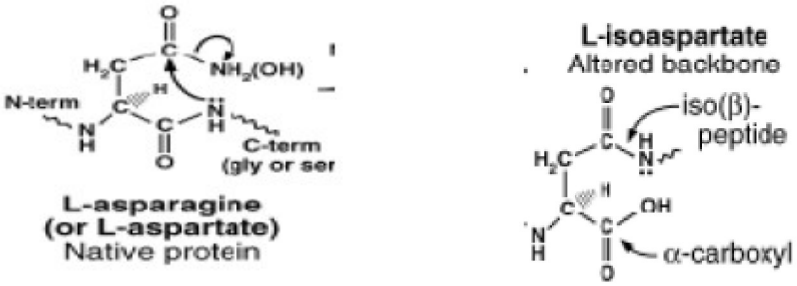


Fig. 2 Structural differences between aspartate and isoaspartate residues (Skinner *et al.*, 2000).

Iso-Asp sequences contain an extra carbon in the peptide backbone as well as an abnormal one-carbon carboxyl 'side chain'. This isoaspartate results in a kink in the backbone of the protein and disturbs its normal conformation. Thus, iso-Asp sites may cause considerable disruption to normal secondary and tertiary protein structures (Johnson *et a*l., 1987). The formation of isoaspartyl residues often results in significant loss of protein function (Skinner *et al.*, 2000).

2.6 PIMT mediated repair of isoaspartate residues

The rearrangement of the back bone during isoaspartate formation is linked to Asp/ Asn deamidation through the formation of a common succinimide intermediate both in case of small peptide (Battersby and Robinson, 1955) and in intact protein (Teshima *et al.*, 1991). The initial step in these processes is nucleophilic attack by the peptide nitrogen on the carbonyl carbon of an asparagine side chain or the carboxylate carbon of an aspartic acid side chain. At either amino acids, nucleophilic attack by the peptide nitrogen leads to the formation of an unstable succinimide intermediate. This succinimide intermediate is susceptible to hydrolytic cleavage by either of the carbonyl groups. Hydrolysis at one carbonyl group leads to formation of native aspargine but the other one forms a deamidated product i. e. the iso aspartate residues (Skinner *et al*., 2000). Mostly the ratio between the restoration of native residue i. e. aspartate and formation of isoaspartate is 1 : 3 (Vigneswara *et al*., 2006).

Experiments performed *in vitro* have demonstrated that incubation of several synthetic L-isoaspartyl containing peptides with PIMT and the methyl donor S adenosyl methionine (SAM) results in the conversion of at least 50 % of the peptide to the normal L-aspartyl containing form. This occurs because methylated L- isoaspartyl residues can form succinimidyl residues much faster than can unmethylated L-isoaspartyl and L-aspartyl residue (Mc Fadden and Clarke, 1987). This cycle of restoration and formation of an unusual residue continues until the backbone acquires its normal conformation.

Various assays have been performed like methanol diffusion assay, radiolabelled transfer assay which showed that SAM is the methyl donor in PIMT mediated reaction (Vigneswara *et al*., 2006).

PIMT also plays role in repair of D-aspartate in case of mammals. In this case the conversion leads from D-aspartyl to L-aspartyl residues (Lowenson and Clarke, 1992). The efficiency of these pathways is dependent upon the affinity of PIMT for the D-aspartyl site (Clarke, 1999). Interestingly proteins from red blood cell membranes of methylation deficient patients with uremic diseases actually have lower levels of D-isoaspartate residues resulted due to the absence of L-isoaspartyl methyltransferase activity (Fuji *et al*., 1994).

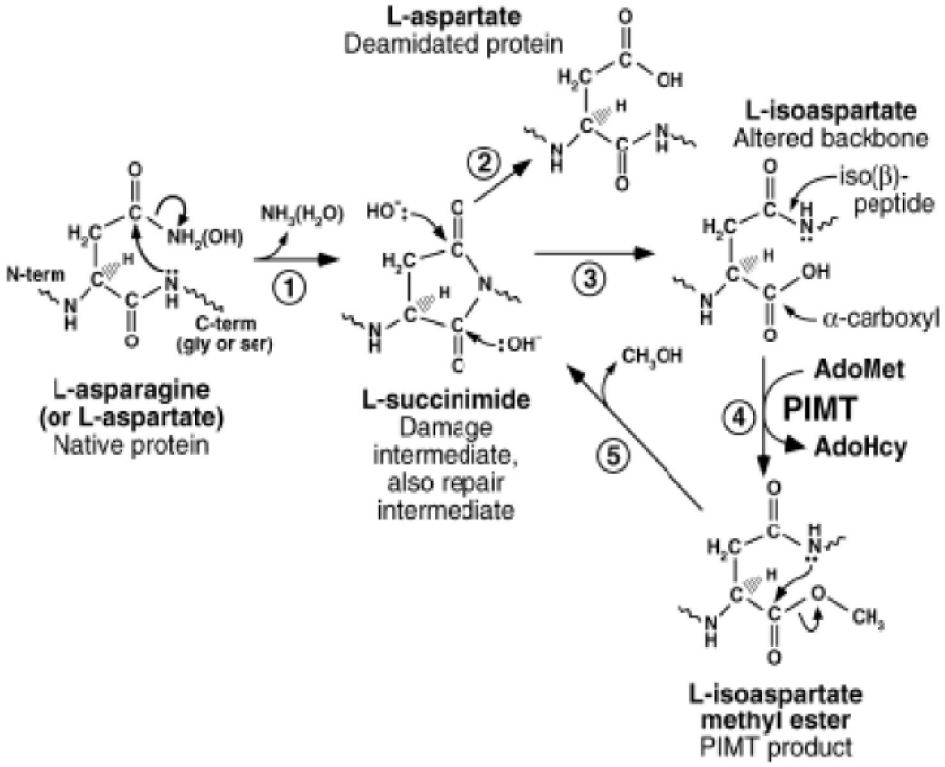


Fig. 3 Structural presentation of PIMT mediated repair pathway of isoaspartate residues (Skinner *et al*., 2000)

2.7 PIMT is specific towards its substrate

PIMT mainly targets two substrates i. e. L-isoaspartyl and D-aspartyl forms. No methylation for the D-iso-Asp and L-Asp has been reported by PIMT. In case of prokaryotes, it only targets L-iso-Asp (Kagan and Clarke, 1995). In case of synthetic peptides, the human enzymes mostly repairs L-iso-Asp (Lowenson and Clarke, 1991). But regarding affinity towards Ado-Met this enzyme does not show any significant variability (Mudget and Clarke, 1993). Considering the structures of these four isoforms i. e. D-Asp, D-iso-Asp, L-Asp and L-iso-Asp, it has been noticed that they all have similar atomic orientation except the N-atom.

So PIMT may require the N-atom to stay behind the plane of the structure like in the case of L-iso-Asp and D-Asp (Clarke, 1999).

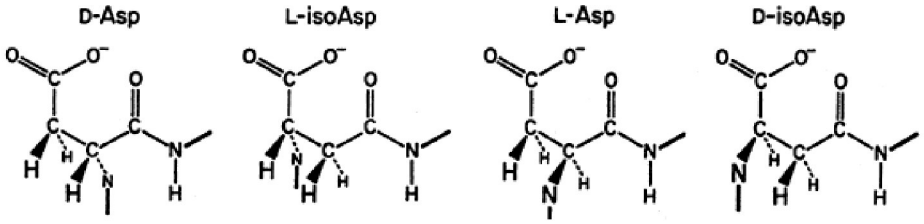


Fig. 4: Structural representation showing atomic orientation of the N-atom in isomer of Asp and iso-Asp. The dotted line shows behind the plane where as the solid line shows above the plane of orientation.

2.8 PIMT: A multitask accomplishing enzyme

Researches in various species suggested that PIMT has significant role in aging, heat stress survival and stationary phase recovery etc. PIMT enhanced the survival of aging *E. coli*, when subjected to secondary environmental stresses i. e. methanol and paraquat (Viscik *et al*., 1998). Also deficiency of PIMT leads to epileptic seizures in case of mice (Yamamoto *et al*, 1998). Deficiency of this enzyme leads to accumulation of altered proteins, retardation of growth, and fatal seizures in mice (Kim *et al*., 1997).

In *C. elegans*, defective response to oxidants like quinone, paraquat, homocysteine and homocysteine thiolactone has been seen in PIMT mutant (Khare *et al*., 2009). An increase in PIMT activity was observed at 42 ˚C in HeLa cells (Ladino and O'connor, 1992). In *A. Thaliana* and *E. coli* mutant *(pimt)* does not survive well upon extended culture into stationary phase or upon heat challenge at 55˚C. Maximum activity of PIMT at this temperature has been seen in *Arabidopsis thaliana* (Villa *et al.*, 2006).

PIMT mediated protein repair plays an essential role in long term stress survival at alkaline pH in *E. coli* and damaged proteins in cells those are recovering from nutrient limitation and also in those cells that are able to divide during long term stationary phase (Hicks *et al*., 2005).

2.9 Targets of PIMT and significance of PIMT mediated repair in the protein function

In bacteria only one PIMT target is known. In *E. coli* PIMT repairs deamidated histidine phosphotransferase (HPr) protein which is a component of phosphoenolpyruvate : sugar phosphotransferase system and thus restores its function (Brennan *et al.,* 1994).

Mostly PIMT targets have been identified in mice brain. For example α,β-tubulin, β,γ-actin, α-internexin, dyanamin-1, aconitase-2, α- enolase and creatine kinase B (Zhu *et al*., 2006).

A separate study reported that α and β synuclein as targets of PIMT (Morrison *et al*., 2012). Mass spectrometric analyses of proteins isolated from mice brain revealed microtubule associated protein-2, calreticulin, clathrin light chains a and b, ubiquitin carboxy terminal hydrolase L1, phosphatidylethanolamine-binding protein and stathmin as the targets of PIMT (Vigneswara *et al*., 2006).

Additionally, few PIMT targets have been found in other tissues. PIMT mediated repair should play an important role in modulation of target proteins. PIMT mediated repair restored the function of deamidated calmodulin (Johnson *et al.,* 1987).

PIMT helps in inhibition of protein synthesis by maintaining the activity of anti translational factor 4E-BP2 (Bidinosti *et al*., 2010).

PIMT negatively regulates the tumor suppressor protein p53 by repairing mdm2, which targets p53 to ubiquitin mediated destruction pathway, thereby suppressing the p53-mediated transcription of target genes. In addition, PIMT depletion up regulates the proapoptotic and checkpoint activation functions of p53 (Lee *et al*., 2012). *Arabidopsis* ATP-dependent DEAD box RNA helicase activity was restored by PIMT (Nayak *et al.,* 2013). PIMT mediated repair of histone H4 protein in mouse brain has been reported (Biterge *et al*., 2014). Na^+ / K^+ ATPase gets repaired via PIMT mediated pathway in humans (Adav *et al*., 2014). In *pimt* knock out mice, impaired repair of iso-Asp residue in creatine kinase B have been reported (Dimitrijevic *et al*., 2014). Impaired Asn deamidation and repair of these residues in human SOD-1 have been observed (Bierczyńska-Krzysik *et al*., 2014).

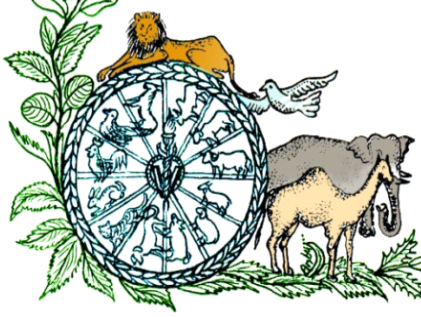
2.10 PIMT in *Salmonella*

Presence of PIMT has been reported in *Salmonella* (Li and Clarke, 1992a). But detail study about its function, targets has not been reported. Cloning, expression and purification of *Salmonella* PIMT has been done in our lab (Dixit, 2013).

2.11 CO-IP can be a suitable method to isolate the PIMT interacting proteins

CO-IP is a technique of isolating a specific antigen bound to another protein moiety. It is basically a modification of immunoprecipitation technique. Targets of PIMT (i. e. α/β-synuclein) from mice brain extract has been isolated by the method of immunoprecipitation (Morrison *et al*., 2012). With the help of this CO-IP, MsrA interacting proteins of *Salmonella* have been isolated in our lab (Rajan, 2014).

Materials and Methods



3 MATERIALS AND METHODS

3.1 *Salmonella* Typhimurium

Initially the culture was obtained from National *Salmonella* center (Veterinary) under Division of Bacteriology and Mycology of our institute (Indian veterinary research institute). In my current research I have used *Salmonella* Typhimurium strain E 2375 maintained in our lab from that initial procurement.

3.2 Δ*pimt* mutant strain of *Salmonella* Typhimurium

Was constructed and kindly provided by Mr. Manoj Kumawat (senior research fellow) of our laboratory.

3.3 Antibodies against PIMT

Anti-PIMT antibody was custom generated by GeNei™, Bangalore and was available in our laboratory. Alkaline phosphatase conjugated with anti–rabbit IgG was procured from Sigma Aldrich, USA.

3.4 Prokaryotic host and vector

For cloning and expression of recombinant PIMT of *Salmonella*, prokaryotic expression vector pET-28c(+) from Novagen® (catalogue no: 69866-3) was used. Competent *E. coli* cells, T7 Express *lysY* (High efficiency, New England BioLabs® Inc, USA) (catalogue no: C30101) was used for production of recombinant proteins.

3.5 Media used for culture of bacteria

Luria Bertani agar (LB agar) (catalogue no: M1151-500G) and Luria Bertani broth (LB broth) (catalogue no: M1245-2.5 Kg) and Hektoen Enteric Agar (catalogue no: M467-500G) were procured from HIMEDIA® laboratories Pvt. Ltd, Mumbai, India.

3.6 Kits

Pierce® Crosslink IP Kit (catalogue no: 26147) and Pierce™ BCA Protein Assay Kit (catalogue no: 23225) were procured from Thermo Scientific, USA.

3.7 Glass wares and Plastic wares

Glass wares were procured from, Riveria™ (India) and Schott Duran (Germany). Plastic beakers were procured from Axiva Sichem Biotech (India). Strict sanitary measures regarding washing and sterilization were taken for these glass and plastic wares. Petridishes and centrifuge tubes along with DNase, RNase and pyrogen free micropipette tips and microfuge tubes were obtained from Tarsons (India). 0.22 µm and 0.45 µm syringe filters were obtained from HIMEDIA® (Mumbai, India) and Nalgene (USA). Vivaspin 6 sample concentrator (3 kDa MWCO) was purchased from GE Healthcare, UK. Chromatographic columns were procured from BIO-RAD. Slide-A-Lyzer® dialysis cassette (MWCO 3.5 kDa) was procured from Thermo Scientific.

3.8 Chemicals used

Unstained protein weight molecular marker (Medium) range was purchased from GeNei™ and pre stained SDS-PAGE standards (Broad range) was procured from BIO-RAD, USA. Lysozyme was procured from Calbiochem, Canada. Skim milk powder was obtained from HIMEDIA® (India). 2-Meracptoethanol (β-ME) was purchased from SIGMA Life sciences, USA. Heterobifunctional cross linker like N-succinimidyl-3-(2-pyridildithio) Propionate was purchased (SPDP) from Thermo Scientific and IPTG (catalogue no: 102101) was obtained from MP Biolaboratories, LLC, France. Reagents for SDS-PAGE like acrylamide, bis-acrylamide, bromophenol blue, Coomasie Brilliant Blue G-250 (CBB G-250), SYPRO® Ruby stain, dialysis tubing, sodium dodesyl sulphate (SDS) , tris base and glycine

were procured from SIGMA Life sciences, USA. Buffer components like disodium hydrogen phosphate, sodium chloride, magnesium chloride, sodium dihydrogen phosphate and some other chemicals like methanol, glacial acetic acid, imidazole, sodium hydroxide, twin 20, were purchased from MERCK, Mumbai, India. Agarose of ultrapure grade was obtained from SIGMA Life sciences, USA. Ammonium per sulphate (APS), dimethyl formamide (DMF), 5-bromo-4-chloro-3' indolyl phosphate (BCIP) and nitro blue tetrazolium chloride (NBT) were procured from SRL, India. Ni-NTA agarose was purchased from Qiagen™, USA.

3.9 Buffers and reagents

The details for buffers and other reagents preparation used in current study have been given in appendix.

3.10 Equipments

SDS-PAGE maxi gel system was purchased from GeNei™, India and Mini-PROTEAN® system was procured from BIO-RAD, USA. Molecular imager® Gel Doc™ XR+, BIO RAD gel documentation system was used and refrigerated centrifuge from Sorvall® RC 5C plus and Eppendorf was used. Nano drop (Model ND 100) Thermo Scientific, USA was used. Distillation water apparatus and water bath were purchased from Jain scientific, India. -80 ºC deep freezer and shaker incubator was procured from New Brunswick Scientific™, USA and -20 ºC freezer were purchased from Cellfrost®, India. Electronic Balance was purchased from (Rad wag®) and magnetic stirrer was obtained from REMI. Spin win micro centrifuge and rocker were purchased from Tarsons. Refrigerated centrifuge (Centrifuge 5430 R) was procured from Eppendorf .Variable pipettes were procured as from Thermo (FINNIPIPETTE® F1) and accupipet from Tarsons. Autoclave and 37 ºC incubator was procured from popular traders, India. UV-visible spectrophotometer was procured from Systronics, India, Ltd. and was used. Biosafety cabinet was procured from Haier, China.

3.11 Expression and purification of *S.* Typhimurium recombinant PIMT

3.11.1 Induction of PIMT

T7 Express *lys*Y competent cells containing PIMT cloned in pET-28c(+) (Dixit, 2013)

was available in our laboratory. The stock culture was taken and streaked on LB plates having kanamycin (30 µg/ ml) and chloramphenicol (10 µg/ ml) and was incubated overnight. Next day morning, isolated colonies were incubated and grown in LB broth containing same antibiotics at 37 ºC for overnight. The overnight grown culture was diluted as 1: 100 in fresh LB broth with antibiotics and was grown to an O. $D._{600}$ of 0.5. IPTG at the final concentration of 1 mM was added and incubated for another three hours at 30 ºC. After harvesting cells at 4 ºC the pellets were washed with chilled PBS and were stored at -80 ºC.

Expression of protein was analyzed in SDS-PAGE. The un-induced and induced pellets were resolved by SDS-PAGE as according to the Laemmli's (1970) protocol. The resolving and stacking gel was made up of 10 % and 3.3 % of acrylamide respectively. The pellets of un-induced and induced were mixed with 5X sample buffer containing β-ME and were boiled for 10 minutes. The lysates were resolved on SDS-PAGE and later stained by CBB G-250. Destaining was carried out with 5 % acetic acid by changing destaining solution several times.

3.11.2 Purification of PIMT

Purification of PIMT was carried out as according to following protocol. Composition of different buffer used is given in appendix..

1. 15 ml of lysis buffer was added to the pellet and it was dissolved properly.
2. 150 µl (100 mg/ ml) of lysozyme was mixed to it and was incubated for 30 minutes on ice.
3. Lysis of cells were done by sonicating the cell suspension as 60 second pulse and 60 second off cycle for 15 cycle with amplitude of 46 Hz.
4. A protease inhibitor (PMSF) was added during lysis.
5. After sonication the lysates were centrifuged at 13,500 rpm for 10 minutes at 4 ºC. The supernatants were filtered through 0.22 µm filter. The filtrate was later used as column load.
6. Pre prepared Ni-NTA column for purification of PIMT was available in our laboratory. Regeneration of column was done with gradient washing with first increasing than

decreasing concentration of ethanol.

7. The column was washed with 10 volumes of lysis buffer

8. The filtrate from step 5 was loaded to the column and let to be flown by gravity flow. The flow through was collected.

9. The unbound proteins were washed by using 10 volumes of wash buffer.

10. Proteins bound to the column were eluted with elution buffer.

11. The fractions collected were stored at -80 ºC.

The purity of protein was checked by SDS PAGE. Staining of gel was done with CBB G 250 and destaining with 5 % acetic acid.

3.11.3 Dialysis of purified PIMT

The protein was subjected to dialysis for five times with different buffers as given below

- First round of dialysis was carried out with 500 ml of buffer having 50 mM NaP (pH 7.5) and 50 mM imidazole (pH 7.5).
- 12 hours later second round of dialysis was carried out with 500 ml of 50 mM of NaP (pH 7.5).

  12 hours later to second round, third round of dialysis with one litre of 50 mM NaP (pH 7.5) was done followed by fourth round with one litre of 50 mM NaP (pH 7.5).
- All the dialysis steps were carried out at 4 ºC in Slide-A-Lyzer® dialysis cassette (MWCO 3.5 kDa).

3.12 Purification of anti-PIMT polyclonal antisera.

Three ml of antisera was diluted in physiological PBS and was subjected to three rounds of precipitation by using saturated ammonium sulfate solution (pH 7.4) at 4 ºC with constant stirring. The precipitated antibody was centrifuged and supernatant was discarded and further re- suspended in physiological PBS and the process was repeated twice. After that the pellet was re-suspended in PBS and was dialyzed five times with chilled 20 mM NaP and

150 mM NaCl to remove excess ammonium sulfate.

3.13 Cross precipitation of anti-PIMT antibodies with the Δ*pimt* strain

One ml of overnight grown Δ*pimt* culture was sub cultured at 1: 100 ratio and was incubated at 37 ºC for 4 hours with constant shaking at 180 rpm. Culture was centrifuged and was re-suspended in 4 ml of PBS. Dialyzed anti PIMT was added to it and was incubated on ice for one hour. The cross precipitated antibody was then centrifuged at 4 ºC and the supernatant was filtered with 0.22 µm filter. The filtered antibody was concentrated to final volume of 1.3 ml by using the Vivaspin 6 concentrator (MWCO 3 kDa). Concentration of antibody was measured by BCA protein assay kit.

3.14 Isolation of PIMT interacting proteins by co-immunoprecipitation

3.14.1 Preparation of cell lysates for co-immunoprecipitation.

Salmonella Typhimurium wild type and Δ*pimt* mutant strains were streaked on HEA plates and after overnight incubation (37 ºC) were inoculated to ten ml of LB broth and again incubated (37 ºC, shaking at 180 rpm) overnight. Both strains were sub cultured at the ratio of 1: 100 in 300 ml of fresh media and were grown up to the O. $D._{600}$ of 0.5. Thermal stress was given to the cultures for two hours at 42 ºC. Cultures were centrifuged at 4 ºC and the pellets were washed with sterile PBS. Then the washing was repeated once with buffer C. The pellets were re-suspended in chilled buffer C and were lysed by sonicating as 30 sec pulse and 30 sec off for 16 cycles at 46 Hz. The supernatants were filtered with 0.22 µm filter and were kept as aliquots at -80 ºC. The concentration of protein in those lysates was measured by using BCA assay method.

3.14.2 Immobilization and cross linking of anti-PIMT antibodies to protein A/G agarose resin.

For immobilization of anti-PIMT antibodies and co-immunoprecipitation of PIMT interacting proteins, the standard protocol provided with pierce crosslink immunoprecipitation kit was followed.

1. To a single spin column provided in kit, 20µl of protein A/G plus agarose resin was poured and was centrifuged at 2000 x g for one minute in order to remove the storage buffer. Four tubes were taken as in duplicates for control and test.
2. 15 µl of antibody (150 µg) was diluted in 85 µl of 1X coupling buffer and was added to each spin column
3. The columns were incubated for one hour at room temperature placed on a shaker.
4. The columns were centrifuged and O. $D._{280}$ of flow through was measured.
5. Washing of columns were carried out thrice with 1X coupling buffer
6. Disuccinimidyl suberate (DSS) diluted in dimethyl formamide (DMF) was added at a final concentration of 2.5 mM to each spin column in order to cross link antibodies to the protein A/G resin.
7. Columns were incubated at room temperature for one hour on a shaker and incubator.
8. Two empty spin were given to remove excess of DSS.
9. 50 µl of low pH elution buffer was added to the column and was centrifuged.
10. The columns were washed twice with 100 µl of elution buffer followed by once with cold IP lysis buffer.

3.14.3 Immunoprecipitation of complex of PIMT and their interacting proteins

The lysates of both wild type and Δ*pimt* were incubated with SPDP (a cleavable cross linker) which facilitated the cross linking of PIMT to its targets. Cross linking in Δ*pimt* served as a control. The reactions were carried out as follows.

S. Typhimurium lysate	700 µl (28 mg)	ΔPIMT lysate	700 µl (28 mg)
SPDP	250 µl (20 mM)	SPDP	250 µl (20 mM)
Purified PIMT	200 µl (200 µg)	Buffer C	200 µl

Incubation of lysates were carried out at 4 ºC for two hours with constant mixing and then at room temperature for 30 minutes. The reactions were quenched by using a tris containing wash buffer. The volume was made up to 1.5 ml using wash buffer.

The diluted lysates were added to the spin columns separately as for *S.* Typhimurium and Δ*pimt* (9 mg protein/ each column). Those columns were incubated at 4 ºC for two hours

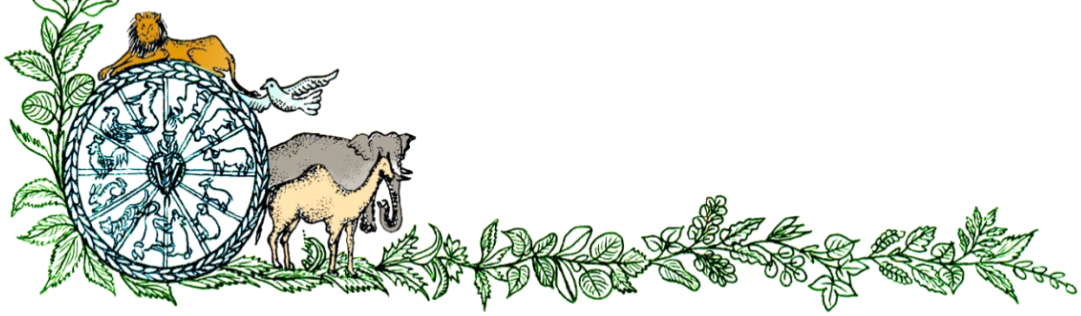
with constant mixing. The columns were washed twice with 200 µl of lysis buffer followed by once with 100 µl of 1X conditioning buffer. After washing the columns were reloaded with the residual volume of lysate and the process was repeated twice. This was done targeting to get maximum yield of protein.

Elution of PIMT-target complex was done as follows:

1. 10 µl of 1M Tris (pH 9.5) was taken in 1.5 ml of eppendorf tubes.
2. The spin columns were transferred to the tubes and were added with 20 µl of elution buffer. It was incubated at room temperature for five minutes.
3. The columns were centrifuged and again 50 µl of elution buffer was added and was incubated for another ten minutes.
4. Centrifuged again and the eluate was stored at -80 ºC.

Staining of the eluted fractions were done by SYPRO® Ruby stain. After staining overnight, it was destained with 10 % methanol followed by 7 % acetic acid solution. The bands were carefully excised under UV light and were submitted for liquid chromatography mass spectrometry (LC-MS) custom analysis at Indian Institute of Science, Bangalore. The data were analyzed by a web based MASCOT server (www.matrixscience.com).

Results



4 RESULTS

4.1 Expression and purification of *S.* Typhimurium PIMT in *E. coli* by Ni-NTA chromatography

PIMT was purified according to the standard protocol (Dixit *et al*., 2013). The stock cultures were streaked on LB plates and incubated overnight. Next day, isolated colonies were inoculated into ten ml of LB broth with antibiotics (Chloramphenicol 10 µg/ ml and Kanamycin 30 µg/ ml) and were incubated overnight at 37 ºC with constant shaking at 180 rpm. The overnight grown culture was sub cultured at a ratio of 1: 100 and grown upto an O. D. $_{600}$ of 0.5. IPTG was added at the final concentration of 1 mM and the culture was incubated for three hours at 30 ºC. SDS-PAGE analysis was done for both un-induced and induced fractions. Staining of the gel with CBB G-250 showed a band in induced lane just above to the 26 kDa of the marker band (Fig. 5), this band was absent in un-induced lane. As our recombinant protein PIMT was fused with a hexa histidine residue, Ni-NTA column was used for protein purification. Elution of protein was done with elution buffer containing 250 mM of imidazole. Ten fractions were collected during elution, the O. D. $_{280}$ was found to be significant in first, second, third, fourth, fifth and sixth fractions. So these fractions (one to six) were pooled and were dialyzed. After final dialysis with 50 mM NaP (pH 7.5), protein was concentrated to three ml with concentrator (Vivaspin 6 columns). The final preparation was stored at -80 ºC in aliquots.

4.2 Purification of anti-PIMT antibodies

Polyclonal sera containing anti-PIMT antibodies were precipitated by three rounds of ammonium sulfate precipitation. In order to avoid non specificity of the anti-PIMT antibodies, it was subjected to cross precipitation with Δ*pimt* strain of *S*. Typhimurium.

4.3 Exposure of *S.* Typhimurium to thermal stress and isolation of PIMT interacting proteins

Generations of isoaspartate residues is a prerequisite for PIMT mediated repair. The high temperature is known for introduction of iso-Asp residues in the proteins (Aswad *et al*., 2000). A link between increase in temperature w.r.t. the formation of isoaspartate has been demonstrated in HPr protein (Sharma *et al*., 1993). Therefore exposure of cells to high temperature enhances the interaction of PIMT to its target proteins. Both wild type and mutant cultures were subjected to thermal stress at 42 ºC for two hours. The cultures were then pelleted and were lysed by sonication. The lysed cells were then pelleted by centrifugation.

PIMT interacting proteins were isolated by co-immunoprecipitation (Alamuri and Maier, 2006 and Rajan, 2014). The Δ*pimt* strain served as a negative control as it did not have PIMT to interact with the iso-Asp containing proteins. This interaction of PIMT and its targets were made covalent by linking them with a cleavable cross linker SPDP. The Spacer arms provided opportunity to proteins which are only as close as 6.8 Aº to get linked covalently to each other. The lysate thus contained PIMT, bound to its targets covalently. The cross precipitated anti-PIMT antibodies were immobilized on a protein A/G agarose resin. The anti-PIMT antibodies was bound to the resin covalently by a non-cleavable cross linker DSS. The complex formed between PIMT and its targets was passed through the anti-PIMT antibodies bound column for CO-IP (Fig. 6). The eluted fractions were boiled with sample buffer containing β-ME. Reduction of disulfide bond between PIMT and its targets rendered them to be resolved in SDS-PAGE.

4.4 Staining with SYPRO® Ruby

Staining of gel with SYPRO® Ruby, a mass spectrometry compatible dye (White *et al*., 2004) gave eight bands in test lane and two bands in control lane. Out of these two bands

in control lane, only one band was overlapping with the test lane (Fig. 7). Bands were excised carefully and were sent for custom analysis by LC-MS to IISC, Bangalore.

4.5 Identification of PIMT interacting proteins by LC MS

LC-MS is a sensitive technique which combines the expertise of both HPLC and Mass spectrometry in order to identify protein from a protein mixture. Out of eight bands sent for analysis only seven were analyzed leaving the overlapping one. The data were obtained in 'mgf' files. An online data bank for mass spectrometry is available as MASCOT server. Data obtained were analyzed in MS/MS ion search category by adjusting the default parameters (Fig. 8). All seven targets gave score more than the thresh hold value. The iterative search continued still with BLASTp analysis to find out the best match. The results of data analysis are being given in table 1. Several targets were found to have $E^{\#}$ score well above the statistical thresh hold ($E^{\#} \leq 0.05$) like (a) yjeP mechanosensitive channel protein (b) napA periplasmic nitrate reductase large subunit (c) ccmA cytochrome c biogenesis ATP binding export protein (d) rplC 50S ribosomal protein L3. Some targets identified were below the $E^{\#}$ score thresh hold value ($E^{\#} \geq 0.05$) as (a) hypothetical protein AK79_23160, partial (b) Tail fiber like protein.

Table 1: LC MS results of PIMT interacting proteins

Name of the protein	E# value	Protein score*	Estimated size(kDa)	% of Asn and Asp	Accession Φ number	Function
yjeP mechanosensitive channel protein	0.052	54	123.64	6.81%	gi|447159489 STM4347	Helps bacteria to combat osmotic stress by opening these channels
rplC 50S ribosomal protein L3	0.012	48	22.33	8.134%	gi|513038519 STM3440	Part of 23S ribosomal subunit. Helps in assembly of 50S ribosomal subunit.
napA periplasmic nitrate reductase large subunit	0.034	56	93.99	10.27%	gi|727823836S TM2259	Detoxifies the NO produced inside cell when cell shifts its metabolism from oxic to anoxic condition
ccmA cytochrome c biogenesis ATPbindingexport protein	0.078	52	23.17	6.05%	gi|446100525	STM3819It is a part of the cytochrome c maturase complex which helps in haem group transfer during the formation of cytochrome c.
Tail fiber like ¥protein	4.7	53	78.27	8.10%	gi|554237183 STM2588	Helps in penetration of bacteriophage by lysis of bacterial receptor
hypothetical protein¥ AK79_23160, partial	4.3	54	45.45	0%	gi|677621978	unknown
hypothetical protein¥ AK79_23160, partial	54	63	45.45	0%	gi|677621978	unknown

#. An E value is an indication of number of times a given protein score can be achieved by incorrect matches from data base search. Inversly related to the repeatability of analyzed results.
*. This is calculated as -10Log (P), where P is the probability indicating that the observed match is a random event.
Φ. Accession number is based on the NCBI data base entry codes.
¥. Proteins having E value less then thresh hold.

The above mentioned proteins were isolated by two different CO-IP experiments performed using the cell lysates of *S.* Typhimurium strain E 2375 serving as test and mutant strain Δ*pimt* as control. Bands were resolved in SDS-PAGE and were sent for LC-MS custom analysis following SYPRO® Ruby staining.

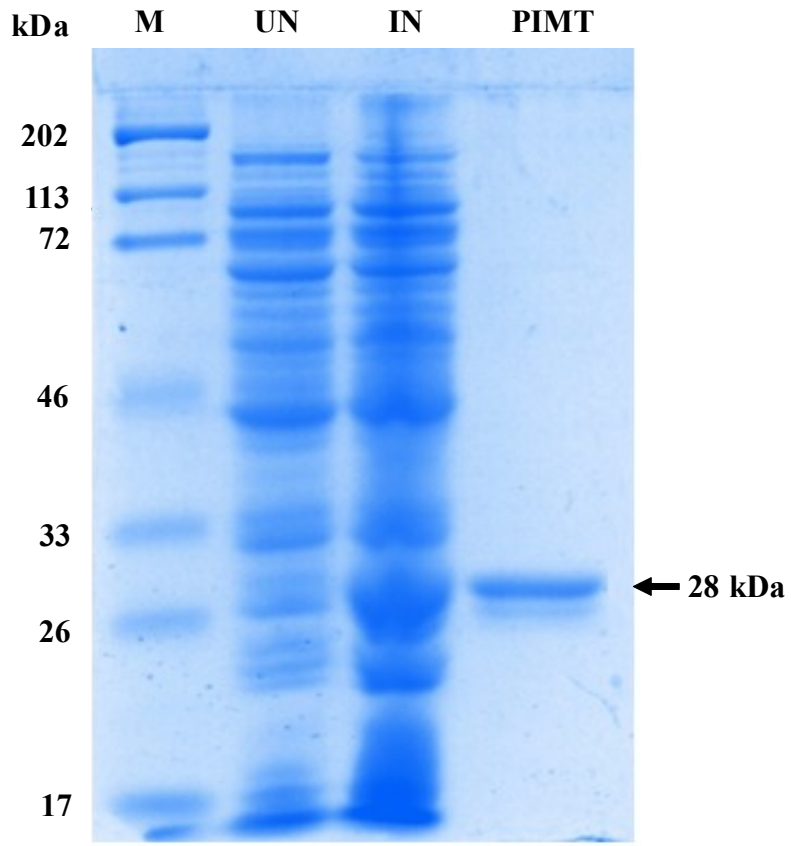


Fig. 5: Purification of PIMT by Ni-NTA chromatography

pET28c(+) plasmid having insert of PIMT gene from *S.* Typhimurium was transformed in T7 Express lysY *E. coli* competent cells. Positive clones were induced using IPTG. lane M contain protein molecular weight marker, lane UN and IN contain un-induced and induced cell pellets respectively. The dark arrow indicates purified PIMT in PIMT lane.

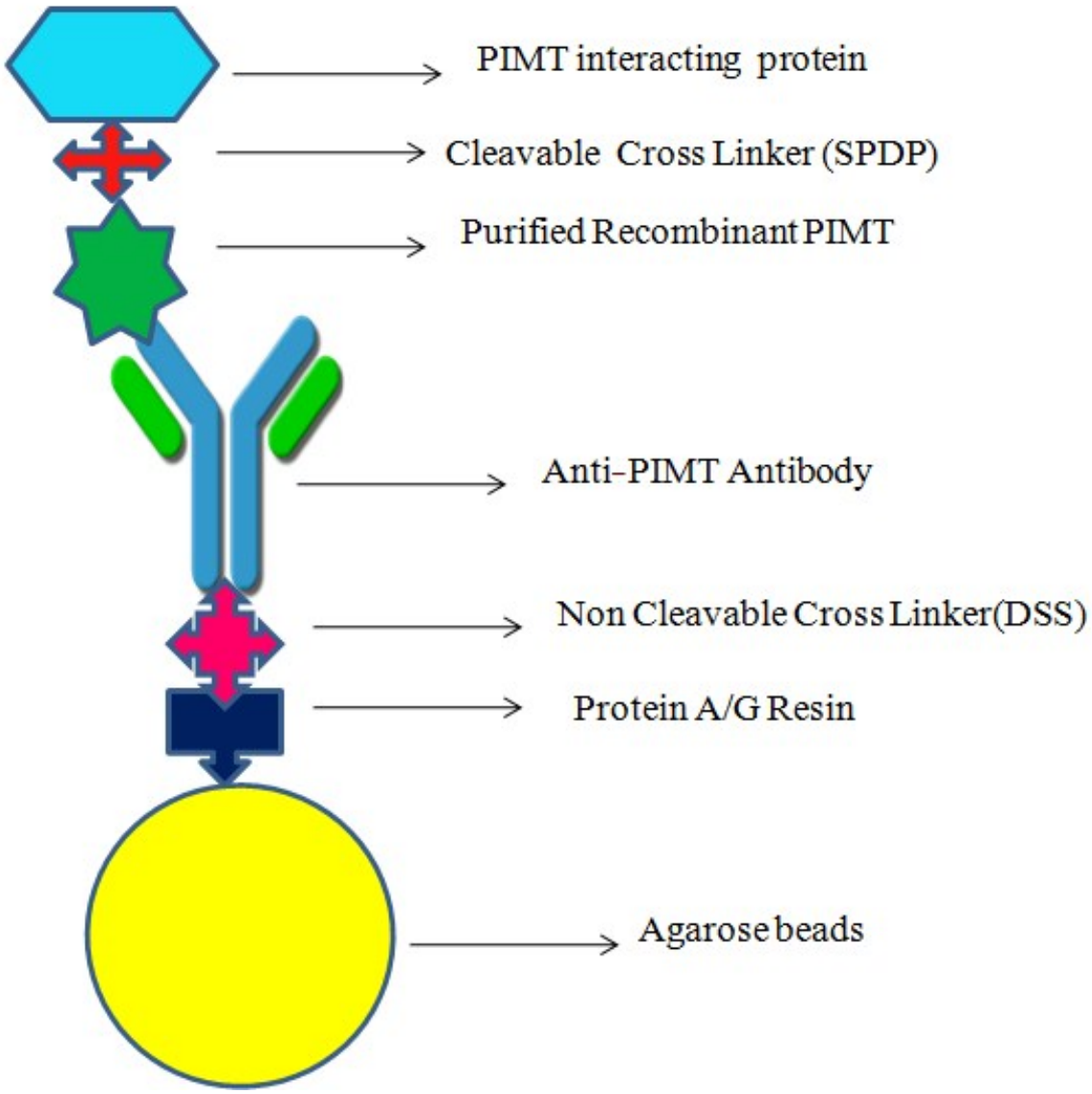


Fig. 6: Schematic representation showing isolation of PIMT interacting proteins from *Salmonella* Typhimurium

PIMT interacting proteins were isolated by co-immunoprecipitation. *S.* Typhimurium cultures were exposed to 42 °C for two hours. The lysates from such cultures were incubated with pure PIMT along with SPDP. The cross-linked PIMT- targets were pulled down by an anti-PIMT antibody bound protein A/G resin coupled to agarose beads. The PIMT-targets adducts were detached by disrupting the disulfide bonds using buffer containing β-ME and analysed on SDS-gels. The pull down from the Δ*pimt* lysates served as control

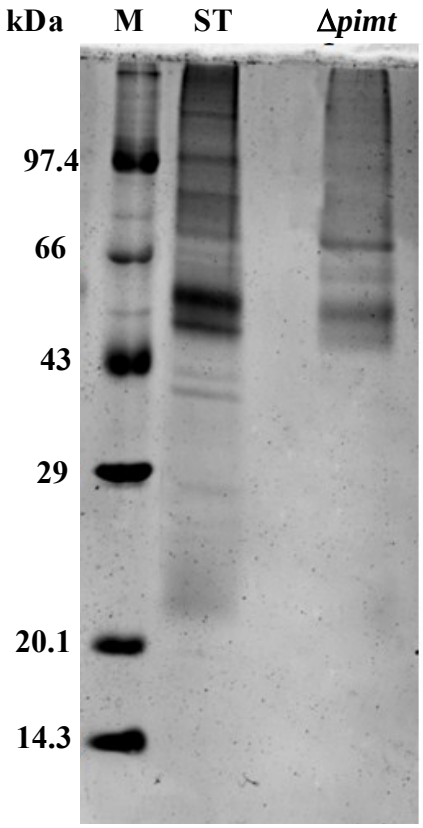


Fig. 7: Staining of CO-IP eluates with SYPRO-Ruby® stain following SDS-PAGE.
The eluates of CO-IP were resolved in SDS-PAGE. Staining of gel was done with SYPRO-Ruby® stain. Lane M is un-stained medium range protein molecular weight marker, lane ST contains CO-IP eluate from *S.* Typhimurium lysates and lane Δ*pimt* contains CO-IP eluate from Δ*pimt* lysates.

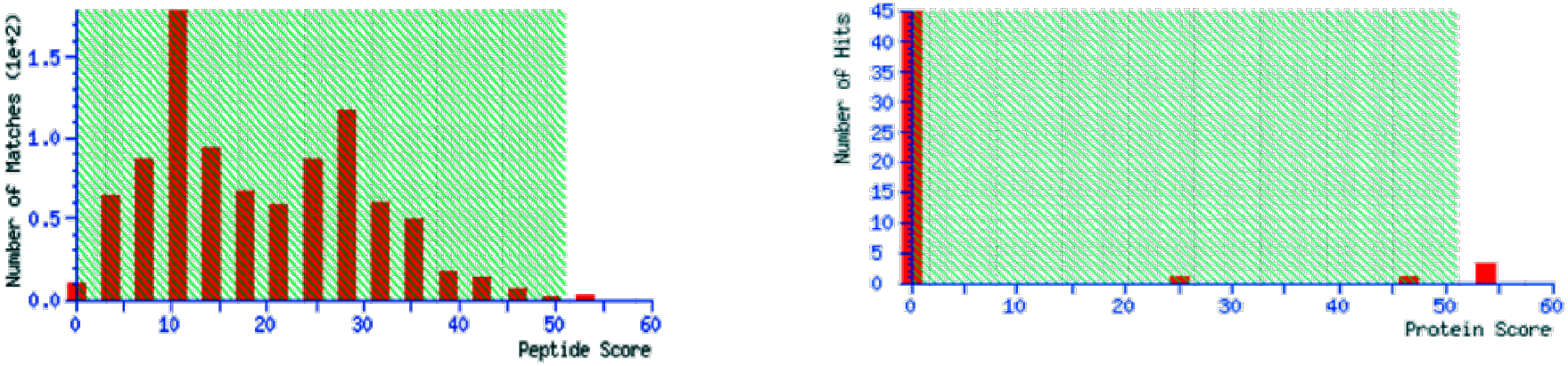


Fig. 8(a): LC-MS spectrum of mechanosensitive channel protein. On average, individual ions scores > 51 (beyond green shading) indicate identity or extensive homology ($p<0.05$)

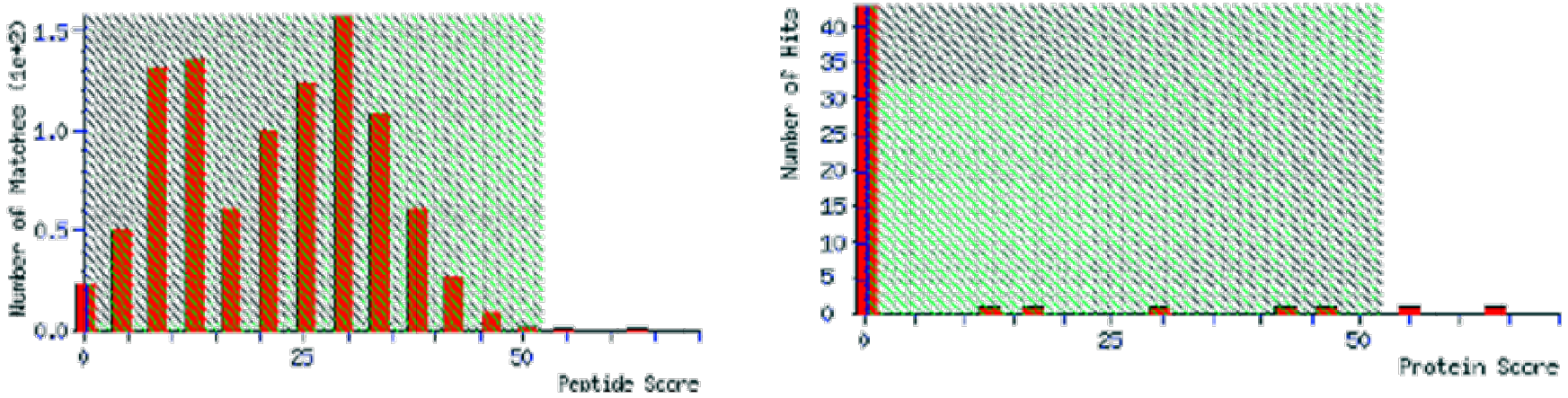


Fig. 8(b): LC-MS spectrum of minor tail protein. On average, individual ions scores > 52 (beyond green shading) indicate identity or extensive homology ($p<0.05$)

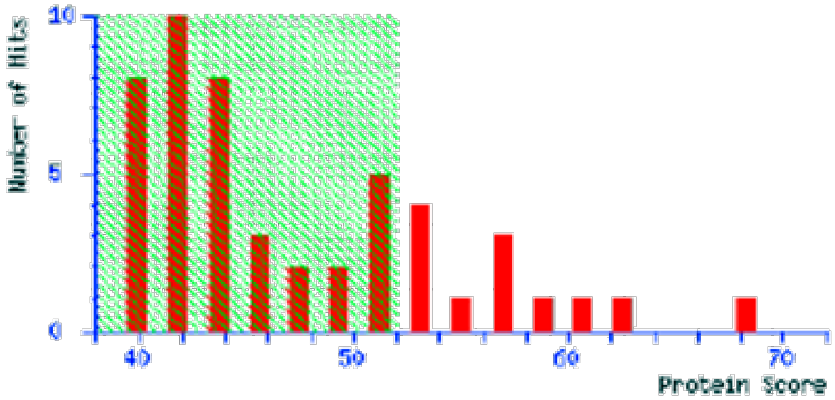


Fig . 8(c): LC-MS spectrum of periplasmic nitrate reductase. On average, individual ions scores > 53 (beyond green shading) indicate identity or extensive homology ($p<0.05$)

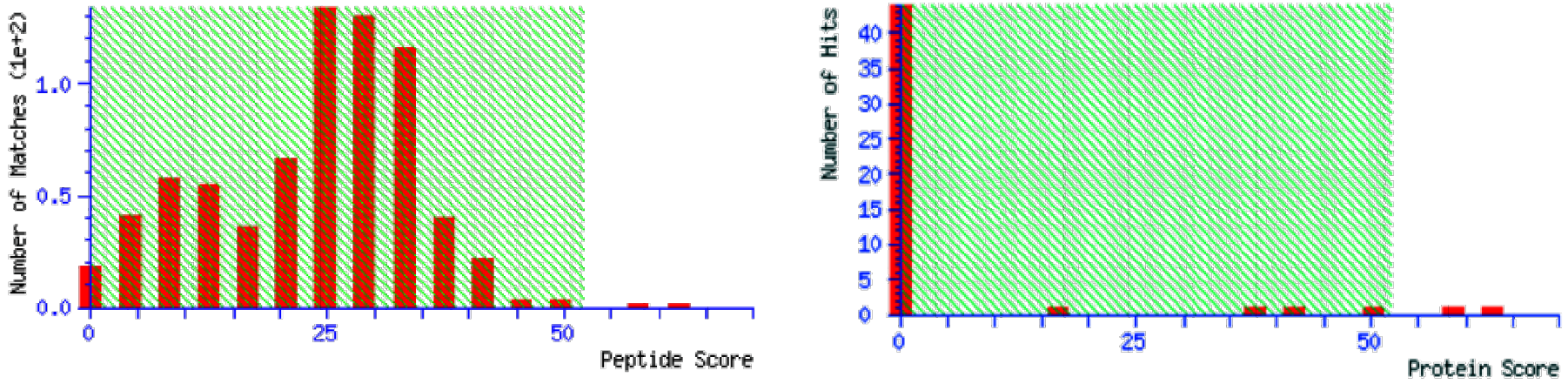


Fig . 8(d): LC-MS spectrum of hypothetical protein AK79_23160, partial. On average, individual ions scores > 52 (Beyond green shading) indicate identity or extensive homology ($p<0.05$)

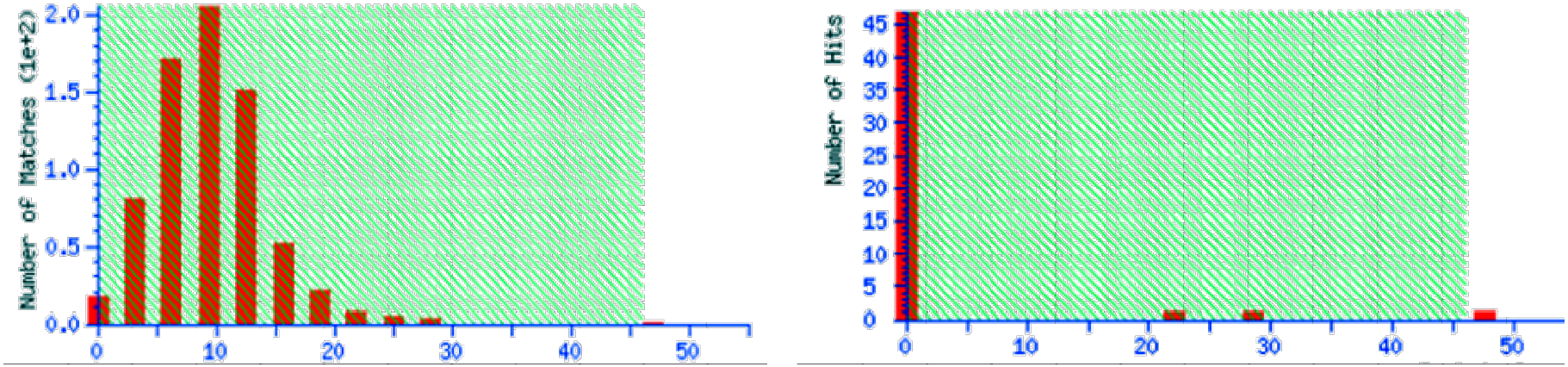


Fig . 8(e): LC-MS spectrum of rplC 50S ribosomal protein L3. On average, individual ions scores > 46 (beyond green shading) indicate identity or extensive homology ($p<0.05$)

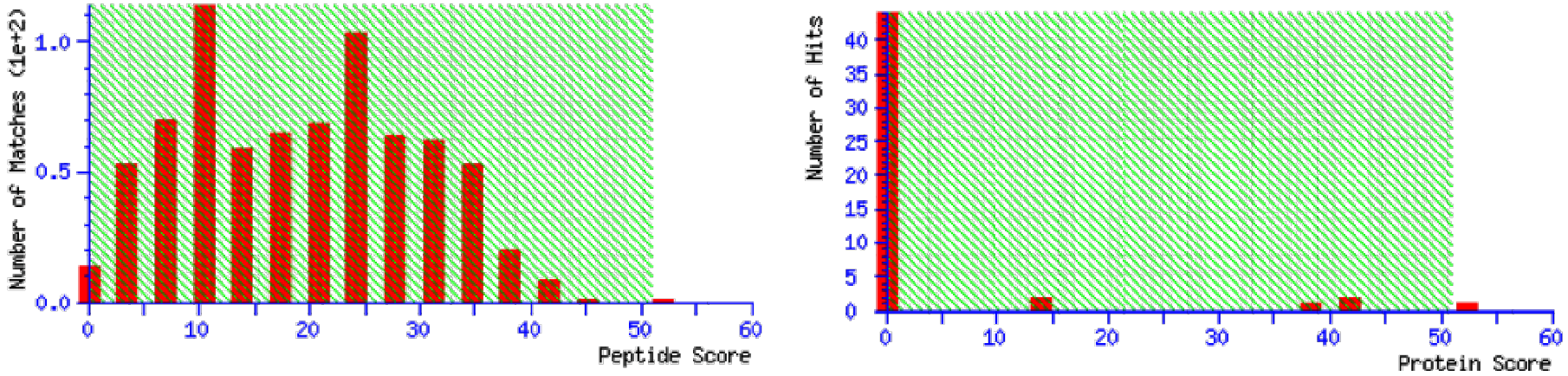


Fig . 8(f): LC-MS spectrum of cytochrome c biogenesis ATPbinding export protein .On average, individual ions scores > 51(beyond green shading) indicate identity or extensive homology ($p<0.05$)

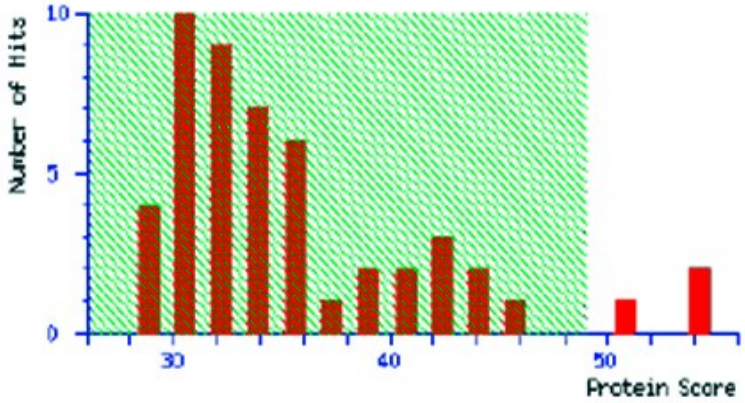
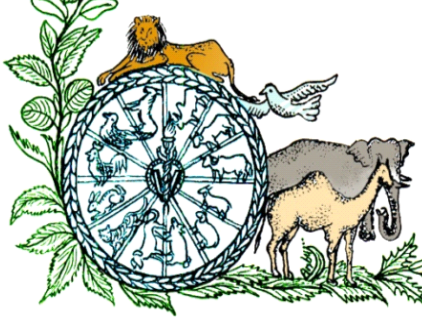


Fig. 8(g): LC-MS spectrum of hypothetical protein AK79_23160, partial. On average, individual ions scores > 49 (beyond green shading) indicate identity or extensive homology ($p<0.05$)

Fig. 8: **LC-MS spectrum of CO-IP eluted samples**

The CO-IP eluates were run in SDS-PAGE and was stained with SYPRO® Ruby. Bands were excised and custom analyzed in LC-MS following trypsin digestion. The data obtained were analysed based on an iterative search method from a web based MS data repository i. e. MASCOT Server. Two different types of graphs have been shown in this experiment. The graph plotted as number of hits against protein score gives idea about non probabilistic basis for ranking protein hits, at the same time graph presenting number of matches against peptide score gives idea about identity or extensive homology. The graphical presentation is an out put of MASCOT Server, so in some cases one of the graphical presentation is missing as per availability of data.

Discussion



5 DISCUSSION

Informational gateway of molecular biology originates from DNA and ends at protein. These mediators are not static rather dynamic in nature which makes them to bring various diversity existing in nature. The functions of macromolecules are multidimensional. They play crucial role in survivability of the organisms in various stress conditions. Stress mediated damages to nucleic acids and their repair has been studied in detail and is well established as compare to protein (Viscik and Clarke, 1995).

Cells handle damaged proteins in two ways, either they destroy them or will repair them. But the fate deciding factor is the economic status of cell with respect to its energy metabolism and distribution. It has been seen that under stress condition cellular protein synthesis becomes severely deprived and if it is expressing any protein then it will be mostly stress specific ones (Neidhart and VanBogelen, 1987). So protein repair systems play important role in cellular survival under various stress conditions.

The damages to proteins may be either conformational or covalent one (Viscik and Clarke, 1995). Conformational change means distortion of the spatial arrangement of the amino acids in the proteins which leads to their unfolding and aggregation. By refolding, chaperones can help in restoring the protein structures and their functions. Roles of different chaperones like DnaK, GroEL, Hsp33 and Prohibitin have been (Ellis, 1987; Wholey and Jakob, 2012; Giannato *et al.*, 2015) documented under various stress conditions.

There are many covalent modifications to amino acids are known but few of them are repairable. Enzymatic systems are present in the cell that can repair disulfide bonds, isomerized prolines (*cis* form) (Lang *et al.,* 1987), modified aspartates (isoasparates) and oxidized Met (Met-SO). These repairs restore the function of proteins thus enhance cellular survival in the stress conditions. Out of these above covalent modifications the conversion of Asp to iso-Asp and its repair is an interesting mechanism. Formation of iso-Asp in a peptide/ protein from an Asn/ Asp residue is a natural process related to aging (Masson *et al.,* 2012). Various stress conditions like temperature, pH (Aswad *et al*., 2000 and Hicks *et al*., 2005), methanol, long term stationary phase (Hicks *et al*., 2005) and consumption of alcohol (Erdozoin *et al*., 2014) also promotes formation of iso-Asp residues.

By repairing iso-Asp to Asp, PIMT is found to be modulating activities of several proteins. For example PIMT plays a significant role in repair of partially deamidated calmodulin protein isolated from bovine brain (Johnson *et al*., 1987).

Few PIMT targets were identified in bovine, mice, Arabidopsis and these were calmodulin (Johnson *et al*., 1987), aconitase 2, enolase, creatine kinase B, synuclein (Zhu *et al*., 2006), Arabidopsis ATP-dependent DEAD box RNA helicase (Nayak *et al*., 2013), CPS-1(Carter *et al*., 2015).

But in case of bacteria only one target has been isolated i. e. HPr in *E. coli* (Brennan *et al.*, 1994). So elucidation of different targets of PIMT in bacterial species may be able to answer a number of limitations in current generation prevention and protection strategies against various bacterial diseases.

In my current study I attempted to find out some of the PIMT interacting proteins of *S.* Typhimurium. This organism is the causative agent of gastroenteritis in human and colonizes in the poultry caecum. The physiological body temperature of poultry is 42 °C and condition of caecum is alkaline. These factors (high temp and pH) are shown to induce iso-Asp residues in the proteins (Aswad *et al*., 2000 and Hicks *et al*., 2005). Therefore it is highly likely that PIMT would play an important role in the *S.* Typhimurium survival in the poultry caecum. Interestingly Δ*pimt* gene knock out strain of *S.* Typhimurium showed defective colonization in

poultry (unpublished observation of our lab). In other words PIMT mediated repair of isoaspartate containing protein is essential for the persistence caecal colonization of *S.* Typhimurium.

The co-immunoprecipitation yielded eight bands in *S.* Typhimurium (Fig. 7, lane ST) lane. Interestingly seven out of these bands were absent in the lane where lysates from Δ*pimt* strain was used for CO-IP (Fig. 7, lane Δ*pimt*). The data obtained after LC-MS analysis and MASCOT based search identified rplC ribosomal L3 protein, periplasmic nitrate reductase (NapA), mechanosensitive channel protein (Mec), cytochrome c biogenesis protein (CcmA), minor tail fiber protein and hypothetical protein AK79_23160 to be interacting with PIMT (Table-1).

Out of seven targets five were of known functions as rplC ribosomal L3 protein, periplasmic nitrate reductase (NapA), mechanosensitive channel protein (Mec), cytochrome c biogenesis protein (CcmA), minor tail fibre protein and two were of unknown functions as hypothetical protein.

Prokaryotic ribosome has been an interesting target to block their replication during the course of infection. In contrast to 80S eukaryotic ribosome prokaryotic 70S ribosome is less complex and has two sub units as 50S and 30S. The 50S is again formed of 23S and 5S rRNA with 30 different proteins (Nomura and Erdmann, 1970). Among these ribosomal proteins most important are L2, L3 and L4, which are involved in peptidyl transferase activity (Franceschi and Nierhaus, 1990). L3 is responsible for 50S ribosomal assembly and binds independently to the 11S 3' fragment of 23S RNA (Spierer *et al*., 1975). L3 has been termed as assembly initiator protein in *E. coli* and its role in assembly of 50S ribosomal subunit has been explored by two separate methods as pulse chase and by reconstituting purified protein (Nowotny and Nierhaus, 1982). The study also suggested that at the excess of rRNA L3 becomes more important as an initiator leading to increase in assembly of dead ends which controls the translation process (Nowotny and Nierhaus, 1982). Single mutation at G52D in the L3 ribosomal protein has shown reduced sporulation in *B. subtilis* (Akanuma *et al.,* 2013). Tiamulin is an antibiotic which inhibits bacterial protein synthesis by binding to peptidyl transferase site at

23S rRNA. A point mutation at 149 Asn/ Asp of ribosomal protein L3 which is near to the peptidyl transferase site confers resistance against tiamulin suggesting the importance of these residues in antibiotic resistance (Bosling *et al*., 2003). In a recent study with different plasmid exchange system mutants of *E. coli* was constructed for L3 gene which showed reduced susceptibility to linezolid and tiamulin (Klitgaard *et al*., 2015). Stress related decrease in expression of ribosomal protein L3 has been studied in fission yeast *Schizosaccharomyces pombe* (Suslu *et al*., 2011).

Looking at the importance of ribosomal L3 protein in ribosomal assembly, it is possible that this protein is a target of host mediated Asp damage. So that translational efficiency of ribosome will compromised and leads to bacterial cell death. However as our data suggests that PIMT interacts with ribosomal L3 protein, so it is possible that PIMT (by repairing iso-Asp to Asp) maintains the translational efficiency of this bacteria under stress.

Salmonella faces several stresses during the infection establishment in poultry. That includes macrophage generated ROS/ RNS, acidic conditions in proventriculus and higher body temperature of poultry. *Salmonella* confronts NO produced from two different sources as exogenous NO, produced in gastric lumen of host derived from dietary nitrate and nitrite (Weitzberg and Lundberg, 1998). Apart from them inflamed intestinal mucosa also contributes to NO pool (Henards and Torrers, 2011). The next source is an endogenous one. Membrane bound nitrate reductase (Nar GHI) produces NO endogenously (Gliberthorpe and Poole, 2008). To deal with this toxic NO, *S.* Typhimurium has developed a complex system consisting of a flavohaemoglobin, flavorubredoxin and a periplasmic nitrate reductase (Muhlig *et al*., 2014). The periplasmic nitrate reductase (NapA) is a non-membrane bound Fe-S containing enzyme which catalyzes following reaction.

$$NO_3^- \xrightarrow{NapA} NO_2^- \xrightarrow{NrfA} NH_4^+$$

NrfA is a nitrite reductase which detoxifies napA produced nitrite by converting it into ammonium ion. Shifting the metabolism in *Salomenlla* from oxic to anoxic condition under nitrate limited environment will up regulate NapA gene. So there is every possibility that this NapA must be playing some important role for *Salmonella* during its survival inside

macrophages. Some other studies supported the importance of NapA; in case of *Campylobacter jejuni napA* gene deletion mutant were less efficient in caecal colonization in poultry (Weingarten *et al*., 2008). In another study, reduction in virulence of *S.* Typhimurium was observed in case of *napA* gene deletion strain of *S.* Typhimurium (Arguello *et al*., 2010). The starvation-stress response (SSR) of *S*. Typhimurium includes gene (NapA) products necessary for starvation avoidance, starvation survival and virulence for this bacterium (Spector *et al.*, 1999). Utilization of the chlorate ion by nitrate reductase enteric pathway (NREP) has been shown to reduce enteric pathogens in chickens (Mc Reynolds *et al.*, 2004).

Third target of PIMT was mechanosensitive channel protein (Mec). Ion channels have been found to be important in various physiological activities of cells starting from ion transport to regulation and maintenance of tonicity. Mechanosensitive (MS) channels respond directly to the membrane tension serving as emergency release valve preventing cells from getting lysed (Zhong and Blount, 2013). In *E. coli,* MS channels consist of mechanosensitive of large conductance (MscL) and mechanosensitive of small conductance (MscS). These mechanosensitive channel proteins were shown to have immense importance in stationary phase survival in *E. coli* (Stokes *et al.*, 2003). The expressions of MscS and MscL were increased following exposure of *E. coli* to osmotic stress or when cells reach to stationary phase (Stokes *et al.*, 2003). In a separate study the effect of *mscS* gene on growth of *E. coli* at varying temperature was evaluated i. e. MscS mutant of *E. coli* showed compromised growth at 42 °C and at 18 °C (Koprowsky *et al*., 2015). *Synechocystis* sp. a cyanobacterium was found to be defective in growth at higher temperature i. e. 44 ºC when its MscL was disrupted. Same study showed that the mechanosensitive channel proteins are essential in controlling the cell volume under thermal stress i. e. 44 ºC (Bachin *et al*., 2015). MscL was reported to be functional in its oligomeric state which comprises of the tetrameric and pentameric forms of MscL. Ratios of tetrameric and pentameric forms in oligomeric MscL is definite for a particular species. The balance of tetrameric and pentameric forms of MscL was found to be modulated at higher temperature.

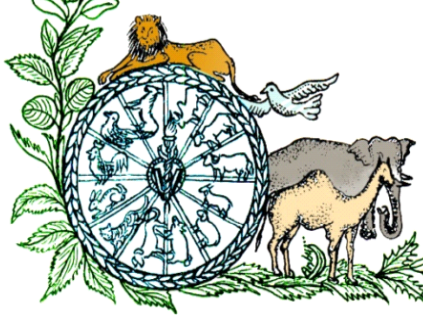
S. Typhimurium encounters temperature stress (42 °C) and chronic colonization (which is just like to be in stationary phase) inside poultry suggesting the importance of PIMT mediated

activity maintenance of Mec for their survival.

Cellular respiration irrespective of prokaryotes or eukaryotes needs different complexes which mediate the transfer of electron from different reducing equivalents in order to generate ATP/ heat. One of these complex is the complex cytochrome c which is a haemoprotein attached covalently to the iron protoporphyrin IX (heme b) (Sanders *et al*., 2010). This covalent attachment by thioether bond is formed between vinyl of heme b and cysteine of apocytochromes as a result of post translational modification. This post translational modification is a function of membrane associated cytochrome c maturase (Ccm) system located in periplasmic space (P side). In Gram -ve bacteria the Ccm system consists of ten Ccm as ABCDEFGHI and CcdA or DsbD (Sanders *et al*., 2010 and Tukarslan *et al*., 2006). Out of these above components CcmA is a peripheral membrane protein with a nucleotide binding domain, conserved walker A, B motifs and ABC signature sequence (Christensen *et al.,* 2007). It is mostly involved in ATP dependent release of holo CcmE. These holo complexes are responsible for final assembly of haem molecule with apocytochrome c. ATP hydrolysis by CcmA is coupled with release of holo CcmE from CcmABCD containing complex to make holo CcmE capable of supplying haem b for cytochrome c generation (Feissner *et al*., 2006). Out of three different modules for cytochrome c formation, CcmA belongs to module I (Verissimo and Daldal, 2014). The module exists in a form of $CcmA_2BC$ which is formed by CcmC, CcmB (Feissner *et al*., 2006). Phenotypic analysis of helABCD deletion strains in plants (analogous to CcmABCD) was shown to be unable to grow under anaerobic photosynthesis (Goldman *et al*., 1997).

The data obtained also gave two other targets which were found to be hypothetical proteins having unknown function and tail fiber like protein helping bacteriophage P22 to penetrate into bacteria.

Summary and Conclusions



6 SUMMARY AND CONCLUSIONS

Cellular stress and the pathways by which cell confronts them has been always an intricate and eclectic mechanism. With the advancement of knowledge and technology we have been able to figure out at least glimpse of those pathways which lead to restoration of normal cellular physiology. Out of various macro molecular damages, damages to protein has been least studied. Various agents, both physical and chemical can bring changes to these macromolecules. Physical agents like UV, temperature and pH along with chemical agents like reactive oxygen species, reactive nitrogen species and unusual metabolites like methanol and ethanol are able to bring changes in protein structure as well as functions. Out of two different types of damages which are common to proteins, repair of covalent modification become a difficult task for chaperones which normally are efficient in restoring the conformational modifications. Different types of covalent modifications have been studied like formation of disulfide bonds, isomerization of proline residues, conversion of Met to Met (SO) and formation of isoaspartate from aspartate/ asparagine residues.

Maintenance of native structure and retention of the overall charge density is essential for proteins to maintain their function. Conversion of aspartate to isoaspartate disturbs both charge and structure of proteins. The isomerization and racemization occurred in this conversion may be due to normal aging process or may be resulted due to various physical and chemical stresses. Peptide bound asparagines and aspartates get converted to isoaspartates via a succinimide intermediate. A constitutively expressed enzyme protein L-isoaspartate (O) methyl transferase converts the iso-Asp residue to its labile methyl ester so that the restoration cycle continues via succinimide intermediate leading to formation of aspartate and iso-Asp in a ratio

of one: three respectively. This cycle continues till the iso-Asp residues get reconverted back to Asp.

Salmonella Typhimurium, a causative agent of gastroenteritis across the globe is transmitted by poultry and its product (OIE, 2010). *Salmonella* Typhimurium inside the poultry encounters various stresses. The intra-phagocytic lifestyle of *Salmonella* Typhimurium exposes it to various ROS/ RNS generated by macrophages. Interestingly body temperature of poultry is 42 ºC which is 5 ºC more as compare to human. These stresses are known to induce iso-Asp in the proteins thus compromising their functions. Out of these stresses temperature contributes significantly in aspartate deamidation, isomerization and racemization. It is conceivable that PIMT mediated repair of iso-Asp in these proteins thus help *Salmonella* Typhimurium to survive in poultry. In current study we attempted to isolate PIMT interacting proteins of *Salmonella* Typhimurium or I could say the proteins of *Salmonella* Typhimurium in which formation and repair of iso-Asp is occurring.

Recombinant PIMT of *Salmonella* was expressed in *E. coli* (Expression system T7 *lysY*). Poly clonal anti-PIMT antisera available in our laboratory were purified by three rounds of ammonium sulfate precipitation followed by cross-precipitation with Δ*pimt* strain. The *Salmonella* Typhimurium was subjected to 42 ºC stress (in order to induce iso-Asp formation). The PIMT interacting proteins from such stressed samples of *Salmonella* Typhimurium were isolated by standard CO-IP protocol, utilizing purified anti-PIMT antibodies. The knockout strain for *pimt* gene (Δ*pimt* strain) was served as a control. The PIMT interacting proteins were resolved on SDS-gels and custom analyzed by LC-MS. The results were analyzed by an online MASCOT server. Proteins having score more than the thresh hold values were considered as targets.

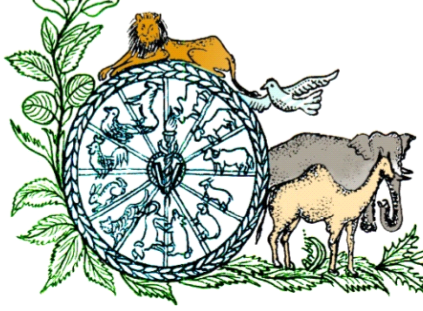
The analysis gave us seven different targets. Five of them were of known function but rests two were found to be of unknown functions. Mechanosensitive channel proteins having importance in stationary phase survival was found to be a target. rplC riobosomal L3 protein which helps in 50S ribosomal complex formation was also found to interact with PIMT. Apart from these two another three targets like periplasmic nitrate reductase having role in

NO detoxification in anoxic conditions, tail like fibre protein, helping phage P22 replication in *Salmonella* and cytochrome c biogenesis protein (CcmA), having role in maturation of cytochrome c were also found to interact with PIMT of *Salmonella*.

Conclusions

- Seven of the PIMT interacting proteins in *Salmonella* Typhimurium were isolated.
- Many targets were found to have $E^{\#}$ score well above the statistical thresh hold like (a) yjeP mechanosensitive channel protein (b) NapA periplasmic nitrate reductase large subunit (c) CcmA cytochrome c biogenesis ATP binding export protein (d) rplC 50S ribosomal protein L3
- The targets were found to contain Asp and Asn residues more than the average percentage of 4-5 %.
- Some of these targets were found to be important for survival as well as virulence of different bacteria like *C. jejuni*, *S.* Typhimurium, *S.* Gallinarum and *B. subtilis.*

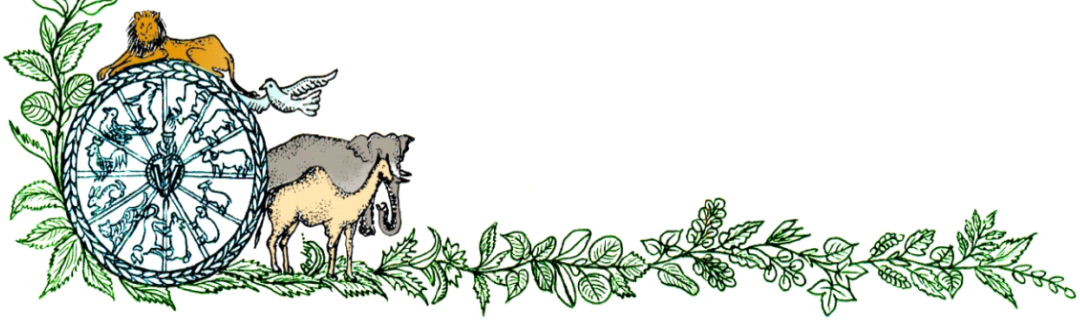
Mini Abstract



7 MINI ABSTRACT

Protein L- isoaspartate (O) methyltransferase (PIMT) repairs the abnormal iso-Asp residues formed via succinimide intermediate in proteins. Various stress conditions are known to be associated with iso-Asp formation like exposure to methanol, oxidants, high temperature, alkaline pH and stationary phase etc. Temperature and pH were found to have significant contribution in generation of iso-Asp residues. These abnormal iso-Asp residues can distort the function of protein by introducing a kink in peptide back bone. Targets of PIMT has been studied in many species including mice, humans, *A. thaliana*,*C. elegans* etc. In contrast targets of PIMT are least studied in bacteria. In the current study we isolated and identified targets of PIMT in *Salmonella* Typhimurium. This bacterium normally colonizes in caecum of poultry. In case of poultry pH of caecum is slightly alkaline and the body temperature is relatively higher i. e. 42 ºC. This relative high temperature must be having some effect in generation of iso-Asp residues. By repairing these abnormal residues, PIMT must be playing some important role in survival of *Salmonella* Typhimurium inside poultry. In order to isolate PIMT interacting proteins, thermal stress was given to both Δ*pimt* and wild type as 42 ºC for two hours and the lysates were co-immunoprecipitated with purified anti-PIMT antibodies. SDS gel of those eluates showed several bands in wild type lane. Those bands were analyzed by LC-MS. Four of those targets were found to be having significant $E^{\#}$ value as (a) yjeP mechanosensitive channel protein (b) periplasmic nitrate reductase large subunit (c) cytochrome c biogenesis ATP binding export protein (d) 50S ribosomal protein L3. Many of them were found to play an important role in survival of various organisms.

लघु सारांश



8 लघु सारांश

प्रोटीन एल. आइसो आस्परटेट (ओ) ओ मिथाइल ट्रान्सफरेज (PIMT) सक्सीनामाइड द्वारा जनित असामान्य आइसो–आस्परटेटअवशेष की मराम्मत करता है। विभिन्न तनावपूर्ण स्थितियाँ जैसेः मिथेनाल एन्टी आक्सीडेन्ट, उच्च तापमान क्षारीय pH निष्क्रिय अवस्था इत्यादि आइसो आस्परटेट के निर्माण में सम्मिलित पायेगये है। तापमान और pH आइसो आस्प के निर्माण में महत्वपूर्ण हैं। यह असामान्य आइसो–आस्प अवशेष पेप्टाइड संरचना को प्रभावित करके प्रोत्स को निष्क्रिय कर देता है। पी.आई.एम.टी. से मराम्मत होने वाले प्रोटीन के बारे में चूहे, मनुष्य ए. थैलीयाना, और सी. एलीगेन्स में विस्तारपूर्वक अध्ययन किया जा चुका है। परन्तु वैक्टीरिया में यह अभी तक सीमित है। वर्तमान की इस अध्ययन मेंसाल्मोनेला में PIMT के द्वारा मराम्मत होनवाले प्रोटीन को पथ्थक करके उनकी पहचान की गई है। यह वैक्टीरिया मुर्गियों के आँत में पाया जाता है। सामान्यतः मुर्गियों में आँत का pH क्षारीय तथा शरीर का तापमान $42^{o}C$ होता है। यह उच्च शारीरिक तापमान आइसो–आस्प अवशेष के गठनमें भूमिका निभाता है। इस तरह सेइन अवशोषों की मराम्मत करके PIMT साल्मोनेला टाइफीमुरियम के जीवन चक्रण में महत्वपूर्ण भूमिका निभाता है। PIMT द्वारा मराम्मत होन्वाले प्रोटीनों को पथ्थक करने के लिए म्यूटेंट *pimt* एवं साधारण साल्मोनेला को दो घन्टे के लिए उच्चताप पर ($42^{o}C$) रखा गया एवं उनके लाइसेट कोशुद्ध एन्टी– PIMT एनटीवॉडी केसाथ को इम्युनोप्रेसीपिटेट किया गया। एस.डी.एस पेज चलानेपर इन प्रोटीन में कई सारे बैण्ड दिखाये । इन बैण्ड के जाँच LC-MS में किया गया है। इनमें से चार बैण्ड अपेक्षाकृत E-value से उपर थे, जैसे कि (क) yjeP mechanosensitive चैनल प्रोत्स (ख) periplasmice नाइट्रेट रिडक्टेस बड़े सबयूनिट (ग) साइटोक्रोम ग जीवजननएटीपी बाध्याकारी निर्यातप्रोत्स (डी) 50 के राइबोसोमल प्रोटीन L3। उनमें से कई, विभिन्न जीवों के अस्तित्व में एक महत्वपूर्ण भूमिका निभाता है।

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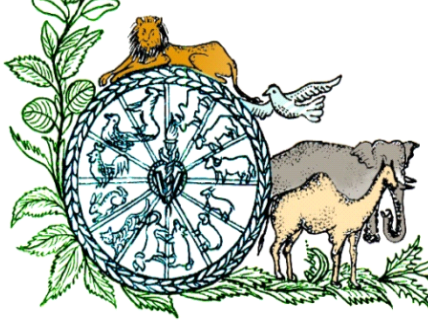
Appendix



APPENDIX

Antibiotic stocks

a) Kanamycin stock (30 mg/ ml)

Kanamycin	45 mg
Autoclaved distilled water	1.5 ml

Finally the solution was filtered by 0.22 µm filter and stored at -20 °C

b) Chloramphenicol stock (1 mg/ ml)

Chloramphenicol	25 mg
100 % ethanol	1 ml
Autoclaved distilled water	24 ml

The solution was filtered by 0.22 µm filter and was stored at -20 ºC

1) Sodium phosphate buffer (0.2 M)

Disodium hydrogen phosphate dihydrate	35.6 g
Sodium dihydrogen phosphate dihydrate	31.2 g

0.2 M solution of disodium hydrogen phosphate and sodium dihydrogen phosphate were prepared by dissolving 35.6 g and 31.2 g respectively in 1000 ml water each. To 800 ml of disodium hydrogen phosphate, sodium dihyrogen phosphate was added till the pH became 7.5. The solution was then filtered by 0.22 µm and stored at 4 ºC.

2) Sodium chloride (1.5 M)

Sodium chloride	87.7 g

Dissolved in 1000 ml of water and was filtered.

3) Imidazole (0.5 M)

Imidazole	34.04 g

Dissolved in 1000 ml of water and was filtered.

4) Potassium chloride (1 M)

Potassium chloride	0.746 g

Dissolved in 10 ml of water and was filtered

5) Magnesium chloride (0.5 M)

Magnesium chloride (hexa hydrate)	1.01 g

Dissolved in 10 ml of water and was filtered

Solutions for induction and purification

1) IPTG (100 mM)

IPTG	23.83 mg
Distilled water	1 ml

The solution was filtered by 0.22 µm syringe filter

Buffers used for purification

a) Lysis or equilibration buffer (Buffer A)

0.2 M Sodium phosphate buffer (pH 7.5)	25 ml
1.5 M Sodium chloride	20 ml
0.5 M Imidazole	8 ml

Final volume made upto 100 ml with double distilled water. The final concentration of the solution is 50 mM Sodium phosphate, 300 mM Sodium chloride and 40 mM imidazole.

b) Elution buffer (Buffer B)

0.2 M Sodium phosphate buffer (pH 7.5)	25 ml
1.5 M Sodium chloride	20 ml
0.5 M Imidazole	50 ml

Final volume was made upto 100 ml with double distilled water. The final concentration of the solution was 50 mM sodium phosphate, 300 mM sodium chloride and 250 mM imidazole.

Co-immunoprecipitation buffer (Buffer C)

0.2 M Sodium phosphate buffer (pH 7.5)	50 ml
0.5 M Magnesium chloride	4 ml
1 M Potassium chloride	10 ml

Final volume was made upto 200 ml with double distilled water. The final concentration of the solution was 50 mM sodium phosphate, 10 mM magnesium chloride and 50 mM potassium chloride.

Solutions for SDS-PAGE

a) Separating gel buffer (200 ml, pH 8.6)

1 N HCl	96 ml
Tris	73.2 g
TEMED	0.46 ml
SDS	1.6 g

pH was adjusted to 8.6 using HCl. The final volume made up to 200 ml with distilled water.

b) Stacking gel buffer (100 ml, pH 6.8)

Tris	12.1 g
TEMED	0.232 ml
SDS	3.2 g

pH was adjusted to 6.8. The final volume was made up to 100 ml with distilled water.

c) Acrylamide-bis-acrylamide solution (30 %)

Acrylamide	29.2 g
Bis-acrylamide	0.8 g

Volume made up to 100 ml with distilled water.

d) Ammonium per sulfate (APS)

20 % solution in distilled water.

e) Stacking gel (3.3 %)

Acrylamide (30 %)	1.5 ml
Stacking gel buffer	1.5 ml
Distilled water	9 ml
APS (20 %)	75 µl

f) Separating gel (10 %)

Acrylamide (30 %)	10.6 ml
Separating gel buffer	4 ml
Distilled water	17.4 ml
APS (20 %)	100 µl

g) Sample buffer (5X) (50 ml, pH 6.8)

Tris	1.89 g
SDS	5 g
Glycerol	25 ml
Bromophenol blue few crystals	

h) Electrode buffer

For 1000 ml : 3.0 g Tris + 14.4 g glycine + 1.0 g SDS

i) Staining solution

CBB G-250	0.5 g
Methanol	250 ml
Acetic acid	50 ml
Distilled water up to 500 ml	

CBB G-250 was added to methanol, mixed on a magnetic stirrer for overnight. Acetic acid was then added and stirred for an additional hour. Finally volume was made up with distilled water and filtered by Whatman filter paper No.1. The solution was stored in an amber colored bottle at room temperature.

j) Destaining solution (5 % acetic acid)

Acetic acid 50 ml Distilled water 950 ml

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