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# Recombinant expression, purification, and refolding to bioactive protein L-isoaspartate-O-methyltransferase of buffalo (*Bubalus bubalus*)

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The cryopreserved semen is used in assisted reproductive technologies. There is ~~a~~ continued oxidative stress during cryopreservation, causing oxidative damage to spermatozoa. The isoAsp formation during oxidative stress results in the loss of protein function. The protein L-isoaspartate-O-methyltransferase (PIMT) repairs back the isoAsp residues to L-aspartyl, restoring the lost functionality of the affected proteins. The quality of cryopreserved semen could be improved by using PIMT as a supplement to semen extender. The objective of the study was to express biologically active buffalo recombinant PIMT (rPIMT). The ~650 bp PCR-amplified PIMT was cloned and sequenced. The protein was consistently expressed as inclusion bodies in *E. coli* T7 Express *lys*S cells and then bulk-purified under denaturing conditions. The protein sequence showed 96.55% identity with the bovine PIMT protein. The purified rPIMT showed a single protein band corresponding to 31 kDa on SDS-PAGE and Western blot. A synthetic isoaspartate-containing peptide served as the substrate to assess enzymatic activity. The effective methylation of iso-aspartate residues in the synthetic peptide substrate served as evidence of PIMT enzyme activity. Thus, the bioactive rPIMT could potentially be added to buffalo semen extender to repair the oxidatively damaged seminal plasma proteins that arise during cryopreservation.

**Keywords**: Buffalo, Enzyme activity, Protein expression, Recombinant PIMT

Cryopreservation is commonly employed to preserve the germplasm of endangered species, elite animals, and terminally ill patients for their subsequent use in assisted reproductive techniques. In veterinary practice, cryopreserved semen is most commonly used for artificial insemination to deliver superior germplasm. During cryopreservation, the semen gets exposed to osmotic shock and cold shock, and there is also an increase in dissolved oxygen levels. Such factors contribute to the overproduction of reactive oxygen species (ROS)[1]. Proteins are among the most common targets of oxidative modifications. Thus, the spermatozoa undergo protein modifications due to the oxidative stress of freezing-thawing which impair their functional lifespan[2].

Proteins need to keep their structural integrity for their biological functions. However, several stressors can cause damage to proteins, resulting in changes in their stability and activity. In proteins, the iso-aspartyl residues formed spontaneously or due to stress by deamidation of asparaginyl or aspartyl isomerization (isoAsp/isoD)[3]. IsoAsp residue formation is linked to the loss of protein function in ageing[4]. These residues act as “hot spots” for the isomerization process, resulting in abnormal isoAsp residue in proteins[5]. The additional methylene group of the isoaspartyl residue forms a β-peptide linkage and generates kinks in the polypeptide backbone further disrupting the existing secondary and/or tertiary structures[6], thus affecting protein functions[7-9]. Protein aggregation, loss of function, and increased susceptibility to proteolytic degradation have all been linked to the buildup of iso-aspartate residues in proteins.

To combat oxidative stress, there are cellular enzymatic and non-enzymatic antioxidant defenses involved in the scavenging of ROS molecules, and repair and/or degradation of the damaged cellular macromolecules[10-12]. The enzymes that serve to repair oxidative protein modifications are methionine sulfoxide reductases (Msr), protein L-isoaspartate methyl transferase (PIMT) and fructosamine 3-kinase (FN3K) *etc*. Protein methyl transferases (PMTs) are broad class of enzymes that catalyze protein methylation[13]. The *PCMT1* gene encodes the PIMT (EC 2.1.1.77) and is placed in the transferase family[14-17]. PIMT catalyzes the conversion of the iso-aspartyl residue back to the L-

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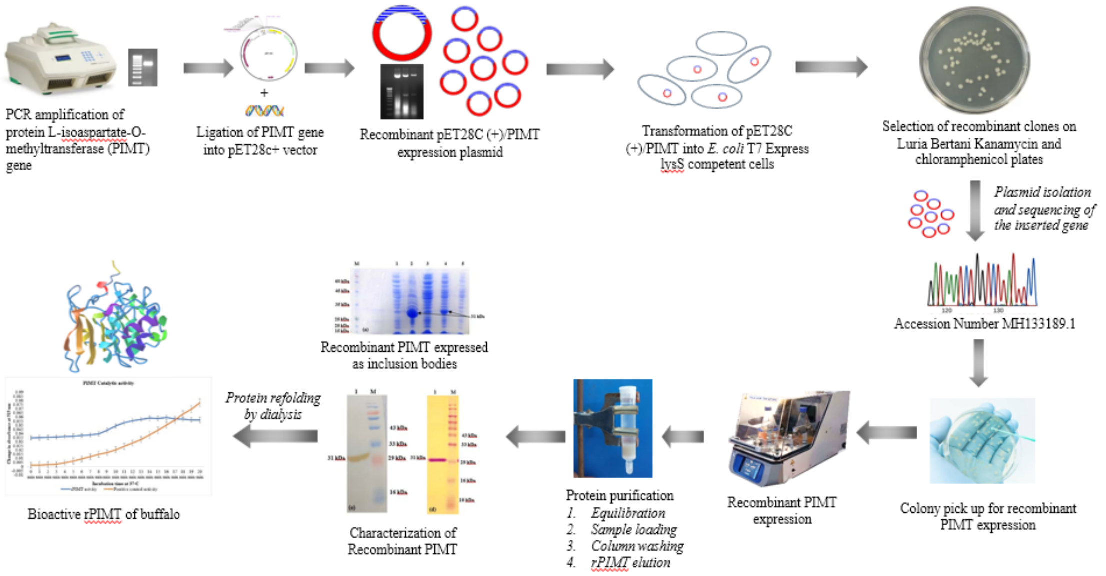

Graphical abstract

aspartate by methyl group transfer from *S*-adenosyl-L-methionine (SAM) to the beta-carboxyl group[18,19]. The PIMT plays a crucial role in intracellular protein repair in both microbial, plants and mammalian systems[12,20,21]. PIMT repairs calmodulin[22], histidine phosphotransferase (HPr) in *E. coli*[23,24], and histone H4 in mouse brain[25]. In Arabidopsis, PIMT restores ATP-dependent DEAD box RNA helicase activity[26].

An antioxidant that lessens oxidative stress and improves cryopreserved semen quality could be of great help. Recently, considerable research interest has been created towards the repair of protein modifications due to oxidation. With increasing information on oxidatively damaged proteins of cryopreserved semen[2], studying the enzymes involved in repairing oxidatively damaged proteins is essential. The enzyme PIMT has attracted lots of interest due to its role in protein repair and possible implications in reducing age-related protein disorders. Under stress, PIMT restores protein function and their survivability[27]. Though PIMT has been found and studied in several taxa's, including plants and animals, its recombinant expression and purification have remained technically challenging. Successful bioactive rPIMT expression would allow its use in repairing protein oxidation and associated diseases. The rPIMT could be added to the buffalo semen extender to repair the oxidatively damaged seminal plasma proteins that arise during cryopreservation. Hence, the aim of the present study was to clone and express the rPIMT in catalytically active form.

## Materials and Methods

### PCR amplification, cloning and sequence analysis of PIMT gene

The research work was conducted at ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly (UP), India. The Institutional Animal Ethics Committee and the Institutional Biosafety Committee approved this study (F. No. 26-1/2015-16/J. D(R) dated 28/02/2019).

Testicular tissue samples from healthy adult buffaloes were collected from a local abattoir (Bareilly, Uttar Pradesh, India) and transported to the laboratory on ice for further processing. The total RNA was isolated from 100 mg of tissue using TRIzol ™ reagent (Invitrogen, USA). About 1 µg of the total RNA was reverse transcribed into cDNA using the iScript™ cDNA synthesis kit (BIO-RAD, USA) following the recommended protocol. Gene-specific primers were designed using Primer BLAST online software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) with 5′ *Hind*III and 3′ *Xho*I restriction sites. The forward 5′-GTCGACAAGCTTCCACTCGGAGCTAATCCACA-3′ (PIMTF4HindIII) and reverse 5′-CACCACCTCGAGCTGGACCACTGCTTTTCT-3′ (PIMTR4XhoI) primers were synthesized (GCC BIOTECH, India). The PCR mixture of 50 µL volume, consisting of 1x Taq buffer with 20 mM $MgCl_2$, 0.4 mM of dNTP mixture, 50 pmol each forward and reverse primers, and 100 ng of buffalo testis cDNA as template and 1 U Taq DNA polymerase enzyme. The reaction mixture was then subjected to initial denaturation of 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 1 min and, extension at 72°C for 1 min 30 sec, and final extension at 72°C for 10 min. After gel purification of the PCR amplicon, the gene was cloned into the pGEMT cloning vector and transformed into DH5α competent cells. Blue-white screening was used to select recombinant clones on Luria Bertani Ampicillin plates (50 µg/mL). Further, one blue and 5 white colonies were picked up and grown overnight in LB broth. Plasmids were isolated from the overnight grown culture and subjected to restriction digestion by adding Fast digest *Hind*III and Fast digest *Xho*I restriction enzymes. The digested mixture was then run on 1% agarose gel, the released insert was then extracted from the gel using a Gene jet gel extraction kit (Thermoscientific, USA) and stored at -20°C for further use.

The gel purified insert DNA was cloned into the pET28C(+) expression vector and transferred into *E. coli* T7 Express *lys*S competent cells. Recombinant clones were selected on Luria Bertani Kanamycin (50 µg/mL) and chloramphenicol (10 µg/mL) plates by their resistance to both antibiotics. One of the recombinant plasmids was custom sequenced for both ~~the~~ strands, and the sequence details are available at GenBank accession number MH133189.

### Sequence homology and 3D structure prediction of buffalo rPIMT

The deduced amino acid sequence from the buffalo rPIMT nucleotide sequence was initially examined for its similarity with other species using the BLASTp software. Thereafter, the PIMT protein sequences of major domestic animals were compared to the buffalo PIMT protein to find homologous sequences and conservation using Clustal Omega software. Thereafter, the buffalo PIMT protein sequence (GenBank: AZQ05603.1) was used for the 3D structure prediction and analysis. The major hindrance in the structure analysis of the buffalo PIMT is the lack of experimental 3D structure in protein data bank (PDB) due to poor annotation and characterization of the buffalo genome. However, this problem was resolved by curating the 3D structure of the buffalo PIMT (AF-A0A3Q9DY96-F1-model_v4), cattle PIMT (AF-P15246-F1) and Human PIMT (PDB ID: 1I1N) from the Alpha Fold and protein database, respectively.

### Secondary structure alignment

Structure alignment of the buffalo, cattle and human PIMT proteins was done by using the DALI web server, which was later used to annotate the structural elements. Further, the elements of the secondary structure of proteins were predicted using the DSSP server, DALI web server and literature curation.

Since the buffalo rPIMT protein sequence showed a significant homology with the human PIMT protein, whose crystal structure with S-adenosyl homocysteine at 1.6-A resolution was available, it was used for further analysis of the buffalo PIMT enzyme structure (https://www.rcsb.org/structure/1I1N). However, we supplemented it by comparing them with human PIMT, the only mammalian PIMT whose experimental structure is available in PDB.

### Expression, characterization and purification of recombinant PIMT

The sequenced recombinant pET28C (+)/PIMT clone was streaked on LB agar plates containing kanamycin (50 µg/mL) and chloramphenicol (10 µg/mL). One of the bacterial clones was picked from the plate and inoculated into an LB broth tube containing 50 µg/mL of kanamycin and 10 µg/mL of chloramphenicol followed by induction with different concentrations of IPTG (0.05 mM, 0.2 mM, 0.5 mM and 1 mM) at different temperatures starting from 37°C, 30°C, and at refrigeration temperature after reaching the absorbance $A_{600}$ of the culture medium to 0.4-0.6 to get protein in the soluble form. The recombinant PIMT consistently formed inclusion bodies. Hence, the expression conditions were optimized having induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 5 h, at 37°C, 180 rpm. Thereafter, 1 mL pellets of each induced and uninduced culture were collected and reconstituted in 160 µL double-distilled water and mixed with 40 µL 5X SDS-PAGE loading dye. The samples thus collected were boiled for 5 m and snap-chilled at -20°C. The protein samples were finally resolved on 12% SDS-PAGE[28]. Protein band visualization was done by staining with Coomassie brilliant blue R-250 staining solution (225 mL 45% *w/v* methanol, 50 mL 10% *w/v* glacial acetic acid, 0.625 g Coomassie brilliant blue R-250 (0.125% *w/v*), and the total volume was made up to 500 mL in double-distilled water). The proteins resolved on the polyacrylamide gel were transferred to the PVDF (polyvinylidene difluoride) membrane for 1 h 20 min[29]. After the

Table 1 — The initial conditions employed for the rPIMT expression in *E. coli* T7 Express *lys*S cells

| S. No. | IPTG final concentration | Temperature | Time of incubation after IPTG induction | Form of rPIMT expression |
|---|---|---|---|---|
| 1. | 1.0 mM | 37°C | 6 h | Inclusion bodies |
| 2. | 1.0 mM | 30°C | 6 h | Inclusion bodies |
| 3. | 0.50 mM | 37°C | 6 h | Inclusion bodies |
| 4. | 0.20 mM | 37°C | 6 h | No expression |
| 5. | 0.05 mM | 37°C | 6 h | No expression |
| 6. | 1.0 mM | 37°C | 1 h | Inclusion bodies |
| 7. | 1.0 mM | 37°C | 2 h | Inclusion bodies |
| 8. | 1.0 mM | 37°C | 3 h | Inclusion bodies |
| 9. | 1.0 mM | 37°C | 4 h | Inclusion bodies |
| 10. | 1.0 mM | 37°C | 5 h | Inclusion bodies |
| 11. | 1.0 mM | 37°C | 6 h | Inclusion bodies |
| 12. | 1.0 mM | Refrigeration temperature | Over night | No expression |

transfer, the membrane was blocked by 3% skim milk powder in PBS at 4°C overnight. The next day, the membrane was washed 3 times with PBS-T (0.05% Tween 20 in PBS) and again the membrane was incubated with His Probe–HRPO conjugate for 2 h (1:5000 dilution, Thermo Scientific, USA). The membrane washed 3 times with PBS-T and developed using 3, 3′-diaminobenzidine (DAB) substrate (DAB-6.0 mg, 50 µL Nickel Chloride (8% *w/v*), 10 µL Hydrogen Peroxide and PBS (pH-7.4) up to10 mL).

The initial rPIMT expression study was carried out by incubating the culture of recombinant clone (*E. coli* pET28C(+)/PIMT) at 37°C for 6 h after inducing with 1 mM IPTG. The rPIMT was expressed as inclusion bodies. Thereafter, several changes were made such as changing the IPTG concentrations (0.05 to 1 mM) and incubation temperatures (4°-37°C) for expressing the rPIMT in soluble form (Table 1). However, rPIMT was not expressed in soluble form. Based on the expression level of the rPIMT visualized on the Coomassie R-250 stained poly acryl amide gel, it was decided to express the recombinant protein at 37°C using 1 mM IPTG for protein induction for 5 h.

A 100 mL broth culture of recombinant clone was induced separately with 1 mM IPTG for 5 h, at 37°C, 180 rpm. After induction, the culture was centrifuged at 2219 × g for 15 m at 4°C. Subsequently, the supernatant was decanted and the resulting pellet was lysed with 10 mL lysis buffer (10 mM Tris HCl pH 7.4, 10 mM imidazole, 1% lysozyme, 3% glycerol, 0.01% β-mercapto ethanol) followed by 3 cycles of freeze-thawing. The resulting pellet was then sonicated for 20 s 12 times with 20 s cooling. The sonicated samples were then subjected to centrifugation at 2219 × g for 30 m at 4°C. The inclusion bodies pellet was washed thrice with urea wash buffer (10 mM Tris HCl, 10 mM imidazole, 1 M Urea, 1% Triton-X100 and pH 7.4). The final wash was given without urea and triton-X 100. Subsequently, the inclusion body pellet was solubilized in 8 M urea lysis buffer (8 M urea, 100 mM $NaH_2PO_4$, 10 mM Tris HCl, 300 mM NaCl, 10 mM imidazole and pH 7.4) and kept on a dancing shaker at room temperature (RT) for 12 h. The resulting samples were centrifuged twice at 15777 ×g for 20 min at 4°C to remove the residual cell debris. The final supernatant was collected and loaded onto a pre-equilibrated Ni-NTA agarose column (10 mL holding capacity) and kept on a dancing shaker for 30 min at room temperature. The flow through was passed down the column for 3 times. The column washed with wash buffer (40 mM imidazole, 100 mM $NaH_2PO_4$, 10 mM Tris-Cl, 8 M urea, pH 7.4). The recombinant protein was eluted in 1 mL fractions using an elution buffer (300 mM Imidazole, 100 mM $NaH_2PO_4$, 10 mM Tris-Cl, 8 M urea, pH 7.4). 12% SDS-PAGE was used to check the purity of the isolated protein[28], and a Western blot[29] with a His Probe-HRP conjugate (1:5000, Thermo Scientific, USA) was used to confirm it.

### Dialysis of recombinant PIMT

The dialysis was performed stepwise to remove urea from denatured rPIMT by using buffers with reducing concentrations of urea, triton X-100, and NaCl (pH 7.5) at 4°C, and buffer changes were done at every 12 h in the following order: 1st change with buffer (25 mM Tris–HCl, 500 mM NaCl, 6 M urea with 1% Triton X-100), 2nd change with buffer (25 mM Tris–HCl, 500 mM NaCl, 4 M urea and 0.5% Triton X-100), 3rd change with buffer (25 mM Tris–HCl, 500 mM NaCl, 3 M urea and 0.25% Triton X-100), 4th change with buffer (25 mM Tris–HCl, 250 mM NaCl, 2 M urea and 0.125% Triton X-100), 5th

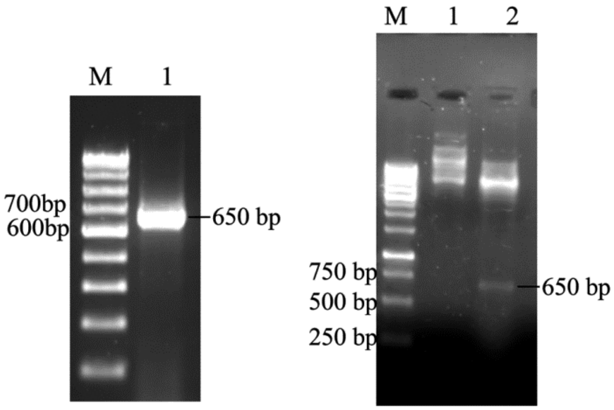

Fig. 1 — (a) 1% agarose gel showing PCR amplicon of buffalo PIMT gene amplified from the cDNA of buffalo testis; lane M: 100 bp marker, lane 1: PIMT amplicon of 650 bp; and (b) Restriction digestion of PIMT clones with fast digest Hind III & Xho I run on 1% agarose gel. lane M- DNA ladder, lane 1- PIMT uncut plasmid, Lane 2- Recombinant PIMT clone showing insert release of 650 bp

change with buffer (25 mM Tris–HCl, 150 mM NaCl, 1 M urea and 0.125% Triton X-100), and 6th and 7th change with buffer (25 mM Tris–HCl and 150 mM NaCl). The dialyzed samples were centrifuged at 15777 × g for 15 min at 4°C to remove the traces of precipitated proteins. The concentration of dialyzed protein was measured by the Bradford method[30]. The protein was stored at -20°C with a Halt protease inhibitor (Thermo Scientific, USA).

#### Enzymatic activity of recombinant PIMT

The enzymatic activity was assayed by using SAM510: SAM methyl transferase kit (Cat. # 786-430, G-Bioscience, USA) following the recommended protocol. In the SAM-methyltransferase assay, the enzyme-coupled activity is continuously monitored. The methyl group removal from SAM results in the formation of *S*-adenosyl homocysteine (AdoHcy) which is rapidly converted to *S*-ribosyl homocysteine and adenine. The adenine deaminase acts on adenine to form hypoxanthine, which in turn is converted to urate and hydrogen peroxide ($H_2O_2$). The rate of $H_2O_2$ production was measured with colorimetric reagent 3,5-diclhoro-2-hydroxybenzenesulfonic acid (DHBS) absorbance recorded at 515 nm.

For enzyme activity measurement, the rPIMT was dialyzed against 0.1 M Tris-HCl, pH 8.0 and used. The assay was performed on an ELISA plate. The acceptor substrate was not supplied with the kit and was procured separately with sequence Lys-Ala-Ser-Ala-L-Isoasp-leu-Ala-Lys-Tyr (GaloreTx Pharmaceuticals Private Limited, Bangalore), dissolved in SAM-methyltransferase assay buffer mix and used at 20 µM concentration. The SAM- methyltransferase assay buffer was equilibrated with the acceptor substrate at 37°C. SAM methyltransferase assay master mix was prepared for 36 wells before use. The background control and positive controls were run simultaneously with the assay. The rate of change in absorbances per minute at 515 nm was recorded at 37°C (approx. 15-30 min). The increasing absorbance plateau was seen by 20 min. The average absorbance of each sample was recorded.

Table 2 — The multiple sequence alignments of PIMT protein against closely related species of live-stock

| S. No. | Species | Percent Identity | Mismatches | Protein accession number |
|---|---|---|---|---|
| 1. | *Bubalus bubalus-1* | 100.00 | 0 | AZQ05603.1 |
| 2. | *Bubalus bubalus-2* | 98.52 | 3 | XP_025150185.1 |
| 3. | *Bubalus bubalus-3* | 98.52 | 3 | XP_006080333.1 |
| 4. | *Bison bison* | 97.04 | 6 | XP_010860841.1 |
| 5. | *Bos mutus* | 97.04 | 6 | XP_005908110.1 |
| 6. | *Ovis aries* | 97.04 | 6 | XP_042109532.1 |
| 7. | *Camelus ferus* | 96.55 | 7 | XP_006185512.2 |
| 8. | *Sus scrofa* | 96.55 | 7 | NP_001182709.1 |
| 9. | *Bos indicus* | 96.55 | 7 | XP-019823139.1 |
| 10. | *Bos taurus* | 96.55 | 7 | XP_005211098.1 |

## Results

Under the study, a ~650 bp PCR product was cloned into a pET-28c (+) vector and transformed into DH5α competent cells. The double digestion of the recombinant plasmid clone with *Hind*III and *Xho*I showed a release of an expected ~650 bp DNA fragment (Fig. 1a & b). The recombinant pET-28c(+)/PIMT clone was sequenced; the sequence data is available in the NCBI GenBank database (Accession Number MH133189.1). Upon analysis, the buffalo PIMT sequence encoded 204 amino acids. The multiple sequence alignments of the bovine PIMT transcript with rPIMT from buffalo were performed using Clustal Omega software (https://www.ebi.ac.uk/Tools/msa/clustalo/). The PIMT is highly conserved across major closely related domestic animals *i.e.,* cattle, sheep, pigs and camels (Table 2). Buffalo PIMT showed 96.55% and 96% identity with the PIMT of cattle and human, respectively. Further, there were 10 amino acid substitutions in buffalo PIMT compared to the human PIMT at positions 80, 93, 94, 111, 136, 140, 184, 194, 199 and 200 in buffalo PIMT (Table 3).

Since the buffalo rPIMT protein sequence showed a significant homology with the human PIMT protein (P22061) whose crystal structure with *S*-adenosyl homocysteine at 1.6-A resolution was available (PDB ID:1I1N) as well as showing closer RMSD to the buffalo 3D structure. Because of this reason, the 3D structure of the human PIMT has been used for the secondary structure annotation and structural comparison with the buffalo PIMT enzyme structure (https://www.rcsb.org/structure/1I1N). In secondary structure annotation, we could observe that the buffalo PIMT protein chiefly consists of six α-helix (α1:2-10 aa, α2:17-24 aa, α3:53-62 aa, α4:82-91 aa, α5:104-123 aa and α6:158-162 aa) and eight antiparallel β-strands (β1:42-50 aa, β2:72-76 aa, β3:96-101 aa, β4:127-131 aa, β4:144- 149 aa, β6:164-174 aa, β7:182-188 aa and β1:194-198 aa) (Fig. 2a & b).

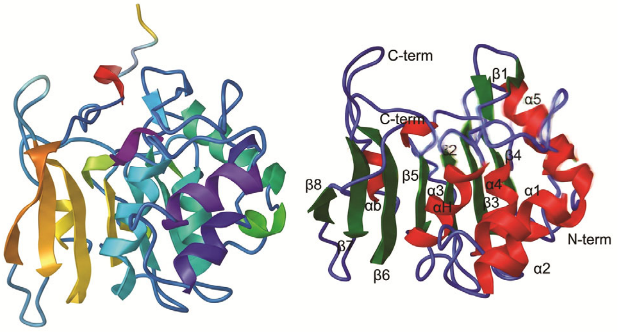

Fig. 2 — (a) Ribbon structure of human protein l-isoaspartate o-methyltransferase (P22061 in iCn3D (nih.gov)); and (b) Predicted 3D structure of buffalo recombinant PIMT (Accession: AZQ05603)

Initially, PIMT expression at different temperatures and different concentrations of isopropyl β-d-1-thiogalactopyranoside (IPTG, 0.05 mM to 1 mM) at 37°C for 6 h were attempted (Fig. 3a). The pellet and supernatant of the bacterial cell lysate were loaded onto 12% SDS-PAGE (Fig. 3b). The rPIMT of 31 kDa was expressed as inclusion bodies in the pellet of

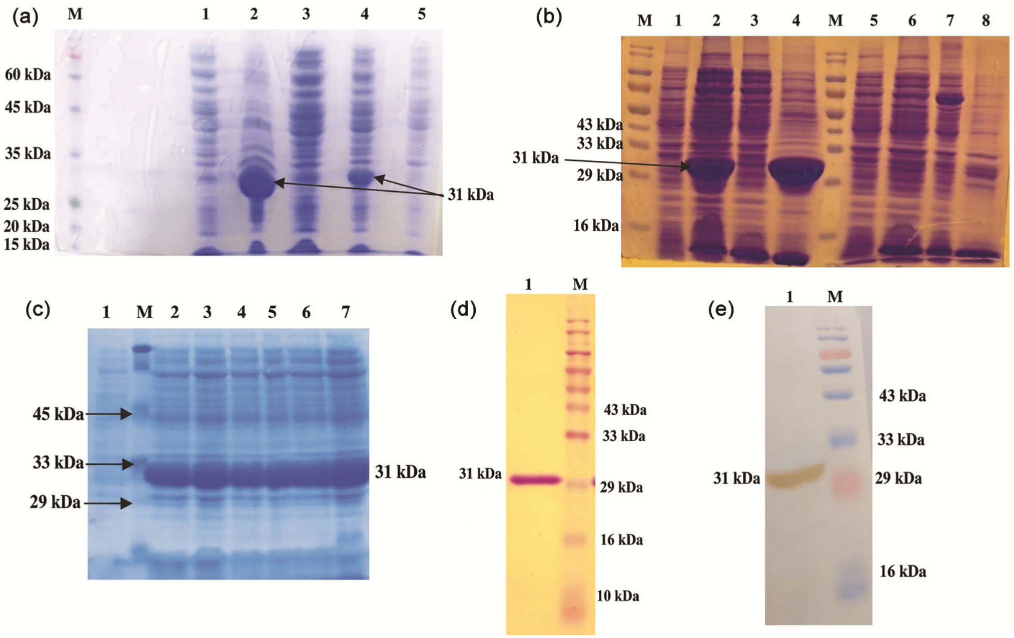

Fig. 3 — (a) 12% SDS-PAGE showing expression of rPIMT incubated at 37°C and 30°C for 6 h using 1 mM IPTG for induction, the culture pellet was lysed by freeze-thawing: lane M- protein marker, lane 1- supernatant from the bacterial lysate incubated at 37°C, lane 2- pellet from the bacterial lysate induced at 37°C, lane 3- supernatant from the bacterial lysate incubated at 30°C, lane 4- pellet from the bacterial lysate induced at 30°C, lane 5- Uninduced whole cell pellet; (b) 12% SDS-PAGE showing expression of rPIMT incubated at 37°C for 6 h using varied concentrations of IPTG for induction, the culture pellet was lysed by freeze-thawing: lane M- protein marker, lane 1- supernatant from the bacterial lysate induced with 0.5 mM IPTG, lane 2- pellet from the bacterial lysate induced with 0.5 mM IPTG, lane 3- supernatant from the bacterial lysate induced with 1 mM IPTG, lane 4- pellet from the bacterial lysate induced with 1 mM IPTG, lane 5- supernatant from the bacterial lysate induced with 0.2 mM IPTG, lane 6- pellet from the bacterial lysate induced with 0.2 mM IPTG, lane 7- pellet from the bacterial lysate induced with 0.05 mM IPTG, lane 8- supernatant from the bacterial lysate induced with 0.05 mM IPTG; (c) 12% SDS-PAGE showing expression of rPIMT incubated at 37°C using 1 mM IPTG for induction for different incubation time: lane M- protein marker, lane 1- uninduced culture pellet, , lane 2-7 - induced culture pellet (1 mM IPTG for 1-6 h, at 37°C, samples collected at every one hour); (d) 12% SDS-PAGE showing purified protein under denaturing conditions using Ni-NTA Agarose column: lane M: protein marker, lane 1: showing purified single protein band of 31 kDa specific to PIMT; and (e) Western blot showing immunoreactive purified rPIMT, probed with anti-his probe antibody, lane M: protein marker, lane 1: purified immune-reactive protein band of 31 kDa

Table 3 — The protein sequence of buffalo PIMT protein (Accession: AZQ05603) showing 10 amino acid substitutions (at positions 80, 93-94, 111, 136, 140, 184, 194, 199-200) and conserved residues (at positions 49, 51, 53-56, 58, 77-79, 101-102, 143) as compared to human PIMT (Uniprot: P22061)

| | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Bubalus bubalis* | | | | | | | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| *Bubalus bubalis* | - | - | - | - | - | - | - | - | H | S | E | L | I | H | N | L | R | K | N | G |
| *Homo sapiens* | A | W | K | S | G | G | A | S | H | S | E | L | I | H | N | L | R | K | N | G |
| *Homo sapiens* | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
| *Bubalus bubalis* | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 |
| *Bubalus bubalis* | I | I | K | T | D | K | V | F | E | V | M | L | A | T | D | R | S | H | Y | A |
| *Homo sapiens* | I | I | K | T | D | K | V | F | E | V | M | L | A | T | D | R | S | H | Y | A |
| *Homo sapiens* | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 |
| *Bubalus bubalis* | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 | *49* | 50 | *51* | 52 |
| *Bubalus bubalis* | K | C | N | P | Y | M | D | S | P | Q | S | I | G | F | Q | A | *T* | I | *S* | A |
| *Homo sapiens* | K | C | N | P | Y | M | D | S | P | Q | S | I | G | F | Q | A | *T* | I | *S* | A |
| *Homo sapiens* | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 | 49 | 50 | 51 | 52 | 53 | 54 | 55 | 56 | *57* | 58 | *59* | 60 |
| *Bubalus bubalis* | *53* | *54* | *55* | *56* | 57 | *58* | 59 | 60 | 61 | 62 | 63 | 64 | 65 | 66 | 67 | 68 | 69 | 70 | 71 | 72 |
| *Bubalus bubalis* | *P* | *H* | *M* | *H* | A | *Y* | A | L | E | L | L | F | D | Q | L | H | E | G | A | K |
| *Homo sapiens* | *P* | *H* | *M* | *H* | A | *Y* | A | L | E | L | L | F | D | Q | L | H | E | G | A | K |
| *Homo sapiens* | *61* | *62* | *63* | *64* | 65 | *66* | 67 | 68 | 69 | 70 | 71 | 72 | 73 | 74 | 75 | 76 | 77 | 78 | 79 | 80 |
| *Bubalus bubalis* | 73 | 74 | 75 | 76 | *77* | *78* | *79* | 80 | 81 | 82 | 83 | 84 | 85 | 86 | 87 | 88 | 89 | 90 | 91 | 92 |
| *Bubalus bubalis* | A | L | D | V | *G* | *S* | *G* | S | G | I | L | T | A | C | F | A | R | M | V | G |
| *Homo sapiens* | A | L | D | V | *G* | *S* | *G* | H | G | I | L | T | A | C | F | A | R | M | V | G |
| *Homo sapiens* | 81 | 82 | 83 | 84 | *85* | *86* | *87* | 88 | 89 | 90 | 91 | 92 | 93 | 94 | 95 | 96 | 97 | 98 | 99 | 100 |
| *Bubalus bubalis* | 93 | 94 | 95 | 96 | 97 | 98 | 99 | 100 | *101* | *102* | 103 | 104 | 105 | 106 | 107 | 108 | 109 | 110 | 111 | 112 |
| *Bubalus bubalis* | T | N | G | K | V | I | G | I | *D* | *H* | I | K | E | L | V | D | D | S | I | N |
| *Homo sapiens* | C | T | G | K | V | I | G | I | *D* | *H* | I | K | E | L | V | D | D | S | V | N |
| *Homo sapiens* | 101 | 102 | 103 | 104 | 105 | 106 | 107 | 108 | *109* | *110* | 111 | 112 | 113 | 114 | 115 | 116 | 117 | 118 | 119 | 120 |
| *Bubalus bubalis* | 113 | 114 | 115 | 116 | 117 | 118 | 119 | 120 | 121 | 122 | 123 | 124 | 125 | 126 | 127 | 128 | 129 | 130 | 131 | 132 |
| *Bubalus bubalis* | N | V | R | K | D | D | P | M | L | L | S | S | G | R | V | Q | L | V | V | G |
| *Homo sapiens* | N | V | R | K | D | D | P | M | L | L | S | S | G | R | V | Q | L | V | V | G |
| *Homo sapiens* | 121 | 122 | 123 | 124 | 125 | 126 | 127 | 128 | 129 | 130 | 131 | 132 | 133 | 134 | 135 | 136 | 137 | 138 | 139 | 140 |
| *Bubalus bubalis* | 133 | 134 | 135 | 136 | 137 | 138 | 139 | 140 | 141 | 142 | *143* | 144 | 145 | 146 | 147 | 148 | 149 | 150 | 151 | 152 |
| *Bubalus bubalis* | *D* | *G* | R | L | G | Y | A | A | E | A | *P* | Y | D | A | I | H | V | G | A | A |
| *Homo sapiens* | *D* | *G* | R | M | G | Y | A | E | E | A | *P* | Y | D | A | I | H | V | G | A | A |
| *Homo sapiens* | 141 | 142 | 143 | 144 | 145 | 146 | 147 | 148 | 149 | 150 | *151* | 152 | 153 | 154 | 155 | 156 | 157 | 158 | 159 | 160 |
| *Bubalus bubalis* | 153 | 154 | 155 | 156 | 157 | 158 | 159 | 160 | 161 | 162 | 163 | 164 | 165 | 166 | 167 | 168 | 169 | 170 | 171 | 172 |
| *Bubalus bubalis* | A | P | V | V | P | Q | A | L | I | D | Q | L | K | P | G | G | R | L | I | L |
| *Homo sapiens* | A | P | V | V | P | Q | A | L | I | D | Q | L | K | P | G | G | R | L | I | L |
| *Homo sapiens* | 161 | 162 | 163 | 164 | 165 | 166 | 167 | 168 | 169 | 170 | 171 | 172 | 173 | 174 | 175 | 176 | 177 | 178 | 179 | 180 |
| *Bubalus bubalis* | 173 | 174 | 175 | 176 | 177 | 178 | 179 | 180 | 181 | 182 | 183 | 184 | 185 | 186 | 187 | 188 | 189 | 190 | 191 | 192 |
| *Bubalus bubalis* | P | V | G | P | A | G | G | N | Q | M | L | M | Q | Y | D | K | L | Q | D | G |
| *Homo sapiens* | P | V | G | P | A | G | G | N | Q | M | L | E | Q | Y | D | K | L | Q | D | G |
| *Homo sapiens* | 181 | 182 | 183 | 184 | 185 | 186 | 187 | 188 | 189 | 190 | 191 | 192 | 193 | 194 | 195 | 196 | 197 | 198 | 199 | 200 |

(*Contd*.)

Table 3 — The protein sequence of buffalo PIMT protein (Accession: AZQ05603) showing 10 amino acid substitutions (bold letters) and conserved residues (italics & bold) as compared to human PIMT (Uniprot: P22061) (*Contd.*)

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Bubalus bubalis* | 193 | 194 | 195 | 196 | 197 | 198 | 199 | 200 | 201 | *202* | 203 |
| *Bubalus bubalis* | S | V | K | M | K | P | Q | K | G | *V* | I |
| *Homo sapiens* | S | I | K | M | K | P | L | L | G | *V* | I |
| *Homo sapiens* | 201 | 202 | 203 | 204 | 205 | 206 | 207 | 208 | 209 | 210 | 211 |

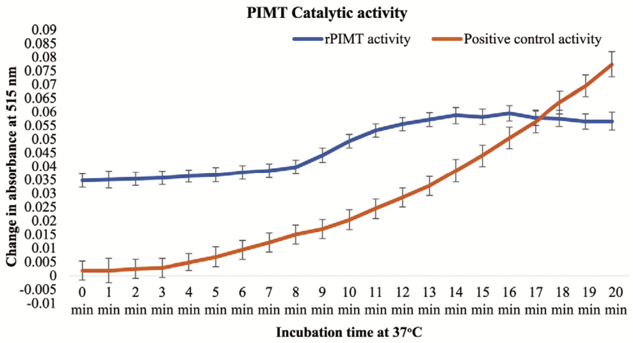

Fig. 4 — The catalytic activity of recombinant PIMT. Data presented show mean ± SD (n=3)

the bacterial lysate at 0.5 mM and 1 mM IPTG concentrations (incubation at 37°C for 6 h) and also at temperatures 30°C and 37°C (1 mM IPTG for induction for 6 h). No rPIMT expression was seen at 0.05 mM and 0.2 mM IPTG concentrations and also at refrigeration temperature (Table 1). Subsequently, based on the level of expression of a specific band of 31 kDa seen on the Coomassie brilliant blue R-250 stained gels (Fig. 3c), it was decided to express the rPIMT at 37°C for 5 h using 1 mM IPTG for induction and purify the rPIMT under denaturing conditions. The rPIMT consistently got expressed as inclusion bodies. The purification of expressed rPIMT protein was carried out under denaturing conditions using a Ni-NTA agarose column, and SDS-PAGE showed a single protein band of 31 kDa (Fig. 3d), which was again confirmed by Western blot using a His probe-HRPO conjugate (Fig. 3e) showing a specific immunoreactive protein band of the expected size. The concentration of the purified protein was about 0.2 mg/mL. Further, the rPIMT protein was checked for its catalytic activity using a SAM510: Sam Methyltransferase colorimetric continuous enzyme assay. The kinetics of enzyme activity rPIMT showed a gradual increase from 0 min to 16 min (Fig. 4). The rPIMT enzyme activity increased (ΔA) from 0.035 to 0.060 absorbance units over 16 min, whereas in the positive control, the activity (ΔA) increased from 0.002 to 0.051. The rate of change in absorbance for rPIMT was 0.0015 compared to 0.003 in positive control measured over 16 min.

## Discussion

Though PIMT has been expressed in different species[12,20,21,31,32]; it is recommended to use the protein from homologous species. In this study, the biologically active enzyme was successfully expressed in a prokaryotic expression system using cDNA derived from buffalo testicular tissue. Upon sequence analysis, buffalo PIMT showed 10 amino acid substitutions, but these are unlikely to affect its catalytic activity, as the sequence homology and *in silico* modelling experiments on buffalo PIMT show structural conservation of the core sheet topology typical to methyltransferases that are required for enzyme activity[33]. The majority of the conserved amino acid residues that are conserved across the species have remained the same in buffalo PIMT also (Fig. 2b). The buffalo *PIMT* gene sequence encoded 204 amino acids and showed a single protein band of 31 kDa on SDS-PAGE. With several approaches and modifications such as IPTG concentration (0.05-1 mM), post-induction incubation time (1 h to overnight) and temperatures (37°C, 30°C, and at refrigeration), the protein was not getting expressed in soluble form and had consistently formed inclusion bodies. This may be due to the protein expression at a very high level overwhelming the capacity of protein-folding machinery, leading to aggregation into inclusion bodies. There is a report of expression of PIMT protein in recombinant form in plants[20].

The expression conditions were optimized at 37°C for 5 h with 1 mM IPTG induction. The expressed protein was purified under denaturing conditions using a Ni-NTA agarose column. It is always very challenging to produce functionally active protein from inclusion bodies.

The renaturation of the recombinant protein from inclusion bodies by dialysis with a stepwise reduction in urea and Triton X-100 resulted in precipitation. However, the addition of 500 mM NaCl to the dialysis buffer and its stepwise reduction to 150 mM could prevent the precipitation. The purified fusion proteins were again confirmed by Western blot using a His probe-HRPO conjugate. The purification procedure was successful in producing rPIMT up to 80-90% homogeneity. The identity and integrity of the expressed enzyme were confirmed by the discovery of a single band on the gel and Western blot of expected molecular weight. This purity is necessary for the precise description of PIMT's enzymatic activity and ensures that observed effects are attributed solely to the target enzyme.

The rPIMT protein was then checked for its catalytic activity by a SAM510: Sam methyltransferase colorimetric continuous enzyme assay. The kinetics of enzyme activity rPIMT showed a gradual increase from 0 m to 16 min. Enzymatic activity was evaluated using a synthetic isoaspartate-containing peptide substrate. The ability of rPIMT to catalyze the transfer of a methyl group to repair aberrant iso-aspartyl residues back to their natural L-aspartate form is confirmed by the successful methylation of iso-aspartate residues in the peptide substrate through the colorimetric assay. It is anticipated that this enzymatic repair route will have a substantial impact on preserving protein stability and reducing protein malfunctions brought on by isoaspartate buildup.

This marks a significant advancement because of the intricacy of the enzyme and its sensitivity to degradation, which made it difficult to produce PIMT in large quantities for their downstream applications. PIMT may contribute to the prevention of protein misfolding, aggregation, and associated cellular dysfunction by enabling the restoration of normal L-aspartate residues. With the expression and purification of biologically active PIMT, the study offers an important new prospective application in biotechnology and medicine.

The cryopreservation of spermatozoa plays an important role in long-term storage of semen for usage in artificial insemination. Previously, the research work was carried out on similar lines using humanin[35-37], regucalcin[38-41] and MsrA[42,43] with improved quality of frozen-thawed semen. During cryopreservation, there is an increased ROS production, resulting in oxidative damage to the biomolecules, especially the proteins[2]. Since, the PIMT has been shown to repair the oxidatively damaged proteins. We assumed that the addition of rPIMT to enhance the frozen-thawed sperm quality. Despite the progress made in this work, examining PIMT's function in biological systems is a significant area for research. Thus, after demonstrating the functionality of rPIMT, it has been used for exogenous supplementation to the semen extender, and had shown beneficial effects on the quality of frozen-thawed spermatozoa[34].

Finally, the molecular expression and purification of bioactive rPIMT was accomplished in this study. The methylation of a synthetic iso-aspartate-containing peptide substrate back to native aspartate by PIMT is suggestive of its possible usage in repairing protein oxidation. With bioactive rPIMT being readily available, this study presents exciting opportunities for novel biological investigation and therapeutic applications in maintaining protein quality.

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## Conflict of interest

All authors declare no conflict of interest.

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