In vitro metal catalyzed oxidative stress in DAH7PS: Methionine modification leads to structure destabilization and induce amorphous aggregation

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A B S T R A C T
The first committed step of the shikimate pathway is catalyzed by a metalloenzyme 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAH7PS), which exhibits vulnerability to the oxidative stress. DAH7PS undergoes inactivation in multiple ways in the presence of redox metal, H2O2, and superoxide. The molecular mechanism and susceptibility of its inactivation might differ in different organisms and are presently unclear. In the present work, we have cloned, expressed and purified a DAH7PS from Providencia alcalifaciens (PaDAH7PS). The oligomeric state and effect of redox metal treatment on its stability were analyzed through the size exclusion chromatography. The FTIR, MALDI-TOF/TOF-MS studies revealed that methionine residues were modified to methionine sulfoxide in PaDAH7PS. During oxidation, PaDAH7PS is altered into partially folded protein and unfolded states as determined by CD and fluorescence studies. A significant loss in enzymatic activity of PaDAH7PS was determined and the formation of amorphous aggregates was visualized using AFM imaging and also confirmed by ThT binding based assay. This is the first report where we have shown a hexameric DAH7PS and the methionine residues of PaDAH7PS get oxidize in the presence of oxidative stress. The partially folded and unfolded oligomeric states with high β-content of PaDAH7PS might be the critical precursors for aggregation.

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1. Introduction

The biosynthesis of essential aromatic amino acids (Phe, Tyr, and Trp) and other important secondary metabolites is mediated by seven different enzyme-catalyzed reactions in shikimate pathway [1,2]. The first enzyme of this pathway is 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAH7PS), a metalloenzyme, which catalyzes the stereospecific condensation of intermediate compounds phosphoenolpyruvate (PEP) and D-erythrose-4-phosphate (E4P) generated from the glycolytic pathway and the pentose phosphate pathway, respectively, and forms 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) and inorganic phosphate (Pi). Since this pathway is employed only in plants, fungi, microbes, and apicomplexans, enzymes of this pathway are potential targets for the development of drug molecules and herbicides. The genetic modifications in the enzymes of this pathway via recombinant DNA technology show high yield of production of the essential amino acids during microbial fermentation at industrial level [3–9]. The shikimate pathway is controlled by three regulatory enzymes: 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAH7PS), chorismate mutase (CM) and prephenate dehydratase (PDT). As a committed enzyme, the regulation of DAH7PS is a key point for the metabolic flux of this pathway [10]. DAH7PS are categorized into two different families: type I (microbial type) and type II (plant type, including few microbial) based on their sequence similarity and molecular masses [11,12]. Despite the very low sequence identity between two families of DAH7PS, they share similarities in the functional and the mechanistic features. All characterized DAH7PS possess a common core catalytic (βα8β) TIM barrel fold and show conservation in the substrate and metal ion interacting key residues [13]. Although, DAH7PS from different sources show

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Abbreviations: AFM, atomic force microscopy; ANS, 1-anilinonaphthalene-8-sulfonic acid; BSA, bovine serum albumin; BTP, bis-tris propane buffer; CD, circular dichroism; DAH7PS, 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase; DTT, 1,4-dithio-D-threitol; E4P, erythrose-4-phosphate; IPTG, isopropyl β-D-thiogalactoside; MALDI, Matrix Assisted Laser Desorption/Ionization; MetSO, methionine sulfoxide; PEP, phosphoenolpyruvic acid sodium salt; Tew-Protease, the tobacco etch virus (TEV) protease; ThT, Thioflavin T; Tris, tris (hydroxymethyl) aminomethane.

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significant differences in their biologically active oligomeric states and additional regulatory extensions [14,83].

Oligomerization of the DAH7PS is essential for their catalytic activity as well as for the interaction of regulatory molecules at the allosteric site. Several studies have shown that microbial DAH7PS are feedback regulated by enzymes through the diverse allosteric mechanism; inhibit either by the end products (Phe, Tyr, and Trp) or intermediate compounds formed in the pathway [14,15]. However, the plant’s DAH7PS show redox regulatory mechanism [15]. The mono-functional isoenzyme of Escherichia coli i.e. DAH7PS, regulated by the end product (Phe), DAH7PS of Thermotoga maritima (Tyr/Phe) and bifunctional DAH7PS of Listeria monocytogenes and Geobacillus sp. are tetrameric proteins, whereas other two isoenzymes of E. coli (Tyr and Trp) and DAH7PS of Saccharomyces cerevisiae (Tyr) exist in the dimeric state [16–20]. The unregulated DAH7PS of Pyrococcus furiosus and Aeropyrum pernix show dimeric as well as tetrameric forms [21–23]. A type II DAH7PS of Mycobacterium tuberculosis is also a tetrameric protein, which is regulated synergistically by all three aromatic amino acids [24].

In 1999, Park and Bauerle reported that phenylalanine regulated E. coli DAH7PS (EcDAH7PS) is a metal catalyzed oxidation enzyme system. The biologically active homotetrameric form of EcDAH7PS dissociates into an inactive monomer form due to the formation of a disulfide linkage, catalyzed by redox metal ions in the absence of substrate (PEP), between the two cysteine residues (Cys61 and Cys328) of the active-site [25]. Recently, Sobota and coworkers have also shown that enzymatic activity of EcDAH7PS is poisoned by hydrogen peroxide and superoxide. Here, in the presence of peroxide an inactivated apoprotein with oxidized cysteine residues was generated due to the removal of prosthetic iron metal, whereas under superoxide stress active site metal was replaced by a non-activating zinc metal [26].

In in-vivo and in-vitro conditions, redox metal catalyzed oxidation, peroxidation, superoxidation, and other environmental oxidative stress conditions are common mechanisms of oxidative damages in the proteins [27,28]. Consequently, amino acids in the proteins undergo a variety of chemical modifications such as isomerization, deamination, peptide bond cleavage, and oxidation which leads to the aggregation and degradation process [29]. EcDAH7PS has shown multiple damage effects under different oxidative stress conditions; however, oxidative damages in DAH7PS of any other organism have not been studied and a clear molecular mechanism is undetermined.

In the present study, we cloned, expressed, and purified a homologous DAH7PS from a Gram-negative pathogenic bacterium Providencia alcalifaciens (PaDAH7PS) which belongs to the family Enterobacteriaceae and commonly causes traveler’s diarrhea and urinary tract infections [30,31]. Here, we determined the oligomeric state and enzymatic activity of purified PaDAH7PS, under different environmental conditions. Furthermore, we investigated the influence of redox metal oxidative stress and its impact on the modification of amino acid residues of PaDAH7PS via FTIR and Mass spectrometry. Moreover, we also characterized the unfolding/partial folding and conformational changes in PaDAH7PS due to the oxidative damage by circular dichroism and fluorescence spectroscopy.

2. Materials and methods

2.1. Materials

All restriction enzymes (BamHI and Xhol), DNA polymerase, and T4 DNA ligase were purchased from New England Biolabs. DNA isolation, plasmid isolation, purification, and gel extraction kits were purchased from Qiagen. E. coli BL21 (DE3), DH5α cells, and pET28c plasmid vector were taken from Novagen. The N-NTA resins, bis-tris propane buffer (BTP), phosphonoalpyruvic acid sodium salt (PEP), erythrose-4-phosphate (E4P) and Thiouavin T (ThT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals such as TCA, periodic acid, sodium arsenite, 1, 4-dithio-β-threitol (DTT), BSA and CuCl₂ were purchased from HiMedia (India). Thiobarbituric acid was purchased from Loba Chemie (Mumbai, India). Trypsin from Pronema, Amicon ultra concentrator, and Millex syringe filters (γ-irradiated, pore size 0.45 μm, filter diam. 33 mm) from Millipore Corporation, Billerica, MA. Dialysis membrane of 3500 Da cutoffs was taken from Pierce, Rockford, USA.

2.2. Methods

2.2.1. Molecular cloning and overexpression of recombinant PaDAH7PS

The genomic DNA of Providencia alcalifaciens (MTCC No. 4430) was isolated by DNA extraction Kit and the gene of 1054 bp (EKT65704.1) was amplified with the forward (BamHI) 5’-GATTCTCGATCCATGAACTATCAGAACGATGACGTC-3’ and reverse (Xhol) 5’-GATTCTCTCGATATTATTTTCCAGGACACATTACTCT-3’ designed primers and further cloned into the pET28c-His-Tev vector. For expression of soluble PaDAH7PS, the cloned and screened recombinant plasmid (pET28c+DAH7PS gene) from DH5α was isolated. Furthermore, transformed into the E. coli BL21 (DE3) competent cells formed by the CaCl₂ method and plated on the LB agar plates containing 50 μg/ml kanamycin. A single colony was inoculated into the LB broth with 50 μg/ml kanamycin for seed culture preparation. Next day, 1000 ml LB broth was inoculated with 1% of seed culture and allowed to grow at 37 °C with 200 rpm until OD₆₀₀ reached up to 0.6–0.8. Subsequently, the culture was induced with 0.4 mM isopropyl β-D-thiogalactosidase (IPTG) and incubated at 18 °C at 200 rpm for the next 16 h. Next day, bacterial cells were harvested by centrifugation at 6000 rpm for 10 min, the supernatant was decanted and pellets were stored at −80 °C.

2.2.2. Purification and cleavage of His-Tag of PaDAH7PS

Bacterial cell pellets were resuspended in 20 ml lysis buffer containing substrate (PEP) and reducing agent DTT (20 mM BTP pH 7.5, 100 mM NaCl, 10 mM Imidazole, 0.1 mM MnCl₂, 1 mM DTT, and 400 μM PEP) and lysed using constant cell disrupter system. The lysate was centrifuged at 12,000 rpm for 60 min at 4 °C. The supernatant was collected and loaded onto the pre-equilibrated Ni-NTA affinity chromatography column and incubated for 30 min. The unbound impurities were removed by two washings with increasing step-wise gradient containing 25 mM and 50 mM imidazole, respectively. Furthermore, PaDAH7PS was eluted with a higher gradient (100 mM to 250 mM) of imidazole. The molecular mass and purity of eluted fractions of protein were determined on the 12% SDS-PAGE with standard molecular weight protein marker (Bio-Rad). Subsequently, PaDAH7PS was incubated with purified Tev-protease at 20 °C for 12 h to remove N-terminal His-tag from the protein. Moreover, PaDAH7PS was buffer exchanged (buffer without imidazole) to remove high concentration of imidazole and to separate out the residual His-tagged protein, PaDAH7PS was again loaded onto the pre-equilibrated column for reverse Ni-NTA affinity chromatography. The non-tagged PaDAH7PS was obtained in the flow through. The cleaved protein was collected and concentrated up to a final volume of 1 ml using a 3 kDa cutoff concentrator (Amicon Ultra-15) and further subjected to size exclusion chromatography. We also followed the same procedure during purification of PaDAH7PS in the oxidizing environment. In oxidized condition, neither the reducing agent (DTT) nor the substrate (PEP) was added to the lysis buffer.
2.2.3. Size exclusion chromatography

To determine the oligomeric state of purified PaDAH7PS in different environmental conditions (reduced and oxidized) size exclusion chromatography was performed. The concentrated protein sample (∼10 mg/ml) was centrifuged at high speed at 4 °C for 15 min and subsequently loaded onto the prepacked Hi-Load/60 Superdex 200/Prep Grade column from GE Healthcare. The column was pre-equilibrated with different buffer compositions, as mentioned in the previous section, in accordance with the purification environmental conditions. Pure fractions of PaDAH7PS (3 ml) were collected by AKTA purifier system with a flow rate 0.5 ml/min and absorbance was recorded at 280 nm. The gel filtration column was calibrated for construction of the standard curve and the molecular mass of PaDAH7PS was estimated by using Gel Filtration HMW Calibration Kit containing ferritin (440 kDa), aldolase (158 kDa), Conalbumin (75 kDa) and ovalbumin (44 kDa) (GE Healthcare).

2.2.4. Glutaraldehyde cross-linking and native-PAGE

Additionally, the oligomeric state of PaDAH7PS was also analyzed by performing cross-linking of protein with glutaraldehyde and the experiment was set up by following the procedure as described by Faduloglo et al. [32]. The oligomerization of PaDAH7PS was also analyzed by native-page electrophoresis. Electrophoresis was performed by using Tris–glycine buffer (pH 8.3) as an electrode buffer at a constant current of 25 mA at 4 °C for 8 h. Subsequently, the gel was stained by using Coomassie Brilliant Blue R-250.

2.2.5. Metal-catalyzed oxidation effect on the oligomeric state of PaDAH7PS

The purified non-tagged PaDAH7PS fractions in all previously described conditions were pooled separately. These were subsequently incubated in the presence of 75 μM CuCl2 for 22 h at 20 °C to investigate the effect of redox metal treatment on the oligomeric state stability of the protein. The PaDAH7PS, incubated with redox metal, was concentrated and further loaded onto the gel filtration column with buffer according to the purification conditions in the presence of CuCl2.

2.2.6. FTIR spectroscopy

FTIR spectrum of oxidized protein (PaDAH7PS-Tetox) was recorded by using Thermo Nicolet Nexus (FTIR) spectrophotometer with a spectral resolution of 2.0 cm⁻¹ in the range 400–4000 cm⁻¹. The spectrum measurement was carried out in KBr pellets and protein sample was concentrated up to ~10 mg/ml. The vibrational peaks present in the fingerprint region of 900–1100 cm⁻¹ range were analyzed.

2.2.7. Mass spectrometric analysis

The oligomerization of both His tagged-PaDAH7PS and non-tagged PaDAH7PS and detection of the removal of extra N-terminal His tag were also analyzed by using sinapinic acid (matrix) through MALDI/TOF mass spectrometer (Bruker Daltonics, Germany).

Additionally, for the identification of methionine modification in PaDAH7PS the peak fractions from each chromatogram were loaded onto the 12% SDS-PAGE and bands were digested with the trypsin as described earlier [33]. Briefly, excised gel pieces were destained by adding the ammonium bicarbonate/acetonitrile (1:1, V/V) and acetonitrile (100%), alternating incubation for 30 min, following trypsin digestion for 16 h at 37 °C. After digestion, the reaction was stopped through formic acid and digested peptides were recollected in extraction buffer (1:2 (V/V) 5% formic acid/acetonitrile) at room temperature. The collected peptides were dried in SpeedVac and stored at −20 °C. The samples were spotted on the MALDI ground steel plate with HCCA (α-cyano-4-hydroxy-cinnamic acid) matrix. Mass spectrometric analysis was carried out on Ultraflextreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) equipped with a 60–Hz nitrogen laser. Mass spectra were acquired in the reverse positive mode. The acquisition of spectra was controlled by flexControl 3.4, spectral data were processed using flexAnalysis 3.4 (both software by Bruker Daltonics). The instrument was calibrated externally using Peptide Calibration Standard (Bruker Daltonics) and to cover masses in the m/z range of 500–3500. Spectra were accumulated from 1200 laser shots. The searches were done using Mascot Server 2.4 (Matrix Science, UK) and employed mass tolerances for precursors and fragment ions of ±200 ppm and ±0.7 Da, respectively. Trypsin was set as a protease with 1 missed cleavage allowed; carbamidomethylation of cysteine was set as a fixed modification and methionine oxidation as a variable modification. Peptide mass fingerprinting (MS), for protein identification and tandem mass spectrometry (MS/MS), for sequencing was done using the Mascot search engine (Matrix Science) and BioTools complemented with NCBI database. The theoretical m/z values for trypsin–digested peptides of PaDAH7PS were assessed using the online Expasy tool [34].

2.2.8. Enzymatic activity of PaDAH7PS

The enzymatic activity of different oligomeric state of PaDAH7PS was estimated by discontinuous AminoPer iodate thiobarbituric acid assay method [35]. The activity of the enzyme was calculated by measuring the amount of product formed by the condensation reaction of phosphoenolpyruvate (PEP) and erythrose-4-Phosphate (E4P) at 37 °C catalyzed by PaDAH7PS. A reaction mixture of total 100 μl with the purified enzyme in 100 mM BTP buffer with PEP in the presence of the MnCl2 was set up at the room temperature and the final enzymatic reaction was started by adding another substrate, i.e. E4P, and the reaction mixture was kept at 37 °C for 5 min. Measurements were carried out at 549 nm with Cary UV—vis spectrophotometer using quartz cuvette. The kinetic parameters (Km, Vmax, κcat) for both substrates (PEP and E4P) were determined by varying their concentration. All reactions were performed in triplicates. The obtained data were fitted using non-linear regression analysis by graph pad prism software [36].

2.2.9. Atomic force microscopic imaging of PaDAH7PS

The morphological features of different oligomeric states of PaDAH7PS were detected by Atomic force microscopy. AFM images of protein were collected in semi-contact imaging mode with SPM NT-MDT Netra system, a facility provided at the Institute instrumentation Centre, IIT Roorkee, India. 10 μl of freshly prepared protein samples in each condition were used for imaging. A silicon nitride coating cantilever of 100 μM and force contact 5.5–22.5 N/m at a frequency of 1.0 Hz with pyramidal geometry was used to generate the images. At least three regions of the sample surface were investigated to confirm the homogeneity. All images were processed by using Nova software (1.026.1424 version) provided with the instrument.

2.2.10. Circular dichroism spectroscopy

The secondary structure assessment of different states of PaDAH7PS was performed by using Jasco 1500 spectropolarimeter equipped with the Peltier system. The purified PaDAH7PS under different conditions (5.0 μM concentration) in 25 mM sodium phosphate buffer (pH 7.5) was filtered through 0.45 μm Millex syringe filter and a baseline with reference buffer was set up before spectrum acquisition. The spectrum of each sample was recorded in the far-UV region (190–240 nm) at 25 °C with a scanning speed of 50 nm/min by using 0.1 mm quartz cell with a bandwidth of 1 nm. The final CD spectra of the different oligomeric states of PaDAH7PS were the averaged and smoothed values of three independent acquisitions. The data were analyzed to estimate the percentages
Fig. 1. Purification profile and oligomeric state characterization of PaDAH7PS. (A) Gel-filtration chromatogram of PaDAH7PS with His-tag (B) Gel-filtration chromatogram of PaDAH7PS after His-tag cleavage (C) 12% SDS-PAGE profile of elution fractions from ∼62 ml peak, Lane 1–5: Single bands of the expected mass of ∼41 kDa of purified PaDAH7PS, Lane 6: Molecular weight marker (kDa) (D) 12% SDS-PAGE profile of both peak fractions, Lane 1 & 2: Single bands from non-oxidized hexameric state peak (62 ml), Lane 3 & 4: Two closely stacked bands with different migration rate from tetrameric state peak (68 ml) fractions which are indicated the oxidation of PaDAH7PS on SDS-PAGE, Lane 5: Molecular weight marker (kDa) (E) Gel-filtration chromatogram profile of the high molecular weight standard markers on the Superdex 200 column.

Table 1
Different purification conditions and their effects on the oligomeric state of PaDAH7PS.

<table>
<thead>
<tr>
<th>Purifications Conditions</th>
<th>Effects</th>
<th>Oligomeric State</th>
<th>Oxidized/Non Oxidized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sr. No.</td>
<td>Substrate</td>
<td>Reducing Agent</td>
<td>Elution Volume</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>PEP, + DTT</td>
<td>∼ 62 ml</td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>–</td>
<td>∼ 76 ml</td>
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</tbody>
</table>

Table 2
Effect of redox metal treatment of the oligomeric state of PaDAH7PS in different purification conditions.

<table>
<thead>
<tr>
<th>Redox metal treatment of PaDAH7PS</th>
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<tbody>
<tr>
<td>Conditions</td>
</tr>
<tr>
<td>Sr. No.</td>
</tr>
<tr>
<td>1</td>
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of secondary structure using the Dichroweb server and spectrum of every sample was represented from 190–240 nm in CD mDeg [37].

2.2.11. Fluorescence spectroscopy
To examine the conformational and structural perturbation in PaDAH7PS due to oxidation intrinsic and extrinsic fluorescence studies was performed using the spectrofluorometer Fluorolog®–3 (Jobin Yvon Inc.USA) with 5 nm emission slits and cuvette of 1-cm path length at room temperature. All protein samples were diluted up to 5.0 µM in 25 mM sodium phosphate buffer (pH 7.5) and intrinsic fluorescence was measured by exciting specifically
Fig. 2. (A) MALDI-TOF MS spectrum of His-tagged PaDAH7PS (black color) showing two peaks corresponding to ∼42097.129 Da (A1) and ∼83539.558 Da (A2). MALDI-TOF MS spectrum of PaDAH7PS without His-tag (red color) showing two intense peaks at ∼38611.796 Da (B1) and ∼77576.566 Da (B2) corresponding to monomer and dimer and both spectra depicting few traces (A3 and B3) of higher oligomeric states of PaDAH7PS (B) PaDAH7PS profile after glutaraldehyde cross-linking, Lane 1: Molecular weight marker, Lane 2: Purified protein not treated with glutaraldehyde (Control), Lane 3 & 4: Protein treated with 0.05% and 0.1% glutaraldehyde respectively showing monomer and cross-linked oligomers of PaDAH7PS (C) The native-PAGE profile of protein purified in reduced condition with substrate, where band O1 indicating the hexameric oligomeric form and bands O2 and O3 the other higher forms (i.e. aggregated form) of PaDAH7PS. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Trp residue ($\lambda_{exc} = 295\, \text{nm}$) recording the emission spectra between 300–500 nm. The extrinsic fluorescence was measured by ANS binding with protein and excitation was performed at $\lambda_{exc} = 380\, \text{nm}$ and emission spectra were collected from 400–600 nm.

2.2.12. **ThT binding assay**

Furthermore, to rule out the possibility of amyloid fibrils formation during aggregation ThT binding was also performed with different oligomeric states of PaDAH7PS. A stock solution of ThT (Thioflavin T) was prepared in distilled water and final 10 μM was added in each oligomeric state of PaDAH7PS and incubated the mixture for 30 min in the dark. The ThT excitation was performed at $\lambda_{exc} = 440\, \text{nm}$ and emission spectra were recorded from 450–600 nm by using the spectrofluorometer.

3. **Results**

3.1. **Cloning, expression, purification, and oligomeric state determination of PaDAH7PS**

The open reading frame of 1054 bp PaDAH7PS gene from *P. alcalifaciens* was cloned and overexpressed with high solubility in *E. coli* BL21 (DE3) cells. The PaDAH7PS protein was purified by affinity chromatography and single band of the expected molecular weight ∼41 kDa with N-terminal His-tag was observed on 12% SDS-PAGE
profile. The expressed protein constitutes ~26% of the total soluble fraction and yielded 14 mg/ml of purified protein from per gram of wet cell mass. Purified PaDAH7PS with His-tag was loaded onto the gel filtration column with the substrate and a prominent peak at ~62 ml elution volume with two partially resolved small peaks (~49 ml and ~54 ml) were observed on the chromatogram, ~62 ml peak corresponds to the hexameric form of PaDAH7PS (Fig. 1A). In contrast, earlier studies have shown that all type I DAH7PS exhibit either a tetramer or a dimeric state [13,18]. Several reports have revealed that His-tag in the recombinant proteins alters the binding, kinetics, structural as well as oligomerization properties of a recombinant protein when compared to the wild-type protein [38–40]. Therefore, His-tag of PaDAH7PS was cleaved and non-tagged protein was again loaded onto the gel-filtration column (Fig. 1S). Non-tagged PaDAH7PS unexpectedly showed two peaks on the chromatogram (Fig. 1B). A prominent peak at ~62 ml (i.e. the hexameric form) similar to the tagged protein and a second peak at ~68 ml elution volume corresponding to the tetrameric form of PaDAH7PS, (Table 1). Moreover, the homogeneity and purity of both peak fractions were checked on the 12% SDS-PAGE (Fig. 1C). The first peak fractions showed single bands of purified PaDAH7PS, while elution fractions of the second peak showed two closely stacked bands (indicated by underline), one migrating at a little slower rate (Fig. 1D). The presence of a slowly migrating upper band on SDS-PAGE profile indicated the increment in molecular weight of PaDAH7PS.

Furthermore, to rule out the possibility of the influence of His-tag on the oligomerization of PaDAH7PS and to verify the proper cleavage of His-tag from PaDAH7PS, we confirmed the molecular masses and the oligomeric states of both tagged and non-tagged PaDAH7PS using MALDI-TOF-MS. His-Tagged PaDAH7PS showed two major peaks with high intensity at ~42097.129 Da (A1) and ~38539.558 Da (A2) with a few traces of higher oligomeric states (A3), while after removal of His-tag two major peaks of ~38611.796 Da (B1) and ~77576.568 Da (B2) were observed in the mass spectrum, consistent with the complete removal of N-terminal His-tag, which correspond to the monomeric and dimeric states of PaDAH7PS respectively (Fig. 1C). All reported DAH7PS exist as non-covalently assembled loose oligomers, consisting two monomeric units in a ‘tight-dimer’. The sinapinic acid matrix used in MALDI-TOF analysis cannot detect the non-covalently attached oligomeric form of a protein [41]. Therefore, the hexameric form of PaDAH7PS was not detected in the mass spectrum as represented by size exclusion chromatogram. Moreover, the presence of oligomeric state was also confirmed through the native-PAGE and glutaraldehyde cross-linking profile of PaDAH7PS as shown in Fig. 2B and C, respectively.

Unexpectedly, PaDAH7PS depicted a non-oxidized hexameric (PaDAH7PS-Hexnox) and an oxidized tetrameric (PaDAH7PS-Tetox) oligomeric state, even in the presence of substrate (PEP) and reducing agent (DTT) during purification. Generally, metalloproteins are targeted by the redox metal ions at a specific site in the oxidative process. The redox metals might have introduced during the purification with contaminated reagents, formulation of buffer solutions, and leaching of metallic containers. Therefore, the effects of different environmental conditions and redox metal oxidation on the oligomeric state and stability of PaDAH7PS were further analyzed.

3.2. Influence of oxidative condition and redox metal treatment on the PaDAH7PS

PaDAH7PS was purified in the absence of both reducing agent and substrate to study the effect of oxidized condition. The oligomeric state of purified PaDAH7PS was analyzed by the size exclusion chromatography and homogeneity was checked on 12%
SDS-PAGE. The purified PaDAH7PS in oxidative condition showed a peak at ∼76 ml elution volume, which corresponds to the dimeric state of PaDAH7PS (Fig. 3A). It also exhibited two closely stacked bands on SDS-PAGE, which clearly indicated the oxidation of PaDAH7PS (Fig. 1B).

Furthermore, the effect of redox metal treatment on the DAH7PS was critical to evaluate the stability of the quaternary structure. Therefore, PaDAH7PS was purified and cleaved in both the previously mentioned conditions and subsequently was loaded onto the Superdex (200) gel filtration column after treating it CuCl2. The chromatograms in both the conditions showed one prominent peak at ∼47 ml and another small peak at ∼76 ml elution volume, which correspond to the higher oligomeric and the dimeric state of PaDAH7PS, respectively (Fig. 3B). Thus, PaDAH7PS, when treated with redox metal, dissociated into a small population of the oxidized dimeric state (PaDAH7PS-Dimox*), whereas, the majority of the protein population transformed into the higher oligomeric state (PaDAH7PS-HOxon*) (Table 2).

This higher oligomeric state could be an aggregated state of PaDAH7PS, which might have occurred due to the oxidative damage. Therefore, we further studied the modifications in PaDAH7PS to gain insight on the effect of oxidation.

3.3. FTIR spectroscopy analysis

The effect of oxidation on PaDAH7PS was identified by investigating the fingerprint region (900–1200 cm⁻¹) in the FTIR spectrum of PaDAH7PS-Tetox. Methionine and cysteine residues in the proteins are the most vulnerable amino acids for oxidation by any agents [42]. In the FTIR spectrum of PaDAH7PS-Tetox, three small peaks in the range of ∼1040, 1088, 1124 cm⁻¹, were observed, indicating the modification of methionine residues to methionine sulfoxide (MetSO) in the oxidized state of PaDAH7PS (Fig. 4).

3.4. Identification of the oxidative modification in PaDAH7PS

Additionally, the oxidized methionine residues in PaDAH7PS were also confirmed through the peptide mass fingerprinting (PMF) analysis by mass spectrometry. As depicted in the theoretical trypsin digestion profile of PaDAH7PS contains total seven methionine residues Met1, Met91, Met113, Met157, Met263, Met279, and Met300 which should be depicted the peptides of expected masses 1154.489, 962.497, 2180.018, 4047.089, 2414.238, 1864.870 and 3000.498 Da, respectively, resulting after the trypsin digestion (Fig. 25). Here, the PMF of PaDAH7PS-Hexrox showed all released peptide peaks of the expected masses and only peptides peaks of 1023.5788, 962.549, 2180.253, Da were depicted with no modification in any methionine residue (Fig. 5 and Table 3). In contrast, on the PMF of PaDAH7PS-Tetox, two additional peptide peaks of ∼2195.933 Da and ∼3016.454 Da were detected (Fig. 6A and Table 3). Thus, the extra generated peptides i.e. ∼2195.933 Da and ∼3016.454 Da with an increment of 16 Da in their masses on the PMF of PaDAH7PS-Tetox correspond to the peptide masses of 2180.0189 Da and 3000.4982 Da, which contain Met113 and Met300 residues respectively. In addition, PMF of PaDAH7PS-Diox showed three more oxidized residues: Met91, Met263, and Met279 along with Met113 and Met300 in Mascot search engine which clearly indicated the occurrence of higher oxidation in the absence
Fig. 6. Mass spectrometric analysis of PaDAH7PS. (A) PMF of PaDAH7PS-Tetox obtained by tryptic digestion (B) PMF of PaDAH7PS-Diox obtained by tryptic digestion; The increment of 16 Da indicated the oxidation of these peptides.
Fig. 7. (A) MS/MS spectrum of 2179.908 Da peptide (B) MS/MS spectrum of 2195.933 Da peptide; The neutral loss of 64 Da in the spectrum is a valuable fingerprint and detection of immonium ion (120) in Mascot search is also the unique characteristic of methionine oxidation.
Fig. 8. (A) MS/MS spectrum of 3000.426 Da peptide (B) MS/MS spectrum of 3016.454 Da peptide.
Oligomeric states of PaDAH7PS

3.5. Enzymatic activity of different oligomeric states of PaDAH7PS

Oligomerization of enzymes is an essential feature, which enables the core biological activity and also facilitates the scaffolds for allosteric regulation of a few proteins, including DAH7PS [44,45]. As the oxidative modification of a protein can alter its specific function, we checked the enzymatic activity of all oligomeric states of PaDAH7PS. The PaDAH7PS-Hexinox had the highest activity, whereas the PaDAH7PS-Tetox showed ∼20% relative enzymatic activity. Contrastingly, PaDAH7PS-Diox and redox metal catalyzed oxidized oligomeric forms possessed negligible activity (Fig. 9A). Furthermore, the kinetics parameters for PEP and E4P (km, kcat, Vmax, kcat/km) of the most active state of PaDAH7PS were also determined and compared with other type I DAH7PS (Fig. 9B and C). The kinetic parameters of PaDAH7PS-Hexinox are represented in the (Table 4).

3.6. Morphological studies of different oligomeric forms of PaDAH7PS

Atomic force microscopy is a well-suited technique to detect the topographical features, oligomeric nature, and various types of intermediate structure formation during protein folding/unfolding, aggregation, and fibrillation [46-49]. The morphologies of the PaDAH7PS oligomeric species were visualized using AFM to get the detailed information. The PaDAH7PS-Hexinox showed the spherical/globular shape particles and the PaDAH7PS-Tetox contained the heterogeneous particles with varied length, height, and shapes (Fig. 10A and B). The magnified 3D images of the nephroid, trefoil, and quadrifoil shapes have been shown in Fig. 10C, D and E respectively. Interestingly, PaDAH7PS-HOmorox clearly exhibited amorphous aggregate type structures, which might be an outcome of the association of the destabilized/dissociated-oxidized species (Fig. 11A and B).
Fig. 10. (A) AFM 2D image of PaDAH7PS-Tetox form of protein; different shape like neproid, trefoil, and quardifoil which are indicated by the white arrows and presence of these different forms suggest that protein aggregates on oxidation (B) 3D image profile of the PaDAH7PS-Tetox form (C, D, & E) Showing the 3D profile of the neproid, trefoil, and quardifoil, respectively. The scale bar is shown at the bottom of image.

Table 3
Methionine residues containing peptides generated by trypsin digestion in different oligomeric states of PaDAH7PS.

<table>
<thead>
<tr>
<th>Sequential position</th>
<th>Mass observed</th>
<th>Mass expected</th>
<th>Methionine containing peptides fragments of PaDAH7PS after trypsin digestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>PaDAH7PS-Hexnox</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>85–92</td>
<td>962.5491</td>
<td>961.5418</td>
<td>85-DSLEIVMR-92</td>
</tr>
<tr>
<td>106–124</td>
<td>2180.2528</td>
<td>2179.2456</td>
<td>106-GLINDPHMDHSFDINEGLR-124</td>
</tr>
<tr>
<td>PaDAH7PS-Tetox</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>85–92</td>
<td>962.4656</td>
<td>961.4583</td>
<td>85-DSLEIVMR-92</td>
</tr>
<tr>
<td>106–124</td>
<td>2179.9079</td>
<td>2178.9006</td>
<td><em>106-GLINDPHMDHSFDINEGLR-124</em></td>
</tr>
<tr>
<td></td>
<td>2195.9326</td>
<td>2194.9253</td>
<td><em>106-GLINDPHMDHSFDINEGLR-124</em></td>
</tr>
<tr>
<td></td>
<td>295–322</td>
<td>2999.4183</td>
<td><em>295-SITGMIESHLVEQNGNLSEGEPLVYGK-322</em></td>
</tr>
<tr>
<td></td>
<td>3016.4536</td>
<td>3015.4463</td>
<td><em>295-SITGMIESHLVEQNGNLSEGEPLVYGK-322</em></td>
</tr>
<tr>
<td>PaDAH7PS-Diox</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>85–92</td>
<td>962.4788</td>
<td>961.4715</td>
<td>85-DSLEIVMR-92</td>
</tr>
<tr>
<td>106–124</td>
<td>2179.9869</td>
<td>2178.9796</td>
<td>106-GLINDPHMDHSFDINEGLR-124</td>
</tr>
<tr>
<td></td>
<td>2195.9868</td>
<td>2194.9795</td>
<td>106-GLINDPHMDHSFDINEGLR-124</td>
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<tr>
<td>251–273</td>
<td>2402.1860</td>
<td>2401.1788</td>
<td>251-EGLINAGLIPMIDSHIPANKSL-273</td>
</tr>
<tr>
<td>2418.1803</td>
<td>2417.1731</td>
<td>2417.1731</td>
<td>251-EGLINAGLIPMIDSHIPANKSL-273</td>
</tr>
<tr>
<td>278–294</td>
<td>1820.7990</td>
<td>1819.7918</td>
<td>278-QMEVATDVSGLSQGDR-294</td>
</tr>
<tr>
<td></td>
<td>1836.7998</td>
<td>1835.7925</td>
<td>278-QMEVATDVSGLSQGDR-294</td>
</tr>
<tr>
<td>295–322</td>
<td>3000.5833</td>
<td>2999.5760</td>
<td>295-SITGMIESHLVEQNGNLSEGEPLVYGK-322*</td>
</tr>
<tr>
<td></td>
<td>3016.5833</td>
<td>3015.5769</td>
<td>295-SITGMIESHLVEQNGNLSEGEPLVYGK-322*</td>
</tr>
</tbody>
</table>

*M* – Modified methionine residue into methionine sulfoxide, # – Peptide sequences were identified further from MS/MS spectrum of selected peptides.
3.7. Monitoring the changes in secondary structures of different oligomeric forms of PaDAH7PS

The changes in secondary structure of different oligomeric states of PaDAH7PS were elucidated by comparing their far-UV CD spectra as presented in Fig. 12A. The most active PaDAH7PS-Hexnox state, exhibited two negative peaks at 208 nm and 222 nm and a positive peak at 195 nm which indicated the β/α secondary structure, as expected in a TIM barrel fold, which is consistent with the other characterized DAH7PS [13,50]. As the PaDAH7PS-Tetox showed the similar CD spectrum so the ratio of ellipticity [(θ)208/(θ)222], which basically depends on the secondary structural elements packing of a protein, was also determined. Remarkably, the values acquired by PaDAH7PS-Hexnox and PaDAH7PS-Tetox were 0.9 and 1.09 respectively. The value 1.09 being closer to the range (1.1–1.4), illustrates a feature unique to the partially folded state of PaDAH7PS. In contrast, the CD spectrum of PaDAH7PS-Homox* depicted a peak at ~203 nm and a significant reduction (~55%) in ellipticity at 222 nm (Fig. 12B). This suggests the presence of the random coil conformation and loss in the helical content of protein [51].

The ratios of ellipticity [(θ)205/(θ)222] and [(θ)208/(θ)222] of all states of PaDAH7PS were also measured to validate the emergence of β-content and enhancement in disorderliness, respectively (Table 5). When PaDAH7PS-Homox* was compared to PaDAH7PS-Hexnox, a noteworthy increment in both the values of the ratios of ellipticity was observed (Table 5). Thus, the increment in the β-structure and random coil conformation content on methionine oxidation is probably associated with the aggregation of PaDAH7PS as also reported in earlier studies on aggregation of proteins [46,52].

3.8. Fluorescence spectroscopic characterization of different oligomeric states of PaDAH7PS

Furthermore, the fluorescence spectroscopy was also performed to analyze the tertiary structural perturbation in all oligomeric states of PaDAH7PS. The intrinsic emission spectra of Trp (λem = 295 nm) residues in all PaDAH7PS oligomeric states were analyzed. The comparative modeling of the PaDAH7PS monomeric structure revealed the existence of total four Trp104, Trp331, Trp159, and Trp215 residues. Between these Trp104 and Trp331 were surface exposed, while Trp159 was located inside the barrel of PaDAH7PS (data not shown). The intrinsic fluorescence spectra of PaDAH7PS-Hexnox, PaDAH7PS-Tetox, and PaDAH7PS-Diox depicted maximum emission at λmax = 346.5 nm, which suggests that the Trp residues are exposed to the hydrophilic environment. Notably, PaDAH7PS-Homox* and PaDAH7PS-Dimox* exhibited a red shift of 6.5 nm and 1 nm, respectively, and a concomitant loss was detected in fluorescence intensity of PaDAH7PS-Homox* (Fig. 13A). The aforesaid red shift indicated that more exposure and interaction of Trp residues occurred with the solvent on oxidation. Thus the occurrence of conformational changes in PaDAH7PS, as denoted by the above analysis, resulted in the alteration of the hydrophobic core of the protein. Moreover, we also probed the nature of partially folded and unfolded states of PaDAH7PS using Anilino-naphthalene-sulfonic acid (ANS) binding dye (λexc = 380 nm) (Fig. 13B). A few plausible hydrophobic sites are present in PaDAH7PS structure, which can accommodate the extrinsic ANS dye owing to which, PaDAH7PS-Hexnox (folded form) contributed noticeably to the fluorescence intensity with ANS. The PaDAH7PS-Tetox exhibited highest intensity of all oligomeric states due to the strong binding of ANS, suggesting the generation of a partially folded state with a destabilized hydrophobic core. In contrast, the PaDAH7PS-Homox* and the PaDAH7PS-Dimox* showed negligible fluorescence intensity, as expected, due to less binding of ANS in more unfolded state of a protein (Fig. 13B).

3.9. ThT binding assay of different oligomeric states of PaDAH7PS

The ThT binding of all forms of PaDAH7PS was analyzed to clarify the presence of the amyloid fibrils. In Fig. 14A, the PaDAH7PS-Hexnox, showed a little binding with ThT may be
Fig. 12. (A) Far-UV CD spectra comparison of different oligomeric states of PaDAH7PS (B) Changes observed in ellipticity at 222 nm in different oligomeric states; A significant loss in ellipticity was noticed in PaDAH7PS-HOmx* form which suggests that enzyme has lost its helical structure content.

Table 4
Comparison of kinetic parameters for PaDAH7PS-Hexnox oligomeric state with its homologs.

<table>
<thead>
<tr>
<th>DAH7PS from Organisms</th>
<th>$K_m^{\text{PP}}$ ($\mu$M)</th>
<th>$K_m^{\text{E4P}}$ ($\mu$M)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m^{\text{PP}}$ (s$^{-1}$ $\mu$M$^{-1}$)</th>
<th>$k_{cat}/K_m^{\text{E4P}}$ (s$^{-1}$ $\mu$M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PaDAH7PS</td>
<td>48.49 ± 3.12</td>
<td>256.8 ± 18.15</td>
<td>7.81 ± 0.89</td>
<td>0.161</td>
<td>0.030</td>
</tr>
<tr>
<td>EcDAH7PS</td>
<td>80</td>
<td>900</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NmDAH7PS</td>
<td>11 ± 1</td>
<td>43 ± 4</td>
<td>25.5 ± 0.5</td>
<td>2.3</td>
<td>0.59</td>
</tr>
</tbody>
</table>

ND- Not Determined, NmDAH7PS-Neisseria meningitidis DAH7PS.

Table 5
Secondary structure content of PaDAH7PS of different oligomeric states.

<table>
<thead>
<tr>
<th>Oligomeric State of PaDAH7PS</th>
<th>Helical content (%)</th>
<th>$\beta$-content (%)</th>
<th>Random Coil (%)</th>
<th>$[\langle \theta \rangle_{215}/\langle \theta \rangle_{222}]$</th>
<th>$[\langle \theta \rangle_{200}/\langle \theta \rangle_{222}]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexnox</td>
<td>~21</td>
<td>~27</td>
<td>~52</td>
<td>0.9472</td>
<td>0.7411</td>
</tr>
<tr>
<td>Tetox</td>
<td>~17</td>
<td>~31</td>
<td>~52</td>
<td>0.9766</td>
<td>0.8214</td>
</tr>
<tr>
<td>Dixo</td>
<td>~19</td>
<td>~27</td>
<td>~54</td>
<td>1.0440</td>
<td>1.5160</td>
</tr>
<tr>
<td>HOMox*</td>
<td>~9</td>
<td>~42</td>
<td>~49</td>
<td>0.9998</td>
<td>0.9210</td>
</tr>
<tr>
<td>Dimox*</td>
<td>~17</td>
<td>~30</td>
<td>~53</td>
<td>1.0584</td>
<td>0.9724</td>
</tr>
</tbody>
</table>

Fig. 13. (A) Intrinsic Fluorescence spectra of different oligomeric states of PaDAH7PS; change in Trp fluorescence intensity was measured at ($\lambda_{exc} = 295$ nm) (B) Extrinsic fluorescence spectra of different oligomeric states of PaDAH7PS and change in ANS fluorescence intensity of at ($\lambda_{exc} = 380$ nm) was measured.
due to the presence of beta sheets in the native protein. Interestingly, PaDAH7PS-Tetox, PaDAH7PS-Diox and PaDAH7PS-Dimox* states depicted more ThT binding intensity as compare to the PaDAH7PS-Hexnox. The PaDAH7PS-HOmx* state exhibited the lowest binding with the ThT (Fig. 14A). This may be occurred due to the presence of exposed hydrophobic surfaces with high β-content in the PaDAH7PS-Tetox, PaDAH7PS-Diox and PaDAH7PS-Dimox* partial folded/unfolded intermediate states of PaDAH7PS. ThT is a benzothiazole dye interacts with a core cross β-sheet ordered structures present in the amyloid fibrils [53]. In PaDAH7PS, these partially folded and unfolded intermediate states might be rapidly interacted between their hydrophobic surfaces and self-associated with intermolecular β ladders, which have no long range ordered cross β-sheet structures like amyloid fibrils. Thus, PaDAH7PS-HOmx* state exhibited lowest binding with ThT and enhanced the process toward amorphous aggregation rather than fibrillation. The relative intensity of ThT at 485 nm with different forms of PaDAH7PS has shown in Fig. 14B.

4. Discussion

The DAH7PS, a well-characterized enzyme from different sources, has shown more diverse quaternary structures and allosteric control. The evolution of quaternary structures of DAH7PS is fundamentally connected with their uniquely controlled allosteric mechanism [14]. The evolutionary link between different types of DAH7PS enzymes has been determined on the basis of various properties such as metal requirement, substrate specificity, and allosteric regulation [54]. In the present study, PaDAH7PS, sequentially homologous (~79% identity) to homotetrameric EcDAH7PS, contains conserved active site and divalent metal binding site residues [13] (Fig. 25). The purified PaDAH7PS exhibits a functional hexameric state rather than the tetrameric state, as in EcDAH7PS, which is shown by size exclusion chromatography, native-page, cross-linking based experiments.

In vitro, instability of EcDAH7PS due to redox metal oxidation is prevented by binding of substrate (PEP) at the active site of DAH7PS and the restoration of thiol (–SH) content is achieved by reducing agent (DTT) [25]. Surprisingly, even in the presence of both the protecting agents (PEP & DTT), the quaternary structure of PaDAH7PS destabilizes into a tetrameric oxidized state (PaDAH7PS-Tetox) with less enzymatic activity. But when both the protecting agents are absent, the protein oxidizes and transforms into an inactive dimeric state (PaDAH7PS-Diox). Moreover, during redox metal treatment, PaDAH7PS does not dissociates completely into a monomeric state, as found in EcDAH7PS, but converts into an unexpected higher oligomeric form (PaDAH7PS-HOmx*) and an oxidized dimeric form (PaDAH7PS-Dimox*).

Proteins are the more susceptible targets for oxidation in biological systems due to their low redox potential [55, 56]. Methionine, cysteine, and histidine are the most vulnerable amino acid residues to get oxidized in the presence of oxidative stress [42, 57]. In PaDAH7PS, the oxidation of methionine residues to methionine sulfoxide is seen through FTIR spectroscopy, where the absorption bands are seen in the fingerprinting region due to the stretching vibration of the sulfoxide (S=O) group. Similarly methionine oxidation in human serum albumin, RNase, and other therapeutic proteins has been detected by FTIR spectroscopy [58–60].

The susceptibility of methionine residues for oxidation depends on their location in the three-dimensional structure of a protein and their solvent accessibility i.e. the more surface exposed residues are more labile for oxidation [42]. In mass spectrometry analysis PaDAH7PS-Tetox, Met113, located on the surface of the protein, as well as Met300, located inside the barrel closer to the active site of the protein, are oxidized. Whereas, in PaDAH7PS-Diox and PaDAH7PS-Dimox* five methionine residues (Met91, Met113, Met263, Met279, and Met300) that get oxidized. Thus, the degree of susceptibility of methionine residues for oxidation increases due to redox metal treatment. The generation of the unexpected higher oligomeric state as well as the oxidation of methionine residues occurs in PaDAH7PS, which is in contrast with the feature of EcDAH7PS, where an inactivated monomeric state with oxidized cysteine residues was depicted. Thus, the unpredicted oxidation of methionine residues with increment in the degree of oxidation and the generation of high oligomeric state encouraged us to further insight the effects on the morphological features, as well as secondary and tertiary structural changes in PaDAH7PS during stress conditions.

Oxidative modifications in proteins usually affect their conformational stability, which can further induce the aggregation and fibrillation process [56, 61]. Additionally, the atomic force microscopic analysis of all forms of PaDAH7PS reveals the presence of amorphous aggregates formation under oxidative stress conditions. Similarly, the oxidation of methionine residues in the light chain of immunoglobulin and other therapeutic proteins also provoked amorphous aggregation [62, 63]. Methionine oxidation also promotes the process of fibrillation in apolipoprotein A-I [64] and k-Casein [65]. In Parkinson and Alzheimer diseases, the metal-
triggers oxidation of methionine residue resulted in the formation of fibril and amyloid β–plaques, respectively [66–70]. In contrast, the fibrillation of α-synuclein, transthyretin, and apolipoprotein C-II was inhibited due to the methionine oxidation [71–73]. Therefore, it suggests that methionine oxidation affects the behavior of different proteins in multiple ways.

The protein aggregation is a critical aspect which is affected by several intrinsic and extrinsic factors [74,75]. The partially folded, unfolded, and misfolded states of a protein can lead to the process of aggregation [76–78]. Generally, protein tends to show the higher beta structure which is responsible for the nucleation process of aggregation [29,79]. Similarly, in the far-UV CD spectra analysis, the ratio of ellipticity [(θ)208/(θ)222] acquired value 1.09 and high binding affinity for ANS of PaDAH7PS-Tetox which clearly indicates that it possesses a unique feature of the partially folded state, which might have caused the oxidation of unexposed methionine residue i.e. Met300, located inside the hydrophobic core of the protein [80–82]. PaDAH7PS-HOMox*, PaDAH7PS-Dimox*, and PaDAH7PS-Diox unveil the clear emergence of β-content in CD spectra. Remarkably, the lack of tertiary structure and reduced binding affinity with ANS in the above three oligomeric states indicate the more unfolding of PaDAH7PS, which increase the degree of its oxidation and aggregation propensity. The ThB binding assay result also supported that the partially folded and unfolded intermediate states might be rapidly interacted and self-associated between their hydrophobic surfaces and enhanced the process of amorphous aggregation. Thus, PaDAH7PS significantly shows the methionine modifications, conformational changes, perturbation of hydrophobic core, and enhancement in the propensity to aggregate in oxidative stress conditions.

5. Conclusion

In this study, we have shown that PaDAH7PS is functional in its hexameric state, which is susceptible to metal–catalyzed oxidation. Here, for the first time, we have identified that methionine residues are vulnerable to oxidation during stress conditions rather than active site cysteine residues in PaDAH7PS. The quaternary structure of PaDAH7PS destabilizes and attains a partially folded and unfolded state with high β-content. These presumably act as the critical precursors, which enable specific intermolecular interactions responsible for the aggregation. To elucidate the molecular mechanism and kinetics of the aggregation process of PaDAH7PS, further studies are required.

Conflict of interest

The authors declare no conflict of interests.

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Supporting information

Table showing the assessment of theoretical trypsin digestion of PaDAH7PS, Modification of methionine of peptides, SDS profile of PaDAH7PS under different conditions and pairwise sequence alignment analysis with homolog are provided as supplementary data.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiomac.2017.08.105.

References


