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Photosystem Proteins Protects by Small Heat Shock Protein in *Cyanobacteria* under Oxidative Stress Condition

Kollimalai Sakthivel*

Department of Marine Biology, Annamalai University, India

ABSTRACT

Chaperone activity of small heat shock proteins HSP with Photosystem I Protein (PsaAB) in the presence of hydrogen peroxide was studied. HspA plays a protective role under oxidative stress in *Synechococcus elongatus* strain ECT16-1, which constitutively expresses HspA. Reference strain ECT, which does not constitutively express HspA, ECT16-1 showed much better chlorophyll in hydrogen peroxide. Photosystem protein PsaAB was degraded on 72 hours incubation in ECT but there was no change in ECT16-1. The PsaAB absorbance reduced at 65% on 0.3% hydrogen peroxide at 30°C for 9 hours incubation. Two degradation product of photo system protein PsaAB (40, 13 kDa) were detected and those bands intensity was increased at higher concentration of hydrogen peroxide. The PsaB one of the major photo system I protein degradation product were also detected by western blott analysis. The degradation of photo system I protein (PsaAB) was observed at the low concentration of hydrogen peroxide (0.15%) in control, and lysozyme mixed samples, α-crystallin is the homologus protein of small heat shock protein protected up to 0.3% and HspA protected at high concentration of hydrogen peroxide on 0.4%. In western Blotting analysis showed the photo system I protein (PsaB) not degraded when the sample mixed with α-crystallin in the presence of 0.3% hydrogen peroxide. Spectrophotometer results shows when photo system I peak was reduced due to the hydrogen peroxide mixed with buffer (control-1) or lysozyme (control-2), but the peak not reduces in HspA mixed photo system I.

Key Words: *Cyanobacteria; Small heat shock protein; Photosystem I; Chaperon; Oxidative stress*

INTRODUCTION

It was suggested that the accumulation of heat shock protein is correlated with thermos tolerance [1]. Subsequently it has been recognized that HSPs have a protective role against a variety of stresses beside high temperature. These include nitrogen starvation [2], hyperosmotic stress, and salt stress [3,4], oxidative stress [5,6]. Oxidative species like superoxide (O_2), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH) are generated as a result of incomplete reduction of oxygen during respiration and photosynthesis. They can damage the cell, and it is conceivable that DNA repair, protein degradation, metabolic energy generation, cell division, and other cellular activities are all coordinately regulated [7,8].

Chloroplasts have arisen from a *cyanobacteria*l ancestor [9]. It is maintained an independent circular genome encoding gene products which are important for primary and secondary processes of photosynthesis. Photosystem I (PSI) is a multisubunit pigment-protein complex of oxygenic photosynthesis located in the thylakoid membrane of chloroplasts and cyano bacteria. The PSI subunits are designated psaA-P, according to the corresponding psaA-P genes [10,11]. A heterodimer of two integral membrane proteins, PsaA and PsaB, forms the core of the PSI reaction centre, which binds the electron transfer components

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Corresponding author Kollimalai Sakthivel, Department of Marine Biology, Annamalai University, India; E-mail: kollimalaisakthivel79@gmail.com.

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P700, Ao, A1, and Fx. It has been reported that adding the CP sHSP 21, to thylakoid membranes in vitro protects against heat inactivation [12]. Photoinhibition in chilling-sensitive plants did occur during chilling stress under low irradiance [13]. It was reported that the activity of PSI decreased about 70-80%, when cucumber leaves were treated at 5ºC in an irradiance of 100 µmol m-2s-1 In *Synechocystis* sp. PCC 6803, PSI genes are actively transcribed under low-light (LL) conditions, whereas their transcription is coordinately and rapidly down-regulated upon shift to HL conditions [14-17]. PsaA and PsaB proteins, the heterodimer subunits of the PSI reaction centre, were degraded by photo inhibition at chilling temperatures [18].

For the present study we purified photosystem I proteins from *Synechococcus* sp. PCC 7942 in order to analyze the role of heat shock proteins in preventing degradation and aggregation under oxidative stress conditions. Hydrogen peroxide is one of the oxidative species that were used for creating the oxidative stress condition artificially. We found that HspA one of the small heat shock proteins, protect and photo system I, from degradation and aggregation during oxidative stress.

MATERIALS AND METHODS

Organisms and Culture Conditions

The *cyanobacteria*l strain *Synechococcus* sp. PCC 7942 was cultured photoautotrophically in BG-11 inorganic liquid medium or on BG-11 plates containing 1.5% (w/v) agar and 0.3% (w/v) sodium thiosulphate. The BG-11 culture medium was modified with 50 µg/ml Na 2CO3 and 5 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid and adjusted to pH 8.0 with KOH. The liquid cultures in glass vessels were incubated at 30°C, continuously aerated and illuminated with a light intensity of 30 µmol photons m-2 s-1.

Preparation of Thylakoid Membrane

Cultures of *Synechococcus* sp. PCC 7942 in the logarithmic growth phase (0.7 OD at 730 nm) were harvested by centrifugation at 3000 rpm for 15 min. The pellet was resuspended in ten volumes of thylakoid buffer 25 mM MES/NaOH, pH 7.0 containing 1 mM aminocaproic acid. The cells were disrupted using anequal amount of glass beads (0.177-0.25 mm in diameter) and vortexed twice for 1 min with 2 min interruption for cooling on ice. Glass beads were washed four times with 200 µl of thylakoid buffer, aliquots pooled and unbroken cells were removed by centrifugation at 1000 rpm for 5 min. The supernatant containing thylakoid membranes was centrifuged at 13,000 rpm for 30 min. Thylakoid membranes were re suspended in thylakoid buffer to a concentration of about 500 µg Chl ml-1. Isolated membranes were highly enriched with thylakoid and stored at -20°C.

Purification of Photosystem Proteins

The thylakoid membranes were diluted in thylakoid buffer to a chlorophyll concentration of 250 µg ml-1 and solubilized with Triton ×100 (2% final concentration) for 15 min onice. Non-solubilized material was removed by centrifugation at 13,000 rpm for 30 min at 4°C. Then the supernatant was centrifuged at 70,000 rpm (HITACHI Himac CS 120 FX, S120AT2-0157 rotor) for 60 min at 4°C. Photosystem II particles were solubilized in the supernatant, while the sediment was enriched in Photosystem I.

Purification of Small Heat Shock Proteins

Small heat shock protein purified from HspA gene amplified *Synechococcus* sp. PCC 7942 chromosomal DNA with two oligonucleotide primers, HspA-F(5-ATATGGCACTCGTTGATTC-3) and HspA-R(5- CTCGAGTCGCTCGCAAGCTTCA G-3) by a previously described procedure. The amplified fragment was cloned into pT7Blue Vector (*Invitrogen*) according to the manufacturer's instructions. This plasmid was digested with NdeI and XhoI and the insert was subsequently cloned into the pET21a expression vector (*Invitrogen*), previously digested with the same restriction enzymes. The vector pET21a-HspA carried a His6 tag which was fused to the C terminus of the hspA gene product. The constructed plasmid was transformed into *E.coli* BL21(DE3). The cells were grown to apparent absorption 0.6 at 540 nm in LB medium containing 100 µg/ml ampicillin at 37°C, and then IPTG was added to a concentration of 1 mM. After a further 3 h at 37°C, the cells were harvested by centrifugation and stored at -80°C. The pellet was suspended TE buffer (50 mMTris/HCl, pH 7.5 and 1 mM EDTA) and lysed by sonification. After removing cell debris the protein in the supernatant was precipitated with 0-40% (w/v) saturated ammonium sulphate. The protein pellet was dissolved in TE buffer and loaded onto DEAE/Toyopearl 650S equilibrated with TE. After washing the column with TE, HspA was eluted with a 0-250 mM NaCl gradient in TE and fractionated. Fractions were examined by SDS/PAGE to identify those containing HspA. Fractions containing HspA were pooled, dialyzed against TE and applied to a column of hydroxylapatite HP40-100 equilibrated with TE. After washing the column with TE, protein was eluted with a 0-400 mM Na-phosphate (pH 7.7) gradient. Fractions containing HspA were pooled, dialyzed against TE and stored at -20°C. α-crystallin source from Bovine Eye Lens, purchased from Stressgen Bioreagent Corp. and molecular weight 20.037 kDa.

Stress Condition

Photosystem protein was mixed with heat shock proteins (1:1), in the presence of 0.05 to 7% hydrogen peroxide and incubated at 30˚C for 3 hours for SDS-PAGE, Western Blotting, absorption and fluorescence spectrophotometry. For characterization studies, photosystem protein and hydrogen peroxide at 0.03-0.3%, temperature 0 and 37˚C incubated for 9 hours and optimum density at 663 nm was measured by a Shimadzu UV-1200 double beam spectrophotometer (Shimadzu, Kyoto, Japan).

BN/SDS-PAGE and Western Blot Analysis

Thylakoid membrane (14 µg Chl.) was solubilized with 2% Triton X-100 and loaded on 7% BN-PAGE at 4˚C. Soluble fraction (40 µg protein) was mixed with equal amount of SDS-sample buffer containing 0.12 M Tris/HCl (pH 6.8), 0.12 M dithiothreitol, 12% sucrose, 4% sodium dodecyl sulfate (SDS) and 0.06% bromophenol blue, denatured at 100˚C for 3 min and loaded on 15% SDS-PAGE.

RESULTS

Presence of Hydrogen Peroxide on the *Synechococcus* elongates Strains ECT and ECT16-1

In order to test whether sHSP plays a role in the oxidative stress response, we compared the effect of hydrogen peroxide on the ECT16-1 strain harboring a *Synechococcus vulcanus* hspA expression vector with that on the ECT strain, the reference strain, which harbors the vector without the hspA gene. Chlorophyll curves for ECT and ECT16-1 in the presence of 1 mM hydrogen peroxide at 30˚C under a light intensity of 30 µE m-2/s-1 are shown in (**Figure 1**) revealed that the ECT strain had much lower chlorophyll than the ECT16-1 strain. These results suggest that HspA enhances cell survival and tolerance to peroxide stress.

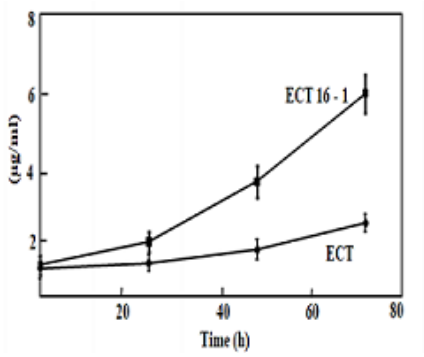

Figure 1: Cell growth of the *Synechococcus* ECT and and ECT16-1 strains incubated in the presence of 1mM hydrogen peroxide for 0, 24, 48 and 72 h. Cells were cultured at 30°C absorbance at 730 nm studied at 0 h. Hydrogen peroxide was added to final concentration of 1 mM after that and culture maintained up to 3 days, absorbance reading has taken every 24 h interval. Results are average of three independent experiments; bars indicate standard deviation.

Samples containing equal amount of chlorophyll were loaded to the sucrose density gradients and the PSI complexes were isolated and separated from ECT and ECT16-1 strains by centrifugation. Three chromophorecontaining bands were observed, which is consistent with a previous report. That report showed that the upper yellow, the middle green, and the bottom green bands contain carotenoids, PSII/PSI monomers, and PSI trimers, respectively (**Figure 2a**). The upper yellow band contained, besides others, the abundant red and orange carotenoid protein. The middle and the bottom bands contained PSI since the PSI subunits PsaA, PsaB, PsaD and PsaF were detected in a CBB-stained gel after SDS-PAGE (not shown). In the bottom band, only PsaAB proteins were detected. They were totally absent in the 72-h hydrogen peroxide treated ECT strain (not shown). The PSI trimer band (the bottom band) became less intense after the 48-h incubation in ECT, indicating that it is sensitive to oxidative stress. All three chromophore-containing bands were greatly decreased after the 72-h incubation in ECT, whereas ECT16-1 retained all three bands at a constant level during the oxidative stress. Photosystem I protein also analyzed by two dimension SDS-PAGE from hydrogen treated ECT16-1 and ECT cells. In first and second dimension results showed, the intensity of Photosystem protein very much reduced on 72 hours treated control ECT cells not in the hspA over expression ECT16-1 cells (Figure 2b). In the photosystem protein such as PsaD and PsaC were detected by second dimension and observed lesser intensity (not shown).

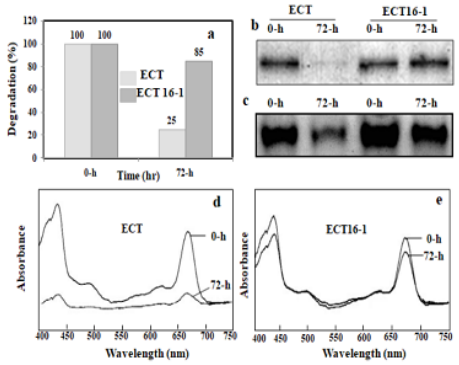

Figure 2: Photosystems protein (PsaAB) isolated by sucrose density gradient centrifugation and BN-PAGE. Cells are harvested from ECT and ECT16-1 strain incubated in the presence of 1 mM hydrogen peroxide for 0 and 72 h, respectively. Thylakoid membrane isolated and equal amount was loaded in each tube for sucrose density gradient centrifugation (250 µg of chlorophyll) and for BN-PAGE (30 µg of chlorophyll). Photosystem protein separated and analyzed by spectrophotometer (a), SDS-PAGE (b) BN-PAGE (c) double wavelength and double beam spectrophotometer for ECT (d), and ECT16-1 (e).

Characterization of Photosystem Protein

The photosystem protein (PsaAB) characters studied in the presence of different concentration of hydrogen peroxide (0.03 to 0.3%) incubated at 4 to 37ºC for 9 h and the optimum density was measured at 663 nm by the spectrophotometer. The result showed three different changes, no change on lower concentration hydrogen peroxide and low temperature. Higher changes were observed on higher concentration of hydrogen peroxide and higher temperature. The optimum density values of the photosystem sample not chanced in the absence of hydrogen peroxide at 4 and 37ºC and 0.03% of hydrogen peroxide at 37ºC. The optimum density values of the samples reduced 20% on 0.3% hydrogen peroxide at 4ºC incubated sample and 0.1% hydrogen peroxide at 37ºC for 9 h incubation samples. There was 65% of optimum density values was reduced when the sample mixed with 0.3% hydrogen peroxide at 37ºC for 9 h incubation (**Figure 3a**).

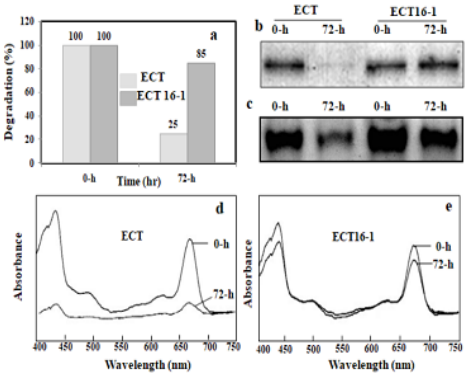

Figure 3: Degradation of PSI in the presence of hydrogen peroxide. PSI proteins were mixed with different concentrations of hydrogen peroxide, incubated at 0 or 37ºC for 9 h. After incubation the samples were centrifuged and supernatant was analyzed for light absorbance. Number 1 for three samples, first sample incubated at 0ºC temperature without addition of hydrogen peroxide, second sample incubated at 37ºC without addition of hydrogen peroxide and third sample incubated at 37ºC temperature with 9.6 mM of hydrogen peroxide. Number 2 for sample incubated at 0ºC temperature with 96 mM of hydrogen peroxide. Number 3 for sample incubated at 37ºC temperature with 32 mM of hydrogen

peroxide. Number 4 sample incubated at 37ºC temperature with 96 mM of hydrogen peroxide (a). PSI proteins were mixed with different concentrations of hydrogen peroxide, incubated at 30ºC for 3 h. Then the samples were centrifuged and supernatant (5 µg of protein) was loaded in each well of 15% SDS/ PAGE with 7 M urea. Lance 1 for marker, 2 for a control without hydrogen peroxide treatment, lanes 3-8 for sample mixed with 16, 32, 48, 64, 80, 96, 112, 128 mM of hydrogen peroxide (b). PSI proteins were mixed with 96 mM of hydrogen peroxide, and incubated at 30ºC for 3 h. Then centrifuged sample (5µg of protein) was loaded in each well of 15% SDS/PAGE with 7 M urea and the PsaB protein was detected by Western blot using antibody. Lane 1 for control, without addition of hydrogen peroxide. Lane 2 for sample with hydrogen peroxide (c).

The photosystem protein PsaAB treated with 0.05 to 0.4% hydrogen peroxide and incubated at 30ºC for 3 h to study the protein degradation and aggregation. Here our results showed the protein degradation correlated with concentration hydrogen peroxide. We found two new degradation product bands at 40 and 13 kDa size respectively, and the intensity of those bands increased in higher concentration hydrogen peroxide (Figure 3b). The degraded photosystem protein by hydrogen peroxide was further detected by western blot analysis using antibody (Figure 3c).

Chaperone Activity between PsaAB and HSP

The photosystem protein mixed with lysozyme for control, α-crystallin (homologus of small heat shock protein) and small heat shock protein HspA in one:one ratio, incubated 30ºC for 3 h in the presence of 0.3% of hydrogen peroxide to study the chaperone activity. Here our results showed control photosystem protein without addition of either hydrogen peroxide nor small shock protein was affected even in the presence very low concentration of hydrogen peroxide 0.05%. In the presence of lysozyme control protein the photosystem protein tolerated upto 0.15% hydrogen peroxide, after that it was degraded. By α-Crystallin approximately 50% of photosystem protein was protected even in the presence of high concentration of hydrogen peroxide (0.4%). Around 80% of the photosystem protein protected by HspA at 0.4% hydrogen peroxide (**Figure 4a**). One of the major photosystem protein (PsaB), degradation was prevented by α-crystallin in the presence of 0.3% hydrogen peroxide at 30ºC for 3 h proved further by western blot analysis (**Figure 4b**).

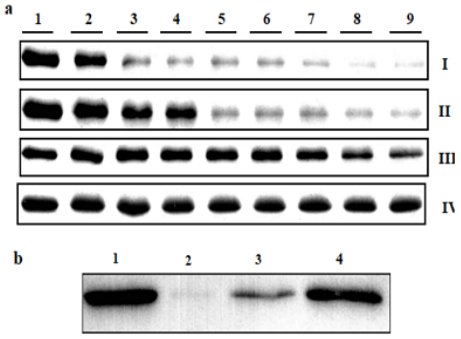

Figure 4: Direct interaction of PsaAB with HSP in the presence of hydrogen peroxide studied by SDS-PAGE (a) and Western Blott (b). PSI was mixed with different concentrations of hydrogen peroxide. Lane 1 for control without addition of hydrogen peroxide, lanes 2-9 addition of hydrogen peroxide 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4%. First row (I) for control (photosystem sample mixed with hydrogen peroxide), Second row (II) for samples mixed with a control protein (Lysozyme), third row (III) for sample mixed with α-crystallin and fourth row (IV)for sample mixed with HspA. Sample mixed with lysozyme, or α-crystallin or HSP in one:one ratio, and incubated at 30ºC for 3 h. The centrifuged samples (5 µg of protein) were loaded on 15% SDS/PAGE with 7 M urea. Photosystem sample mixed with BSA or α-crystallin in one:one ratio, incubated at 30ºC for 3 h, centrifuged sample (5µg of protein) were loaded on 15% SDS-PAGE with 7M urea then photosystem protein (PsaB) detected by western blott analysis. Lane 1 for control sample without addition of hydrogen peroxide, lane 2 for sample with hydrogen peroxide, lane 3 for sample mixed with BSA and lance 4 for sample mixed with α-cyrstallin (b).

The above results showed that PSI proteins were protected by α-crystallin and HspA. These results were further confirmed by light absorbance studies. The PSI proteins were mixed with lysozyme or α-crystallin or HspA in one:one ratio, incubated at 37ºC for 2 h in the presence of 96 mM hydrogen peroxide to study the interaction of PSI and HSPs by a double wavelength and double beam spectrophotometer. In the case of the PSI proteins mixed with HspA (**Figure 5a**) or α-crystallin (Figure 5b), The Chlrophyll absorption at 680 nm was kept at the same level as the control sample (without addition of hydrogen peroxide or α-crystallin or HspA, incubated at 0ºC). The control protein lysozyme did not protect just like no addition sample (without addition of α-crystallin or HspA, only hydrogen peroxide).

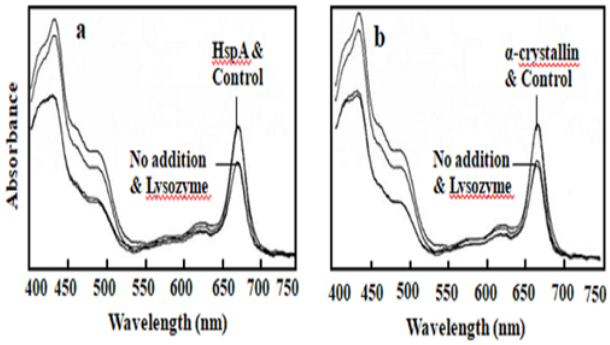

Figure 5: Light absorbance studies were undertaken for interaction of PSI and HPS in the presence of hydrogen peroxide. PSI was mixed with one:one ratio of lysozyme or a HSP, such as HspA (a) and α-crystallin (b) in the presence of 96 mM of hydrogen peroxide, incubated at 37ºC for 2 h. Then the samples were centrifuged and the supernatant analyzed for light absorbance. Control sample without addition of hydrogen peroxide and no addition sample contained only hydrogen peroxide. In the figure (a) number 1 for sample mixed with HspA and control without addition of hydrogen peroxide, number 2 for addition of hydrogen peroxide, but without addition of any protein and addition of control protein lysozyme. On the figure (b) number 1 indicates for sample mixed with α-crystallin and control sample without addition of hydrogen peroxide, number 2 indicates for addition of hydrogen peroxide, but without addition of protein and sample mixed with control protein lysozyme.

DISCUSSION

Several heat shock proteins were induced by hydrogen peroxide. Heat stress stimulates hydrogen peroxide generation in plants [19]. Moreover, heat shock proteins are involved in enhancing survival following oxidative stress in yeast, animals, and plants [20-22]. Thus, the induction of genes encoding heat shock proteins and a heat shock transcription factor by hydrogen peroxide may lead to increased tolerance of further oxidative stress, as in tomato (*Lycopersiconesculentum*) cells [20], as well as contributing to tolerance of other stresses such as high temperature [19]. It was reported that treatment with hydro-

gen peroxide was more effective in inducing the synthesis of sHSPs that treatment with MV [20]. Hydrogen peroxide is able to diffuse freely through membrane [23].

Oxidative stress causes the proliferation of peroxisomes [24]. A dense population of peroxisomes might be highly efficient in scavenging of Reactive Oxygen Species (ROS), especially hydrogen peroxide, which diffuses into peroxisomes from the cytosol. Under normal growth conditions, the production of ROS in cells is low (240 µM S-$1O_2$- and a steady-state level of 0.5 µM hydrogen peroxide in chloroplasts), many stresses that disrupt the cellular homeostasis of cells enhance the production of ROIs (240-720 µM S-1 O_2- and a steady-state level of 5-15 µM hydrogen peroxide) [25].

Photosystem Protein (PsaAB)

Photosystem proteins are very much damaged by oxidative stress condition. Small Hsp prevents irreversible aggregation reactions and keeps protein on the protective folding pathway. During stress, the photosynthetic apparatus might be suppressed or damage. In the present study when we mixed the photosystem sample with α-crystallin the aggregation was prevented after 48 hours dialysis, but not in the sample mixed with Bovin Serum Albumin. The PsaA protein had a significantly higher degradation rate than its partner in the PSI reaction center heterodimer, PsaB. So far, it has been shown that in higher plants and algae, the stability of the PsaB protein is more critical than that of PsaA. For example, in *Chlamydomonas reinhardtii*, it has been demonstrated that in the absence of the synthesis of the PsaB protein, PsaA cannot be detected whereas in the absence of the synthesis of PsaA, one can still detect the PsaB protein in the thylakoid membrane [26].

CONCLUSION

Sonoike demonstrated specific degradation of the PsaB protein during photoinhibition of PSI in spinach thylakoid membranes. In the present study the PsaB intensity was very much reduced by lyzosome control protein mixed sample, but not by the HspA mixed sample. Our data demonstrate that under oxidative stress condition induces partial damage of the PSI complex in the Cyanobacterium *Synechococcus* sp. PCC 7942 control strain.

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