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Detection of in vivo protein tyrosine nitration in petite mutant of *Saccharomyces cerevisiae*: Consequence of its formation and significance

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ABSTRACT

Protein tyrosine nitration (PTN) is a selective post-translational modification often associated with physiological and pathophysiological conditions. Tyrosine is modified in the 3-position of the phenolic ring through the addition of a nitro group. In our previous study we first time showed that PTN occurs in vivo in *Saccharomyces cerevisiae*. In the present study we observed occurrence of PTN in petite mutant of *S. cerevisiae* which indicated that PTN is not absolutely dependent on functional mitochondria. Nitration of proteins in *S. cerevisiae* was also first time confirmed in immunohistochemical study using spheroplasts. Using proteosomal mutants Rpn10Δ, Pre9Δ, we first time showed that the fate of protein nitration in *S. cerevisiae* was not dependent on proteosomal clearing and probably played vital role in modulating signaling cascades. From our study it is evident that protein tyrosine nitration is a normal physiological event of *S. cerevisiae*.

1. Introduction

The formation of 3-nitrotyrosine (3-NT) in proteins occurs as an in vivo post translational modification. Tyrosine is modified in the 3-position of the phenolic ring through the addition of a nitro group. It is believed that tyrosine nitration involves a two-step process where the initial step is the oxidation of the phenolic ring of Tyr to yield the one electron oxidation product, Tyr radical (Tyr·). Several one-electron oxidants known to occur in vivo such as $CO_3^{\cdot -}$, ·OH, $^{\cdot}NO_2$ or compound I of peroxidases can accomplish this task. The second step involves the addition of $^{\cdot}NO_2$ to the Tyr· in a radical termination reaction [1–6]. There are two proximal nitrating agents that account for nitration in vivo. One nitrating agent is peroxynitrite which is formed by the fast reaction between nitric oxide (NO) and superoxide ($O_2^{\cdot -}$). The other proximal nitrating agents involve hemeperoxidases such as myeloperoxidase or eosinophil peroxidase in the presence of hydrogen peroxide (H_2O_2) and nitrite (NO_2^-) [7,8]. Collectively, the published work revealed the existence of multiple biochemical pathways (peroxidases, hemeproteins) leading to nitration. Nitrated proteins have been detected in a number of physiological and pathological settings. Both in vivo and in vitro data support a selective process in which the local structural environment of specific tyrosine residues governs the selectivity. Advancements in analytical biochemistry have also enabled the discovery of specific proteins modified by nitration in vivo using monoclonal and polyclonal antibodies. There are three major effects on protein function that can be observed due to protein nitration: (1) No changes in protein function, (2) loss of function and (3) gain of function [6–8]. A series of recent publications have extensively analyzed biologically relevant nitration under different conditions and environments and its pathological relevance [9–15]. However, the significance of protein nitration in protein turnover, protein localization and signal transduction processes is under investigation.

Yeast has become a very important model organism to study biochemistry and molecular biology of mammalian cells and to answer fundamental questions concerning the mechanisms of many processes [16–21]. The origin of NO in yeast cells is still a matter of debate essentially because of the lack of mammalian nitric oxide synthase (NOS) orthologues in the yeast genome. NO mediated apoptosis and NOS like activity in yeast have been demonstrated [22]. From our laboratory we first time showed that protein tyrosine nitration (PTN) occurs in vivo in *Saccharomyces cerevisiae* cells grown under respiratory proficient conditions [18]. Yeast flavohemoglobin is known to play important role both in oxidative stress and nitrosative stress [23,24]. We observed many immunopositive spots both in cytosol and in mitochondria from wild type as well as in flavohemoglobin deleted strain of *S. cerevisiae* using monoclonal anti-3-nitrotyrosine antibody [18].

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Furthermore, results from our group also showed a novel role of catalase in peroxynitrite detoxification in *S. cerevisiae* [20]. Generation of either peroxynitrite or nitrite is essential for protein tyrosine nitration in *S. cerevisiae*. Concomitant release of NO and $O_2^{\cdot-}$ is prerequisite for peroxynitrite formation. Superoxide anion $O_2^{\cdot-}$ is mainly generated from the leakage of electrons from the mitochondrial respiratory chain as a normal consequence of aerobic respiration [18,22]. Hydrogen peroxide (H_2O_2) is produced during the detoxification of superoxide anion catalyzed by superoxide dismutases and also in yeast during the oxidation of fatty acids in the peroxisome [25]. In the present study, we addressed two questions: (1) Can PTN is dependent on functional mitochondria? (2) What is the fate of nitrated proteins? In this study we first time show that PTN occurs in petite mutants of *S. cerevisiae*. This indicated that protein tyrosine nitration can occur even in the absence of functional mitochondria of *S. cerevisiae*. An investigation on protein tyrosine nitration in *S. cerevisiae* might provide insights and knowledge to elevate protein tyrosine nitration from a biomarker to a biologically important post translation modification.

2. Materials and methods

2.1. Strains and media used

Strains of *Saccharomyces cerevisiae* used in the studies were Y190 (MATa gal 4 gal 80 his 3- Δ200 trp 1-901 ade 2-101 ura 3-52 leu 2-3,112 URA3::GAL1-LacZ LYS2::GAL4 (UAS)::HIS3 cyhR, wild type) and isogenic ΔYhb1 (Y190 yhb1::G418R) cells. These are generous gift from Dr. Jonathan Stamler [23]. BY4742 (MATα his 3- Δ1 leu2- Δ0 lys2- Δ0 ura3- Δ0) and isogenic ΔRpn10, ΔPre9 strain were generous gift from Dr. S. Sirkar, Assistant Professor, Bose Institute, Kolkata. The cells were grown in Yeast Peptone Dextrose (YPD) broth [1% yeast extract (Difco), 2% bactopeptone (Difco), 2% dextrose] at 30 °C under shaking condition. For isolation and screening of petite mutants, YPG broth [1% yeast extract (Difco), 2% bacto peptone (Difco), 3% glycerol, pH-7.0] was used. ΔYhb1 strains were grown in presence of 200 mg/L G418 sulphate (Amresco, Ohio, USA). BY4742 (MATα his 3- Δ1 leu2- Δ0 lys2- Δ0 ura3- Δ0) and isogenic ΔRpn10, ΔPre9 strains were grown in synthetic complete media with yeast nitrogen base (0.67% w/v, 2% dextrose with necessary supplements).

2.2. Measurement of cell growth

For growth measurement experiments, mid-log phase culture was used as inoculum following dilution in fresh media to O.D. 600 nm = 0.07–0.10. Cell growth was monitored tubidimetrically by measuring the absorbance at 600 nm at every two-hour interval.

2.3. Construction of petite mutants

Petite mutants of *S. cerevisiae* were generated according to Goldring et al. [26]. For petite mutant construction, mid log phase cells were incubated in dark at 30 °C with 10 μg/ml concentration of ethidium bromide for 24 h. It was then plated in YPD agar plates. The colonies unable to grow in presence of glycerol, a non-fermentable carbon source were isolated. It was regrown in YPD medium and re plated in YPD agar.

2.4. Cell lysis and protein isolation

Yeast cells were lysed using glass bead lysis method. Cells were suspended in 10 mM Tris–HCl with protease inhibitors cocktail (Sigma). Acid washed glass beads were added and vortexed for 1 min followed by immediately keeping the lysate in ice for 1 min. This cycle was followed at least six times until sufficient numbers of cells were lysed. Protein concentrations were measured using Bradford assay [27].

2.5. Preparation of peroxynitrite

A 2 M solution of H_2O_2 in 2 M NaOH, 2 mM EDTA was stirred vigorously with amyl nitrite (4:1, V/V) for 3 h on ice. The aqueous layer was separated and washed thrice with ice-cold hexane. Then the aqueous layer was stirred with activated MnO_2 for 30 min on ice. Finally it was aliquoted and stored at 80 °C. Peroxynitrite concentration was calculated using extinction coefficient 302 nm = 1760 M^{-1} cm^{-1} [28].

2.6. Western blot analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) was performed as mentioned previously [18]. Twenty micrograms of protein was separated in 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Protein was partially transferred to poly vinylidene difluoride (PVDF) membrane (200 mA, 1 h.) membrane according to standard protocol using wet transfer (Bio-Rad Laboratories Inc., Hercules, CA, USA) apparatus. PVDF membranes were blocked overnight using blocking buffer (0.019 M Tris, 0.136 M NaCl, 0.1% V/V Tween 20 and 3% W/V nonfat dry milk) at 4 °C. Membranes were then probed with anti-3-nitrotyrosine monoclonal antibody (Abcam, Cambridge, UK) or anti nitrotyrosine monoclonal antibody (Sigma) at 1:2000 dilutions in TBST (0.019 M Tris, 0.136 M, 0.1% V/V Tween 20). The membranes were washed three times in TBST for 10 min for each wash. After that the membranes were probed with a HRP conjugated goat anti mouse IgG antibody at 1:5000 dilutions for 30 min at room temperature. After that membranes were washed for six times in TBST and six times in TBS (0.019 M Tris, 0.136 M NaCl) for 10 min for each wash. Then the immunopositive spots were visualized by using chemilluminiscent reagent (Thermo Scientific Pierce, Rockford, IL, USA) as directed by the manufacturer.

2.7. Immunofluorescence microscopy

Immunofluorescence microscopy was performed following a modified protocol of Pringle et al. [29]; briefly, 10 ml log phase *S. cerevisiae* culture was taken, washed with 50 mM phosphate buffer, pH 6.5 (solution A). For fixation of the cells, they were re suspended in 5 ml phosphate buffer with 3.7% formaldehyde and kept at room temperature for 1 h. It was then washed twice with 1.2 M sorbitol in 50 mM phosphate buffer, pH 6.5 (solution B). Permeablization of the cell wall was done by re suspending the cells in 1 ml solution B and adding Lyticase enzyme (300 U/ml, Sigma) in presence of 2-mercaptoethanol. It was incubated at 37 °C for 1 h when high proportions of cells were spheroplasts. Immunofluorescence microscopy was performed using anti-3-nitrotyrosine antibody (mouse monoclonal, 1:100 dilution, Abcam). Alexa Fluor 488 goat anti-mouse IgG (Molecular Probe, Life Technologies) was used as secondary antibody at 1:300 dilutions. For control experiment, primary antibody was preincubated with 5 mM 3-nitrotyrosine (Sigma) for 15 min and then the mixture was added to spheroplast cells of *S. cerevisiae*. Immunostained cells were double-stained with DAPI (1 mg/ml). Light intensity and exposure times were kept constant for a given set of experiment and collection modalities for Alexa fluor 488 green fluorescence (excitation 495 nm; emission 519 nm).

3. Results

3.1. Protein tyrosine nitration occurs in petite mutant of S. cerevisiae

In our previous study, we assessed protein tyrosine nitration under respiratory proficient condition in wild type as well as flavohemoglobin deleted mutants of *S. cerevisiae*. We identified eight mitochondrial proteins, common to both wild type and mutant strains of *S. cerevisiae* which undergo protein tyrosine nitration in vivo [18]. To investigate the role of mitochondria in protein tyrosine nitration, petite mutants of wild type and flavohemoglobin deleted strain of *S. cerevisiae* were generated. The petite mutants were unable to grow in respiratory proficient media containing glycerol as sole carbon source indicated the absence of functional mitochondria (Supplementary Fig. S1). Immunoblot analysis was done in Y190, ΔYhb1, Y190 Petite and ΔYhb1 Petite cells grown in fermentative media (YPD media) using monoclonal anti-3-nitrotyrosine antibody. The clone 1A6 monoclonal antibody is commonly used for immunoblotting because it is less prone to problems of nonspecific recognition and batch to batch variability than a polyclonal antibody. We tested two commercially available monoclonal anti-3-nitrotyrosine antibodies independently for this study. We did not observe any significant difference regarding immunopositive band patterns between them. Surprisingly, petite mutants of *S. cerevisiae* also showed protein tyrosine nitration like the wild type and ΔYhb1 indicating that even in the absence of functional mitochondria this post translational modification can occur in *S. cerevisiae* (Fig. 1A and B).

3.2. Detection of nitrated protein in S. cerevisiae in immuno histochemical study

Immunohistochemistry was carried out in wild type strain of *S. cerevisiae* (Y190) using monoclonal anti-3-nitrotyrosine antibody to visualize the distribution of protein tyrosine nitration in yeast cell. Bright fluorescent spots were very much prominent in spheroplasts of *S. cerevisiae* cells treated with primary monoclonal anti-3-nitrotyrosine antibody followed by Alexa fluor 488 conjugated secondary antibody. All the *S. cerevisiae* cells showed similar bright green fluorescence which corroborated well with the previous western blot experimental results. Fig. 2A–H represents fluorescence micrograph of spheroplasts generated from Y190, ΔYhb1, Y190 Petite, ΔYhb1 Petite of *S. cerevisiae*. To check the specificity of the observed fluorescence, spheroplasts of *S. cerevisiae* cells were treated with anti-3-nitrotyrosine antibody preincubated with 5 mM 3-nitrotyrosine for 15 min which showed decrease in fluorescence (Supplementary Fig. S2).

To check the specificity of protein nitration, Y190 cells were grown in fermentative media (YPD) up to the log phase of growth and cell lysates were subjected to incubate with different concentrations of peroxynitrite for 20 min under in vitro conditions followed by gel electrophoresis and immunoblot analysis with monoclonal anti-3-nitrotyrosine antibody. Protein tyrosine nitration profile of Y190 cells grown in YPD medium was gradually increased with increasing concentrations of peroxynitrite (Fig. 3A and B).

3.3. The fate of protein tyrosine nitration in S. cerevisiae is not dependent on proteosomal clearing

The ubiquitin–proteasome system is the main nonlysosomal apparatus for intracellular protein degradation that is conserved in all eukaryotes from *S. cerevisiae* to mammals [30]. Short-lived proteins as well as abnormal proteins are mostly recognized by the ubiquitin system and are tagged with ubiquitin chains as degradation signals. The polyubiquitinated proteins are then targeted for degradation by 26S proteasomes. Rpn10 is a subunit of the 26S proteasome that recognizes polyubiquitinated proteins [31,32]. A number of publications have shown that proteins exposed to oxidants like hydrogen peroxide, superoxide and hydroxyl radical suffer enhanced degradation [33–35]. Much less work has been done in relation to tyrosine-nitrated proteins. We were interested to investigate the fate of protein tyrosine nitration in *S. cerevisiae*. For this we used Rpn10Δ and Pre9Δ and its corresponding wild type BY4742 of *S. cerevisiae* to follow the protein tyrosine nitration accumulation in defective proteasome containing system. Growth rate of Rpn10Δ and Pre9Δ strain of *S. cerevisiae* was much slower than its corresponding wild type in synthetic media. Interestingly, there was no significant difference in protein tyrosine nitration pattern among the Rpn10Δ, Pre9Δ and BY4742 of *S. cerevisiae*.

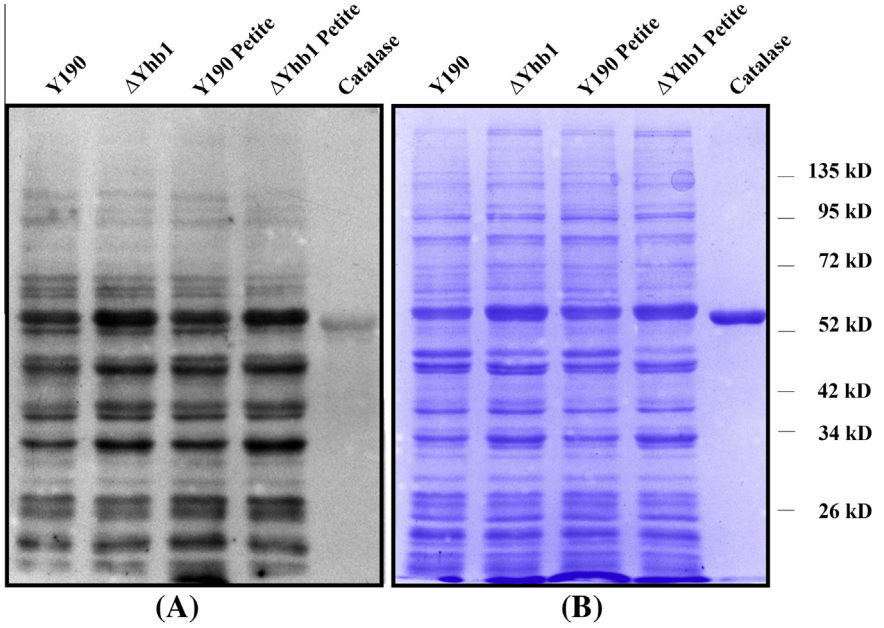

Fig. 1. PTN profile of *S. cerevisiae* under in vivo conditions. Cell lysates of wild type Y190, Δ Yhb1 and their corresponding petite mutant strains of *S. cerevisiae* were subjected to western blot analysis using monoclonal anti nitro-tyrosine antibody (1A). 1B represents the corresponding coomassie stained gel picture.

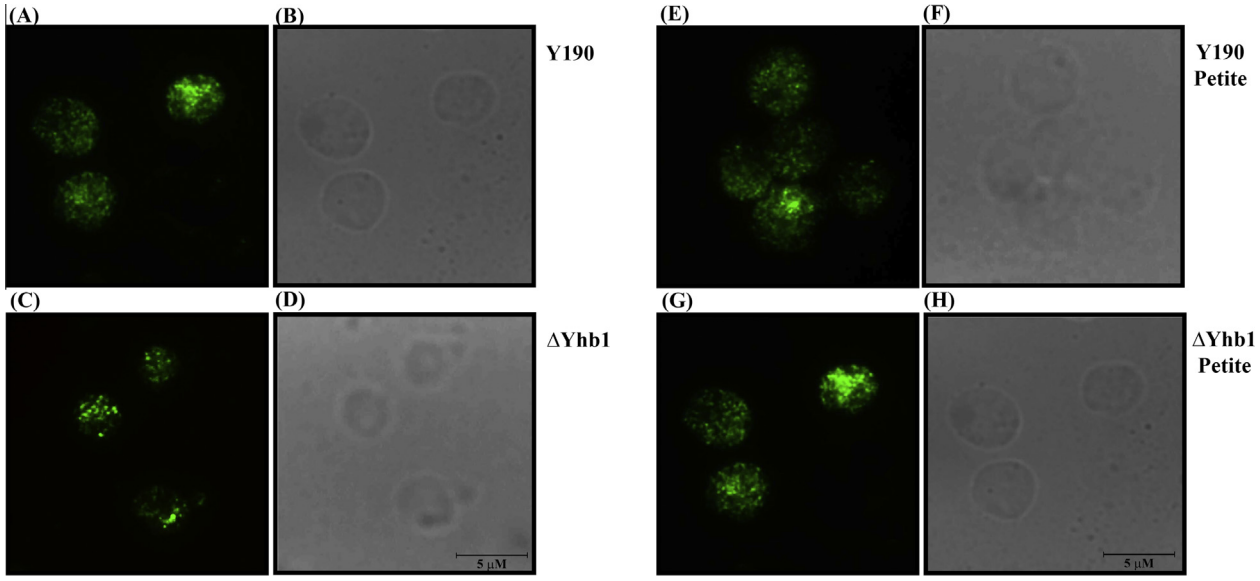

Fig. 2. Immunofluorescence microscopy of nitrated proteins in petite mutants of *S. cerevisiae*. Wild type Y190, ΔYhb1 and their corresponding petite mutant strains of *S. cerevisiae* were subjected to immunofluorescence microscopy to visualize in vivo nitrated proteins using monoclonal anti nitro-tyrosine antibody and Alexa flour 488 conjugated secondary antibody as green color (excitation at 495 nm and emission at 515 nm). Bright green spots indicate nitrated proteins in Y190 and ΔYhb1 strain of *S. cerevisiae* (A and C) as well as their corresponding petite mutants (E and G). (B) and (D) are the corresponding brightfield images of Y190 and ΔYhb1 and (F) and (H) are the bright field images of their corresponding petite mutants. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

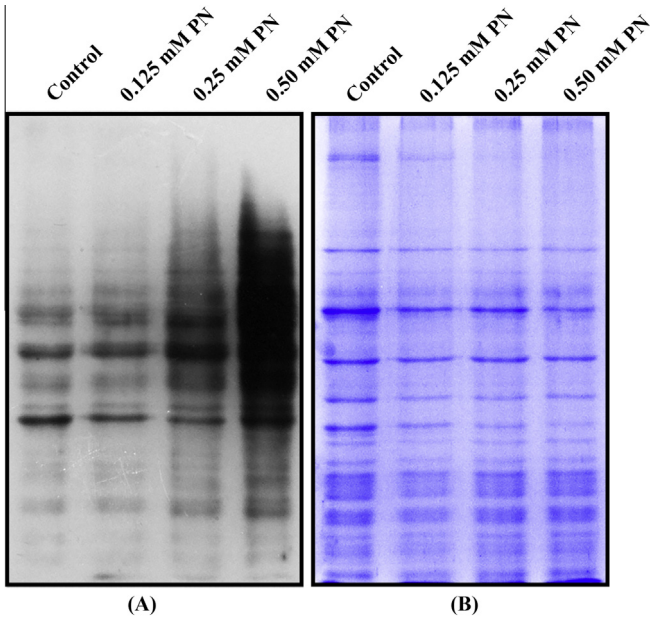

Fig. 3. PTN profile of *S. cerevisiae* under in vitro conditions with increasing concentrations of peroxynitrite (PN). Wild type Y190 cell lysates were treated with increasing concentrations of peroxynitrite for 15 min in vitro. (A) and (B) represents Western blot profile and the corresponding coomassie stained gel respectively.

However, the protein nitrated band intensities of Pre9Δ were high compared to BY4742 of *S. cerevisiae*. On the other hand protein nitrated band intensities of Pre9Δ were high compared to BY4742 of *S. cerevisiae* which indicated that the fate of protein tyrosine nitration in *S. cerevisiae* is not dependent on proteosomal clearing (Fig. 4A and B). Canavanine is an arginine analog that is incorporated into protein competitively with the corresponding natural amino acid [36]. Canavanine differs from arginine in the structure of its side chain and it is capable to induce protein misfolding. Canavanine (0.1 mM) treated BY4742, Rpn10Δ, Pre9Δ strain of *S. cerevisiae* showed an altered misfolded protein profile. We stained the PVDF membrane as well as the corresponding gel to visualize the protein expression profile. Interestingly, there was no significant change in the protein profile between the wild type and the proteosomal mutants. PTN profile indicated that 0.1 mM canavanine treated BY4742, Rpn10Δ, Pre9Δ strain of *S. cerevisiae* produced misfolded proteins which can also be nitrated at the 3 position of the Tyr residue of a protein.

4. Discussion

Yeast is an excellent system to study protein nitration because it is possible to grow the microorganisms both in respiratory proficient conditions as well as in fermentative conditions. It is postulated that nitric oxide may also act as a signaling molecule in unicellular eukaryotes like the yeast [22,37,38]. Mitochondrion is the main endogenous source of intracellular free radicals and various reactive oxygen and reactive nitrogen species in eukaryotic cells. Mitochondrial catalase is an important component of *S. cerevisiae* antioxidant defense [39]. Under respiratory growth conditions in the presence of a non-fermentable carbon source, mitochondrial respiration is maximally induced, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are rapidly accumulating and their detoxification by catalase in vivo would be advantageous for cells. It is conceivable that ROS and peroxynitrite accumulation would become much less in fermentative media in *S. cerevisiae.* So, in petite mutants, protein nitration mechanism may involve either nitrite or peroxynitrite. Recently published results showed that sulfite efflux permease Ssu1 and Ssu2 are also able to excrete nitrite and nitrate. The role of nitrite excretion out of the *S. cerevisiae* cells could be the response of yeast cells to protect it from the nitrite toxicity. It has also been shown that when Ssu2 deleted strain of *H. polymorpha* and *S. cerevisiae* cells were grown in presence of nitrate, nitrite efflux rate was increased not the nitrate indicating nitrite was found to be more toxic to cell than the nitrate [40]. At present it is not clear whether Ssu1 and Ssu2 are involved in controlling protein tyrosine nitration in *S. cerevisiae* or not.

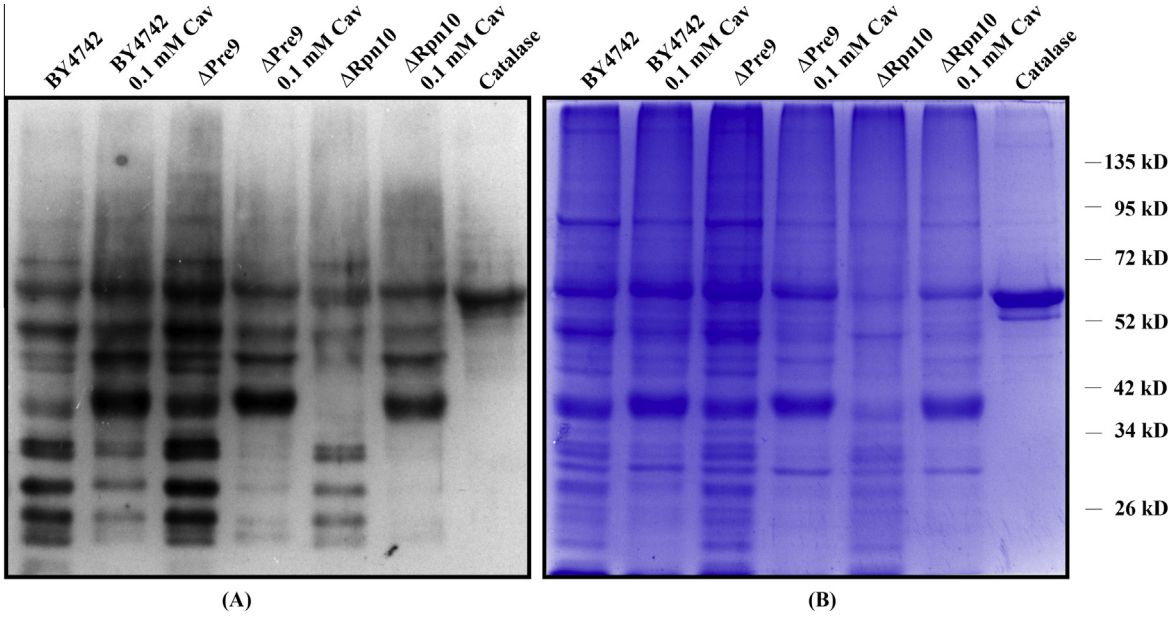

Fig. 4. PTN profile of proteasomal defective mutants of *S. cerevisiae*. Wild type BY4742, its isogenic proteasomal defective mutant ΔPre9 and ΔRpn10 were grown in presence of 0.1 mM canavanine (Cav). Cell lysates of the control and treated were subjected to western blot analysis using monoclonal anti nitro-tyrosine antibody. (A) and (B) represents Western blot profile and the corresponding coomassie stained gel respectively.

The degradation of ubiquitinated proteins is mediated by 26S proteasome which contains the 20S proteasome plus a 19S regulatory complex. Peroxynitrite treatment of aconitase augmented its degradation by the 20S proteasome. However, supraphysiological concentrations of peroxynitrite induced a decrease of the enzyme degradation by the proteasome [6,7]. The nitration of Tyr108 of bovine CuZnSOD has been shown to double the speed of degradation by the 20S proteasome when compared with the unmodified SOD [6,7]. In a number of neurodegenerative diseases an accumulation of 3-nitrotyrosine in the form of insoluble aggregates is observed, like the Lewy bodies in Parkinson's disease which indicates that aggregated protein became a poor substance of the proteasome. There is also evidence that it could be a decrease in the activity of the ubiquitin–proteasome system in Parkinson's disease [41]. Our study indicated that that the fate of protein tyrosine nitration in *S. cerevisiae* is not dependent on proteosomal clearing.

In conclusion, protein tyrosine nitration is a protein modification characteristic of peroxynitrite and other NO-related oxidants, but the functional consequences of this modification are still unclear. Our immunoblotting and immunolocalization data first time provide a sound foundation to further investigate the mechanisms of in vivo protein tyrosine nitration in *S. cerevisiae.*

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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.bbrc.2014.08.011.

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