

Intracellular scavenging activity of Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) in the fission yeast, *Schizosaccharomyces pombe*

Ismail Hamad,
Nazlı Arda¹,
Murat Pekmez¹,
Semian Karaer¹,
Güler Temizkan¹

Department of Biochemistry, Upper Nile University, P.O. Box 1660 Khartoum, Sudan

¹Department of Molecular Biology and Genetics, Istanbul University, Vezneciler 34118, Istanbul, Turkey

Address for correspondence:

Dr. Ismail Hamad, Department of Biochemistry, Upper Nile University, Faculty of Medicine and Health Sciences, P.O. Box 1660 Khartoum, Sudan. E-mail: ismailhamad@yahoo.com

DOI: 10.4103/0976-9668.71667

Abstract

The ability of Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), a water-soluble vitamin E analogue, to prevent oxidative damages is well characterized, but the mechanisms underlying it remain unclear. The protective effect of Trolox pre-treatment on H₂O₂-induced toxicity might be attributed to the decreased cellular permeability to H₂O₂ or *in vitro* scavenging activity of Trolox, induction of antioxidant enzymes or the direct scavenging activity of Trolox. The results obtained rule out the first and second possibilities and intracellular scavenging activity was found to be the mechanism whereby Trolox confers protection. This was confirmed by measuring protein oxidation (levels), and the observed decrease in proteasomal activity indicated that the decrease in protein carbonyls was due to Trolox scavenging activity rather than proteasome activation. In conclusion, the intracellular scavenging activity of Trolox is a key protective mechanism against H₂O₂. These findings obtained in *Schizosaccharomyces pombe*, a good model organism for eukaryotic cells, can be used as standard protocols for investigating the antioxidant activity of pure or complex potential antioxidants.

Key words: Fission yeast, hydrogen peroxide, oxidative stress, protein oxidation, *Schizosaccharomyces pombe*, Trolox

INTRODUCTION

Reactive oxygen species (ROS) are produced as normal by-products of cellular metabolism. These species are also derived from external environmental factors such as redox active drugs, radiation and heavy metals. The major source of ROS is the mitochondrial respiratory chain, which accounts for 85–90% of the oxygen consumed by the cells.^[1] ROS can cause damage to proteins, lipids and nucleic acids and thereby compromise cell viability. Under normal physiological conditions, cellular damages are prevented by antioxidant defenses that neutralize the ROS.^[2] These include ROS-scavenging molecules (e.g., superoxide dismutase, catalase), oxidative damage-repair enzymes (e.g., methionine sulfoxide reductase) and mechanisms such as the S-thiolation of oxidation-susceptible proteins, which prevents oxidation by forming reversible mixed-disulfide bonds with glutathione/thiol.^[3] However, under specific stress conditions, the levels

of ROS exceed the antioxidant capacity of the cells and the cells face an oxidative stress. This unbalanced situation can result from: (i) a decrease in antioxidants, due to depletion of such defenses (e.g., by xenobiotics that are metabolized by conjugation to glutathione or due to mutations that affect antioxidant defenses), (ii) an increased production of ROS (e.g., by exposure to hyperoxia, compounds that generate ROS or due to excessive activation of systems that produce ROS) or (iii) both.^[4]

Such “oxidative stress” is associated with several human pathologies, including cancer, cardiovascular diseases, Down’s syndrome, Friedreich’s ataxia, rheumatoid arthritis, autoimmune diseases and acquired immunodeficiency syndrome. Oxidative damage is also emerging as an important factor in mutagenesis, tumorigenesis, ageing and age-related disorders such as Parkinson’s and Alzheimer’s diseases.^[5]

Protein oxidation is defined as the covalent modification of protein induction, either directly by ROS or indirectly by a reaction with secondary by-products of oxidative stress. Oxidative damages to proteins can lead to diverse functional consequences, such as inhibition of enzymatic and binding activities, protein aggregation and enhanced susceptibility to proteolysis.^[6] Protein oxidation serves as a useful marker for assessing oxidative stress. The most commonly used marker of protein oxidation is the protein carbonyls. The use of protein carbonyl groups as biomarkers of oxidative stress has some advantages in comparison with the measurement of other oxidation products because of the relative early formation and the relative stability of carbonylated proteins. Most of the assays for detection of protein carbonyl groups involve derivatization of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH), which leads to the formation of a stable dinitrophenyl (DNP) hydrazone product. This then can be detected by various means, such as spectrophotometric assay, enzyme-linked immunosorbent assay (ELISA) and one- or two-dimensional electrophoresis followed by Western blot immunoassay.^[7]

Proteasomes are large protein complexes inside all eukaryotes and archaea as well as some bacteria. In eukaryotes, they are located in the nucleus and in the cytoplasm. The proteasome is present in two major forms, the 20S and the 26S proteasomes. The former is a multimeric proteolytic enzyme in a cylinder-like shape, whereas the latter is a complex consisting of two 19S regulatory complexes and one 20S proteasome unit. The 26S proteasome degrades various kinds of excess proteins that have been ubiquitinated with the expense of ATP. This proteasome plays essential roles in the regulation of the cell cycle by specific ubiquitin-mediated proteolysis.^[8]

Besides targeted degradation of regulatory proteins, an important function of the proteasome is the degradation of oxidized and aberrant proteins.^[9] Increased accumulation of highly oxidized and cross-linked protein aggregates within the cell observed during aging has been attributed to decreased proteasome function.^[10]

Natural antioxidants like vitamin C and E, carotenoids and polyphenols are claimed to protect against cancer and cardiovascular diseases, and there is an increasing interest in the use of natural and synthetic antioxidants as functional food ingredients or as food supplements. However, on the other hand, at high doses, toxic pro-oxidant action may become important.^[11]

Vitamin E is the name for a group of biologically active substances including tocopherols and tocotrienols.^[1]

α -tocopherol shows the highest biological activity and is the most common form found in the human body. Tocopherols inhibit lipid peroxidation by scavenging lipid peroxy radicals much faster than these radicals can react with adjacent fatty acid side chains or membrane proteins. In addition, both tocopherols and tocotrienols quench and react with singlet oxygen and slowly react with superoxide anions.^[12]

Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) is a water-soluble analogue of the free radical scavenger α -tocopherol. Trolox has advantages over α -tocopherol, which is lipid soluble because it can be incorporated in both the water and the lipid compartments of cells. Satoh *et al.* have claimed that the antioxidant property of Trolox surpasses that of α -tocopherol.^[13] Many studies investigated the protective effect of Trolox,^[14-16] but the mechanisms underlying these effects remain unclear.

The fission yeast, *Schizosaccharomyces pombe*, is well known for its contribution to the understanding of molecular mechanisms of cell cycle in eukaryotes. It is probably closer to higher eukaryotes than *Saccharomyces cerevisiae*, and represents a good model organism for mammals.^[17] Here, it was used as a model system to evaluate the mechanism of Trolox-mediated protective effects against ROS.

We showed that Trolox was able to prevent or reduce some of the H₂O₂-induced toxic effects in *S. pombe*. In conclusion, the intracellular scavenging activity of Trolox was found to be the key protective mechanism against H₂O₂.

These findings obtained in *S. pombe* can be used as standard protocols for investigating the antioxidant activity of pure or complex potential antioxidants.

MATERIALS AND METHODS

Organisms and growth conditions

The strain used in this work was wild type *S. pombe* (972h). Yeast cells were grown in YE medium (0.5% yeast extract, 3% glucose) at 30°C on a rotary shaker at 180 rpm under aerobic conditions. Yeast cells were pre-treated with 1 mM Trolox for 2 (short-term) and 14 h (long-term) in YE medium. Later, they were collected by centrifugation at 1,200 g, washed with sterile distilled water and re-suspended in YE medium. Exponentially growing cells at 1 X 10⁷ cells/ml were treated with 1 mM hydrogen peroxide for 30 min.

Viability determinations

To calculate cell viability, appropriate dilutions of the cultures were spread on plates with solid YEA medium

(0.5% yeast extract, 3% glucose, 2% agar). These plates were incubated at 30°C and the colony forming units were counted at the end of the third day.

Estimation of hydrogen peroxide in culture media

The H₂O₂ assay was adapted from the methods of Pick and Keisari.^[18] Immediately prior to the assay, phenol red and horseradish peroxidase were added to 1 ml of assay buffer (10 mM potassium phosphate, pH 7 and 40 mM NaCl) at a final concentration of 0.1 mg/ml and 8.5 U/ml, respectively. A 500 µl aliquot of the culture media was then added and the solution was mixed and incubated at 25°C for 5 min. After the reaction was stopped by adding 10 µl of 1 N NaOH, optical density was measured at 610 nm using a Biotek µQuant Microplate Spectrophotometer (Winooski, VT, USA). H₂O₂ concentrations were calculated from a standard curve evaluated for each assay from dilutions of 30% H₂O₂.

Measurement of ROS generation

Intracellular oxidation level was measured as described previously.^[19] After pre-incubation of the yeast cells (10⁷ cells/ml) in YE medium with 40 µM DCFH-DA at 30°C for 60 min, the cells were treated with 1 mM H₂O₂ for 30 min and then washed and re-suspended in 100 µl phosphate-buffered saline (PBS). Fluorescent intensity of the cell suspension was measured using a Bio-Tek FL800 fluorescence microplate reader (Winooski, VT, USA) with excitation at 480 nm and emission at 530 nm. The relative fluorescent intensity was expressed as arbitrary units/10⁷ cells.

Preparation of crude cell-free extract

Cells were collected by centrifugation and re-suspended in 200 µl lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1 mM PMSF, 1 mM DTT). Cells were disrupted by vortexing with acid-washed glass beads (diameter 425–600 µm) for 10 min at 60 s intervals, interspersed with periods of cooling in an ice bath. Cellular debris was removed by centrifugation at 15,000 g at 4°C for 20 min.^[20] The supernatant was collected and the protein concentration was determined according to Waterborg (2002) using bovine serum albumin as a standard.^[21]

Catalase assay

Catalase activity was determined spectrophotometrically by monitoring the disappearance of H₂O₂ at 240 nm as described previously.^[22] The mixture containing 680 µl of 50 mM potassium phosphate buffer (pH 7.2) and 480 µl of 40 mM H₂O₂ was incubated at 30°C for 2.5 min. Enzymatic reaction was initiated by adding 40 µl of the cell extract. The decrease in absorbance at 240 nm was monitored. Catalase activity was expressed as ΔA₂₄₀/min/mg protein.

Immuno-detection of protein carbonyls

As a result of protein oxidation, carbonyl groups are introduced into protein side chains by a site-specific mechanism. We used an OxyBlot kit (Millipore, Billerica, MA, USA) to immunodetect these carbonyl groups in oxidatively modified proteins as described by the manufacturer. Briefly, DNPH derivatization was carried out for 15 min on 15 µg of protein. Five micrograms of protein was separated on 10% SDS polyacrylamide gels, transferred to nitrocellulose membrane and stained by Red Ponceau to check for equal transfer. The membrane was probed with first antibody, specific to the DNP moiety of the proteins. The next step was incubation with horseradish peroxidase-antibody conjugate directed against the primary antibody. Immunoblots were visualized using the ECL-Plus Western Blotting Detection system supplied by (GE healthcare, Piscataway, NJ, USA), with exposure times between 30 s and 2 min. A second gel containing duplicate samples was run and stained with the silver staining technique.^[23]

Measurement of 20S proteasome activity

Quantitative *in vitro* analysis of 20S proteasome activity was performed by measuring the hydrolysis of the fluorogenic peptidyl substrate Suc-Leu-Leu-Val-Tyr-AMC.^[24] Cells were disrupted by vortexing with acid-washed glass beads into buffer containing 0.25 M sucrose, 25 mM HEPES, pH 7.8, 10 mM MgCl₂, 1 mM EDTA and 1 mM DTT. Lysates were centrifuged at 14,000 g for 30 min at 4°C. Cell lysates were diluted with proteolysis buffer (50 mM Tris, pH 7.8, 5 mM magnesium acetate, 20 mM KCl, 0.5 mM DTT) to a protein concentration of 50 µg/ml. The peptidase activity was measured by the addition of 90 µl of proteolysis buffer and 10 µl of Suc-Leu-Leu-Val-Tyr-AMC (4 mM stock solution in DMSO) to 100 µl of the diluted cell lysate. The mixture was incubated at 37°C for 1 h. The reaction was stopped by addition of an equal volume of ice-cold ethanol and 1.6 ml of 0.125 M sodium borate (pH 9.0). The fluorescence determination was performed at 380 nm excitation and 440 nm emission using free AMC as a standard. Results were presented as nM AMC/mg protein/min.

Statistical analysis

All data were represented as mean ± SD. The statistical significance of the difference between the control and the treated sample was assessed by one-way ANOVA and Dunnett's multiple comparison test. Results were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

The objective of the current study was to evaluate the mechanism of Trolox-mediated protective effect against H₂O₂-induced oxidative damages in the fission yeast *S. pombe*.

Determination of sub-lethal hydrogen peroxide and non-toxic trolox concentration

To assess the consequences of H₂O₂-induced oxidative stress and the protective effect of Trolox in *S. pombe*, we first determined the sub-lethal concentration of H₂O₂ and non-toxic dose of Trolox. Cells were treated with H₂O₂ in the concentration range of 0.2–5 mM. The sub-lethal H₂O₂ concentration was found to be 1 mM, which resulted in 29% increased cell mortality following 30 min of incubation. To establish the maximum non-toxic concentration of Trolox, cells were treated with 0.005–1 mM Trolox. 1 mM Trolox was used throughout the experiments as it increased cell survival significantly (data not shown).

The impact of 1 mM H₂O₂ and 1 mM Trolox on cell growth in the mid-exponential growth phase was additionally tested. As indicated in Table 1, pre-treatment of *S. pombe* cells with 1 mM Trolox for 14 h followed by exposure to 1 mM H₂O₂ for 30 min showed the strongest protective effect compared with cells treated with H₂O₂ alone.

Our results with Trolox are in agreement with those obtained by Raspor *et al.*,^[15] demonstrating that Trolox treatment increased cell viability, decreased intracellular ROS formation and suppressed DNA oxidation in *Saccharomyces cerevisiae*. Additionally, it has been reported that vitamin E in the concentration range of 50 µM to 100 mM did not have any genotoxic effect on the yeast cells.^[25]

Once the non-genotoxic potential of Trolox was confirmed, its protective effect on H₂O₂-induced toxicity was further investigated.

Effect of trolox on H₂O₂ uptake, ROS generation and catalase activity

The protective effect of Trolox pre-treatment on H₂O₂-induced toxicity might be attributed to (i) change in cellular uptake of H₂O₂ or *in vitro* scavenging activity, (ii) induction of antioxidant enzymes (glutathione peroxidase, catalase) or (iii) direct scavenging activity of Trolox. For these reasons,

extracellular H₂O₂ concentration, intracellular oxidation level and catalase activity were determined.

H₂O₂ level in the medium revealed that protection against H₂O₂ could not be attributed to reduced H₂O₂ uptake and/or to the *in vitro* scavenging activity of Trolox, because no significant difference between the H₂O₂ concentrations of Trolox pre-treated and of non-treated cultures were observed [Table 1].

We analyzed whether the Trolox-protective effect was due to scavenging of intracellular ROS in *S. pombe* using a fluorescent dye, DCFH-DA [Table 1]. Trolox pre-treatment suppressed ROS generation and a significant decrease in peroxide production was detected when the cells were treated with Trolox for 14 h. These results were consistent with the findings of Peus *et al.*, on human keratinocytes, in which Trolox was found to decrease the intracellular H₂O₂ generation in a dose-dependent manner.^[26]

A significant increase in catalase activity was detected in cells pre-treated with Trolox for 2 h but not in the cells pre-treated for 14 h [Table 1]. Susa *et al.*, also found that a 20 h pre-treatment with 0.5 mM vitamin E did not affect the activities of antioxidant enzymes, including superoxide dismutase, catalase, glutathione peroxidase and reduced glutathione level in rat hepatocytes.^[27] According to Raspor *et al.*, after prolonged exposure, such enzymatic activities tend to go to normal level.^[15]

It was concluded that the low level of H₂O₂ is due to Trolox-mediated increased intracellular scavenging ability rather than alteration in H₂O₂ transport, *in vitro* scavenging activity or induction of antioxidant defense system.

Effect of trolox on protein oxidative modification and proteasome activity

If Trolox decreases the level of intracellular ROS, it would be expected to decrease damage to biomolecules. We used protein carbonyls as a marker for protein oxidation. Protein carbonyls are generated by a variety of mechanisms^[7]

Table 1: Effects of Trolox pre-treatment on the viability, H₂O₂ uptake, generation of ROS, catalase activity and proteasome activity in the fission yeast, *S. pombe*

Groups	Viability assay (CFU/ml) × 10 ⁵	H ₂ O ₂ conc. in the culture medium (mmol/ml)	Intracellular ROS (U 10 ⁻⁷ cells)	Catalase activity (Δ ₂₄₀ /min/mg protein)	Proteasome activity (nM/mg protein/min)
C ₁	119 ± 7.94	NA	703.5 ± 6.4	0.042 ± 0.0121	1.65 ± 0.11
C ₂	106 ± 7.02**	NA	643.6 ± 132.4**	0.056 ± 0.014**	1.12 ± 0.18*
C ₃	140 ± 10.00*	NA	441.5 ± 80.8*	0.044 ± 0.013**	0.80 ± 0.25*
T ₁	75 ± 11.36	17.67 ± 5.5	1408.0 ± 45.0	0.067 ± 0.015	1.76 ± 0.055
T ₂	122 ± 13.11*	13.00 ± 2.00**	675.3 ± 10.3*	0.174 ± 0.035*	0.91 ± 0.11*
T ₃	146 ± 7.64*	13.83 ± 3.25**	766.8 ± 40.5*	0.073 ± 0.015**	0.96 ± 0.42*

The yeast cells were cultured with Trolox at 30°C for 2 and 14 h and then exposed to 1 mM H₂O₂ for 30 min. C₁: untreated control, C₂: cells pre-treated with Trolox for 2 h, C₃: cells pre-treated with Trolox for 14 h. T₁: cells treated with H₂O₂ alone, T₂: cells pre-treated with Trolox for 2 h and then exposed to H₂O₂, T₃: cells pre-treated with Trolox for 14 h and then exposed to H₂O₂. The results are means ± SD of three independent experiments. **P > 0.05 and *P < 0.01 using one-way ANOVA (C₂ and C₃ were compared with C₁ while T₂ and T₃ were compared with T₁).

and are sensitive indices of oxidative injury. The results obtained from protein carbonyl assays [Figure 1] indicated that pre-treatment of the *S. pombe* cells with Trolox for 2 and 14 h decreased the oxidative damage to proteins induced by 1 mM H₂O₂ for 30 min.

It has been amply documented that the 20S proteasome degrades oxidatively damaged proteins.^[28,29] The protective effect of Trolox against H₂O₂-induced protein damage was further confirmed by measuring the 20S proteasome activity. The results obtained here indicated that the protective effect of Trolox pre-treatment on protein carbonyls was due to the increased intracellular scavenging ability rather than Trolox-mediated activation of the proteasome [Table 1].

This study provides proof that intracellular scavenging activity is the mechanism by which Trolox treatment provides prevention against oxidative damages induced by

H₂O₂ in the fission yeast, which represents a good model for mammalian cells. The methods presented can be used as standard protocols for investigating the antioxidant activity of pure or complex potential antioxidants.

REFERENCES

- Halliwell B, Gutteridge CM. Free radicals in biology and medicine. 3rd rev. ed. London: Oxford University Press; 2004. p. 936.
- Cadenas E. Biochemistry of oxygen toxicity. *Annu Rev Biochem* 1989;58:79-110.
- Weissbach H, Resnick L, Brot N. Methionine sulfoxide reductases: History and cellular role in protecting against oxidative damage. *Biochim Biophys Acta* 2005;1703:203-12.
- Costa V, Moradas-Ferreira P. Oxidative stress and signal transduction in *Saccharomyces cerevisiae*: Insights into ageing, apoptosis and diseases. *Mol Aspects Med* 2001;22:217-46.
- Halliwell B. Antioxidants and human disease: A general introduction. *Nutr Rev* 1997;55:S44-9.
- Shacter E. Quantification and significance of protein oxidation in biological samples. *Drug Metab Rev* 2000;32:307-26.
- Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R. Protein carbonyl groups as biomarkers of oxidative stress. *Clin Chim Acta* 2003;329:23-38.
- Davies KJ. Degradation of oxidized proteins by the 20S proteasome. *Biochimie* 2001;83:301-10.
- Grune T, Davies KJ. The proteasomal system and HNE-modified proteins. *Mol Aspects Med* 2003;24:195-204.
- Stadtman ER. Protein oxidation in aging and age-related diseases. *Ann N Y Acad Sci* 2001;928:22-38.
- Rietjens IM, Boersma MG, de Haan L, Spenkelink B, Awad HM, Cnubben NH, *et al.* The pro-oxidant chemistry of the natural antioxidants vitamin C, vitamin E, carotenoids and flavonoids. *Environ Toxicol Pharmacol* 2002;11: 321-33.
- Birgelius-Flohe R, Traber MG. Vitamin E: Function and metabolism. *FASEB J* 1999;13:1145-55.
- Satoh K, Kadofuku T, Sakagami H. Effect of Trolox, a synthetic analog of alpha-tocopherol, on cytotoxicity induced by UV irradiation and antioxidants. *Anticancer Res* 1997;17:2459-63.
- Distelmaier F, Visch HJ, Smeitink JA, Mayatepek E, Koopman WJ, Willems PH. The antioxidant Trolox restores mitochondrial membrane potential and Ca²⁺-stimulated ATP production in human complex I deficiency. *J Mol Med* 2009;87:515-22.
- Raspor P, Plesnicar S, Gazdag Z, Pesti M, Miklavcic M, Lah B, *et al.* Prevention of intracellular oxidation in yeast: The role of vitamin E analogue, Trolox (6-hydroxy-2,5,7,8-tetramethylkroman-2-carboxyl acid. *Cell Biol Int* 2005;29:57-63.
- Poljsak B, Gazdag Z, Pesti M, Filipic M, Fujs S, Farkas N, *et al.* Role of the vitamin E model compound Trolox in the prevention of Cr(VI)-induced cellular damage. *Toxicol Environ Chem* 2006;88:141-57.
- Pekmez M, Arda N, Hamad I, Kig C, Temizkan G. Hydrogen peroxide-induced oxidative damages in *Schizosaccharomyces pombe*. *Biologia* 2008;63:151-5.
- Pick E, Keisari Y. A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. *J Immunol Methods* 1980;38:161-70.
- Okai Y, Higashi-Okai K, Machida K, Nakamura H, Nakayama K, Fujita K, *et al.* Protective effect of antioxidants against *para*-nonylphenol-induced inhibition of cell growth in *Saccharomyces cerevisiae*. *FEMS Microbiol Lett* 2000;185:65-70.
- Forsburg LS, Rhind N. Basic methods for fission yeast. *Yeast* 2006;23:173-83.
- Waterborg JH. Protein protocols handbook. Chapter 2, The Lowry method for protein quantitation. 2nd ed. New Jersey: Humana Press; 2002. p. 7-9.
- Cho YW, Park EH, Lim CJ. Catalase, glutathione S-transferase and thioltransferase respond differently to oxidative stress in

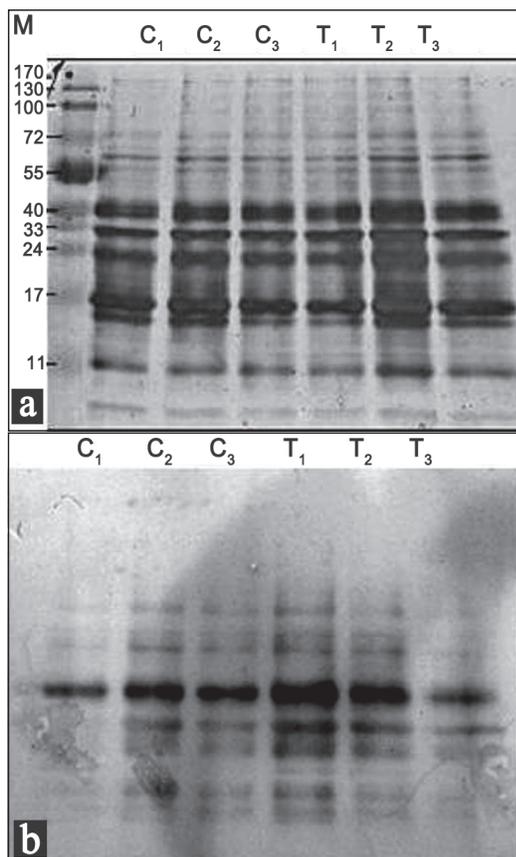


Figure 1: Pattern of oxidatively damaged proteins of *Schizosaccharomyces pombe* pre-treated with Trolox and stressed with H₂O₂. Samples were prepared as described under Materials and Methods. The protein stain was shown in (a). Major oxidatively damaged proteins were indicated in (b). C1: untreated control, C2: cells pre-treated with Trolox for 2 h, C3: cells pre-treated with Trolox for 14 h. T1: cells treated with H₂O₂ alone, T2: cells pre-treated with Trolox for 2 h and then exposed to H₂O₂, T3: cells pre-treated with Trolox for 14 h and then exposed to H₂O₂.

- Schizosaccharomyces pombe*. J Biochem Mol Biol 2000;33:344-8.
23. Dunn MJ. The protein protocols handbook. Chapter 33 Detection of proteins in polyacrylamide gels by silver staining. 2nd ed. New Jersey: Humana Press; 2002. p. 265-72.
 24. Reinheckel T, Grune T, Davies KJ. Stress response: Methods and protocols. Methods in Molecular Biology. Chapter 5, The measurement of protein degradation in response to oxidative stress. Vol. 99. New Jersey: Humana Press; 2000. p. 49-60.
 25. Bronzetti G, Cini M, Andreoli E, Caltavuturo L, Panunzio M, Croce CD. Protective effects of vitamins and selenium compounds in yeast. Mutat Res 2001;496:105-15.
 26. Peus D, Meves A, Pott M, Beyerle A, Pittelkow MR. Vitamin E analog modulates UVB-induced signaling pathway activation and enhances cell survival. Free Radic Biol Med 2001;30:425-32.
 27. Susa N, Ueno S, Furukawa Y, Sugiyama M. Protective effects of vitamin E on chromium (VI)-induced cytotoxicity and lipid peroxidation in primary cultures of rat hepatocytes. Arch Toxicol 1996;71:20-4.
 28. Inai Y, Nishikimi M. Increased degradation of oxidized proteins in yeast defective in 26S proteasome assembly. Arch Biochem Biophys 2002;404:279-84.
 29. Reinheckel T, Ullrich O, Sitte N, Grune T. Differential impairment of 20S and 26S proteasome activities in human hematopoietic K562 cells during oxidative stress. Arch Biochem Biophys 2000;377:65-8.

Source of Support: Nil, **Conflict of Interest:** None declared.

Author Help: Reference checking facility

The manuscript system (www.journalonweb.com) allows the authors to check and verify the accuracy and style of references. The tool checks the references with PubMed as per a predefined style. Authors are encouraged to use this facility, before submitting articles to the journal.

- The style as well as bibliographic elements should be 100% accurate, to help get the references verified from the system. Even a single spelling error or addition of issue number/month of publication will lead to an error when verifying the reference.
- Example of a correct style
Sheahan P, O'leary G, Lee G, Fitzgibbon J. Cystic cervical metastases: Incidence and diagnosis using fine needle aspiration biopsy. Otolaryngol Head Neck Surg 2002;127:294-8.
- Only the references from journals indexed in PubMed will be checked.
- Enter each reference in new line, without a serial number.
- Add up to a maximum of 15 references at a time.
- If the reference is correct for its bibliographic elements and punctuations, it will be shown as CORRECT and a link to the correct article in PubMed will be given.
- If any of the bibliographic elements are missing, incorrect or extra (such as issue number), it will be shown as INCORRECT and link to possible articles in PubMed will be given.