Journal of Life Science **2009** Vol. 19. No. 4. 429~435 ©JLS / ISSN 1225-9918

Increased Antioxidative Activities against Oxidative Stress in *Saccharomyces cerevisiae* KNU5377

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Received January 14, 2009 / Accepted March 11, 2009

Oxidative stress is a consequence of an imbalance of the defense system against cellular damage generated by reactive oxygen species (ROSs) such as superoxide anions (menadione; MD). Most organisms have evolved a variety of defense systems to protect cells from adverse conditions. In order to evaluate stress tolerance against oxidative stress generating MD, comparative analyses of antioxidant capacity, or free radical scavenger ability, were performed between *S. cerevisiae* KNU5377 (KNU5377) and three wild-type *S. cerevisiae* strains. In a medium containing 0.4 mM MD, the KNU5377 strain showed higher cell viability and antioxidant ability, and contained higher levels of trehalose, superoxide dismutase, thioredoxin system, glucose-6-phosphate dehydrogenase, and some heat shock proteins. The KNU5377 strain also produced a lower level of oxidative stress biomarker than the other three yeast strains. These results indicate that *S. cerevisiae* KNU5377 has a higher level of tolerance to oxidative stress due to the increased expression of cell rescue proteins and molecules, thus alleviating cellular damage more efficiently than other *S. cerevisiae* strains.

Key words : *Saccharomyces cerevisiae* KNU5377, oxidative stress, antioxidant enzymes, heat shock protein, stress tolerance

Introduction

Oxidative stress is generated from the toxic levels of oxygen-derived reactive oxygen species (ROSs) such as singlet oxygen, superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical (OH^-) via the metal-catalyzed Haber-Weiss and Fenton reaction [5,12]. These ROSs are the result of normal metabolism, including the energy generation process of aerobic respiration, and the β-oxidation of fatty acids. The toxic materials are also generated through the metabolism of foreign stimulants such as high temperature, osmotic pressure, irradiation and chemicals including ethanol, H_2O_2, menadione (MD) and xenobiotics or the host immune system [21]. The produced ROSs can lead to damage of a wide range of cellular biological molecules: DNA fragmentation and mutations, lipid peroxidation, the disassembly of iron-sulfur clusters, disulfide bond formation, and other types of protein oxidation [6].

Under normal physiological conditions, antioxidant defense systems are well organized to adjust ROSs at a basal level and to repair cellular damage or degrade oxidized molecules. However, an imbalance between oxidants and the ability of detoxification is introduced under oxidative stress [12]. In order to overcome transient or continuous ROS challenges, cells have evolved a variety of enzymatic and non-enzymatic antioxidant defense systems, which are capable of removing free radicals and their by-products leading to the repair of stress-induced cellular damage, and therefore can protect the cellular constituents [7,12,19].

As the industrial yeast, *Saccharomyces cerevisiae* has been used in fermentation and brewing industries for more than 8,000 years for favorite characteristics including high yields of ethanol, production of good flavor, and resistance to ethanol and toxic materials such as sulfite [11,22]. Today, the impact of yeasts on industry extends beyond the original food and beverage production as to production of ingredients for food processing such as B vitamins, proteins, peptides, amino acids and trace minerals, biocontrol of spoilage microorganisms, and biotherapeutic and probiotic agents [26]. For example, *S. cerevisiae* var. *boullardii* has been successfully used over the last 20 years as an oral biotherapeutic agent to treat patients with severe cases of diarrhea and other gastrointestinal disorders [23,26]. *S. cerevisiae*

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KNU5377 (KNU5377) was isolated from sewage [15] for industrial applications such as alcohol fermentation under high temperature and stressful conditions in order to reduce the cost of ethanol production. The KNU5377 was characterized as having thermotolerant and stress tolerances against various types of environmental stressors including hydrogen peroxide, ethanol, high osmotic pressure, and sulfuric acid than that of reference strains such as *S. cerevisiae* S288C (S288C), *S. cerevisiae* W303-1A (W303-1A), and an ethanol-tolerant yeast *S. cerevisiae* ATCC 24858 (ATCC24858) [13,14].

As a model eukaryote, laboratory strain *S. cerevisiae* has been extensively studied to oxidative stress response. However, at present the stress responses to peroxide or superoxide stresses in wild industrial *S. cerevisiae* are not described, especially to superoxide stress. Therefore, understanding the oxidative stress response could give insights into how microorganisms survive in detrimental environments such as extreme temperature, oxidative stress, and osmotic shock. As a first step towards comprehending the defensive mechanisms, the stress response against superoxide anion by menadione was investigated in *S. cerevisiae* KNU5377, and the levels of induction of protective cell rescue proteins containing antioxidant enzymes and antioxidant molecules in stress response.

Materials and Methods

Strains and growth conditions

The KNU5377 was isolated from sewage in Korea [15]. ATCC 24858 as an ethanol tolerant strain, W303-1A and S288C was purchased from the American Type Culture Collection (ATCC), Euroscarf (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/) and Korean Culture Center of Microorganisms (KCCM), respectively. Yeast cells were aerobically grown in a nutrient-rich YPD media (1% yeast extract, 2% peptone, 2% dextrose) for 20 h at 30°C, with shaking at 160 rpm. To monitor cell viability, the mid-log cultured cells (OD_{600}=1.0) following pre-culture at 30°C overnight were exposed to 0.4 mM of menadione (MD) for 1 h at 30°C in YPD liquid media. Cultures were properly diluted, spread on YPD agar plates and then incubated for 24 hr at 30°C. Cell viability was calculated by colony numbers. For growth kinetics, cells were inoculated in YPD media supplemented 40 μM MD, and then cell growth was monitored at 600 nm using a spectrophotometer. For analysis of stress sensitivity, the mid-log cultured cells were exposed to a variety of concentration of MD for 1 hr at 30°C. The cells were properly diluted, spotted onto YPD agar plates, and incubated at 30°C.

Measurement of hydroperoxide, carbonyl content and malondialdehyde

The intracellular hydroperoxide levels were determined by ferrous ion oxidation in the presence of a ferric ion indicator, xylenol orange [13]. Carbonyl contents were measured via the spectrometric method [13]. Malondialdehyde (MDA) levels were examined via thiobarbituric acid (TBA) assay [13].

Trehalose and glycogen assay

Cell pellets (4-10 mg) were put on ice, washed twice with 10-20 volumes of cold-distilled water, resuspended in 0.25 ml of 0.25 M sodium carbonate, and incubated at 95°C for 4 hr. The pH of the mixture was brought to 5.2 by adding 0.15 ml of 1.0 M acetic acid and 0.6 ml of 0.2 M Na-acetate (pH 5.2). One half of the suspension was incubated overnight with trehalase (0.05 U/ml) at 37°C for measurement of trehalose content and the second half with *Aspergillus niger* amyloglucosidase preparation (1.2 U/ml) at 57°C under constant agitation for measurement of glycogen content. After incubation, these suspensions were centrifuged for 3 min at 5,000× *g* [20]. The resulting glucose was measured by the Somogy-Nelson method. The trehalose and glycogen contents were defined as nmol/mg protein.

Protein extracts

Crude cellular protein extracts were prepared by the glass bead method. Briefly, cells were washed with saline (0.85% of NaCl) three times, and resuspended in a lysis buffer (20 mM HEPES, 10% glycerol, 1 mM PMSF, 2 μM pepstatin A and protease inhibitor cocktails) with an equal volume of glass beads (425-600 microns; Sigma, USA). After vigorously vortex-mixing for 1 min 5 times with 2 min interval on ice, the cell extract was collected by centrifuging at 12,000 rpm for 10 min at 4°C. The protein concentration was determined by the Bradford method (Bio-Rad, USA).

Immunoblot assay

Electrophoresis via protein extract for SDS-PAGE and two-dimensional gel electrophoresis was performed by a previously employed protocol [13,14]. Briefly, 40 μg of dena-

tured proteins were analyzed in a 10% or 15% polyacrylamide gel and electrophoretically transferred to a PVDF membrane (Bio-Rad, USA) in a transfer buffer (25 mM of Tris-base, 192 mM of glycine and 20% methanol). The PVDF membranes were blocked for 60 min at room temperature in a TTBS buffer (0.05% Tween-20, 10 mM of Tris-HCl, pH 7.6, 150 mM of NaCl) containing 5% non-fat skim milk and 0.02% sodium azide. The blotted membranes were incubated overnight at 4°C with the primary antibodies: rabbit anti-glucose-6-phosphate dehydrogenase (Sigma, St. Louis, USA), rabbit anti-Hsp104, mouse anti-Hsp60 and rabbit anti-alcohol dehydrogenase (Stressgen, Canada), rabbit anti-hexokinase (Rockland, USA), rabbit anti-Hsp90, rabbit anti-Ssa and rabbit anti-Ssb (kindly provided by Dr. Elizabeth A. Craig, USA), rabbit anti-Tsa1p (kindly provided by Dr. Park Jeen Woo, Korea), rabbit anti-Trx2p, Trx3p and anti-Trr1p (kindly provided by Dr. Kim Kang-Hwa, Korea), and rabbit anti-Cpr1p (kindly provided by Dr Joseph Heitman, USA). After washing four times with a TTBS, the membranes were incubated for 90 min with a secondary antibody such as anti-rabbit IgG (H+L) HRP Conjugate (Promega, USA) or anti-mouse IgG (Amersham Biosciences, Sweden), then washed four times with a TTBS, developed by enhanced chemilluminescence (ECL kit; Amersham Biosciences), and processed.

Results

Viability and growth assay against menadione

To determine the sensitivity of yeast strains against menadione (MD), ATCC24858, KNU5377, W303-1A and S288C cells were challenged with 0.4 mM of MD for 1 hr, and subsequently spread onto YPD agar plates. After MD treatment all of the percent viability was decreased in all four strains. From 30 to 40 % decrements were observed in three strains, ATCC24858, W303-1A and S288C however, only about a 10 percent decrement in KNU5377 could be observed (Fig. 1A). Although three strains could not grow under 40 μM of MD, KNU5377 could do so after a delayed lag phase, for 8 hr (Fig. 1B). These results were strongly supported by a stress sensitivity assay. As shown in Fig. 1C, the KNU5377 strain exposed to a variety of concentrations of MD did grow at 1.0 mM of MD, whereas three reference strains did not grow at this concentration. Therefore, ATCC24858 of a high ethanol tolerance strain, W303-1A of a wild type strain and S288C of a general *S. cerevisiae* strain displayed greater sensitivity than that of KNU5377 against MD.

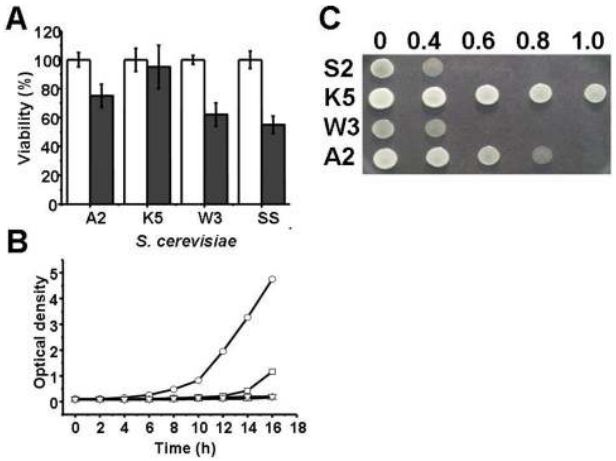

Fig. 1. Cell viability and growth rate of *S. cerevisiae* KNU5377 and three reference strains. For cell viability, aerobically grown cells (A_{600}=1.0) were exposed to 0.4 mM menadione (MD) for 1 h at 30°C, properly diluted, spread on YPD agar plates, and incubated for 24-36 h at 30°C. Cell viability was calculated by colony numbers. A2: *S. cerevisiae* ATCC24858; K5: *S. cerevisiae* KNU5377; W3: *S. cerevisiae* W303-1A; S2: *S. cerevisiae* S288C; Open bar: without MD; Closed bar: with MD (A). For growth kinetics, cells were cultured in liquid YPD media with 40 μM MD and then, optical density was measured at 600 nm. Values are shown for one representative of at least three independent experiments. ATCC24858 (□); KNU5377 (○); KNU5377; W303 (△); S288C (▽) (B). Aerobically grown cells (A_{600}=1.0) were exposed to a variety of concentration of menadione (MD) for 1 h at 30°C, properly diluted, spotted on YPD agar plates, and then incubated for 24 h at 30°C. S2: S288C; K5: KNU5377; W3: W303-1A; S2: S288C.

Hydroperoxide, carbonyl and malondialdehyde assay

To examine intracellular oxidative biomarkers by Ros, hydroperoxide, carbonyl and malondialdehyde concentrations were measured after 0.4 mM MD treatment for 1 hr. Hydroperoxide was measured during MD treatment with and without adaptation by ferrous ion oxidation in the presence of a ferric ion indicator, xylenol orange. As shown in Fig. 2A, the levels were increased after treatment in four strains, however the lowest increment of KNU5377 could be observed in the strains. Patterns of oxidatively damaged proteins were measured by carbonyl content. Although the contents were also increased after treatment, it could be observed that the intrinsic cellular carbonyl content of

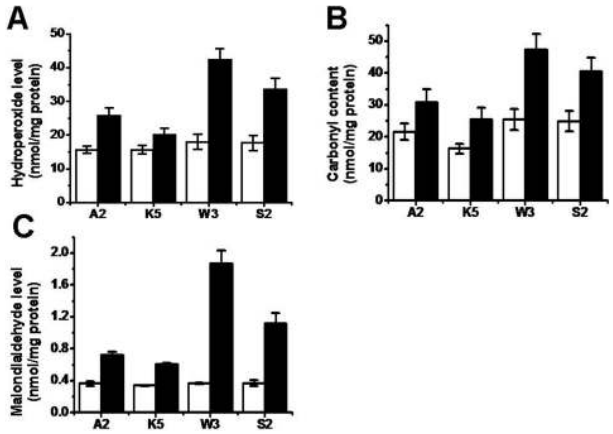

Fig. 2. Cellular levels of hydroperoxide, carbonyl content and malondialdehyde upon exposure to menadione (MD) stress. Cells grown to the mid-log phase were exposed to 0.4 mM MD for 1 hr at 30°C with shaking. Then the cells were collected to measure cellular hydroperoxide (A), carbonyl content (B), and malondialdehyde (C). Values are shown from at least three independent experiments. A2: ATCC24858; K5: KNU5377; W3: W303-1A; S2: S288C; Open bar: without MD; Closed bar: with MD.

KNU5377 was the lowest of four strains (Fig. 2B). Over 2-fold induction of malondialdehyde as a product of lipid peroxidation were observed in W303-1A and S288C however, the increment of the reactive intermediate was not significant in KNU5377 (Fig. 2C).

Trehalose and glycogen accumulation after menadione stress

Intracellular storage dicarbohydrates, trehalose and glycogen concentrations were various depending on *S. cerevisiae* strains. Different accumulation patterns were observed between ATCC24858 and KNU5377, and W303-1A and S288C. The former strains showed lower intrinsic dicarbohydrate levels than those of the latter strains however, cellular accumulation of these two components were well induced in the latter strains after MD treatment (Fig. 3). Moreover, over 2-fold inductions of the two components were observed in KNU5377.

Expression changes of antioxidant enzymes during menadione stress

Immunoblot analysis was performed in order to detect general antioxidant enzymes and molecules including Sod1 (cytosolic Cu/Zn Sod), Sod2 (mitochondrial Mn Sod), Trx3 (thioredoxin 3), Tsa1 (cytosolic thioredoxin peroxidase), Ssa

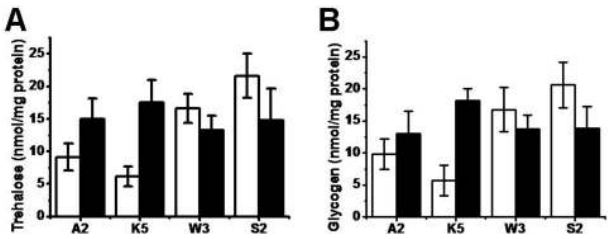

Fig. 3. Cellular level of trehalose and glycogen to menadione. Cells were aerobically grown in nutrient rich YPD media at 30°C with shaking. Once mid-log phase to OD_{600} of 1.0 is reached, the cells were challenged with 0.4 mM menadione for 1 hr at 30°C. Cell cultures were collected to analyze trehalose content (A) and glycogen content (B). The data of the trehalose and the glycogen concentrations are mean values±SD from three independent experiments. A2: ATCC24858; K5: KNU5377; W3: W303-1A; S2: S288C; Open bar: without MD; Closed bar: with MD.

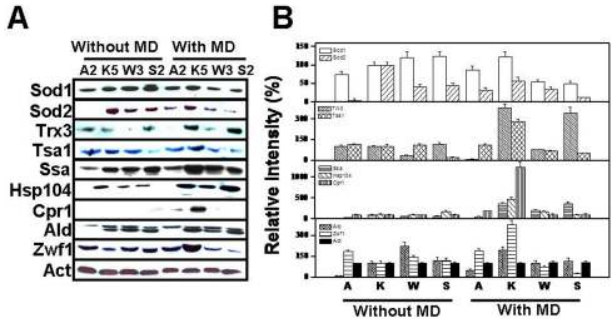

Fig. 4. Immunoblot analysis of cell rescue proteins and antioxidant molecules. Cells were aerobically grown in nutrient rich YPD media at 30°C with shaking. Once mid-log phase to OD_{600} of 1.0 is reached (without MD), the cells were challenged with 0.4 mM menadione (MD) for 1 hr at 30°C (with MD). Then proteins from different strains were extracted and loaded (40 μg) on SDS-PAGE gels (10, 12, and 17%). The gels were transferred to a PVDF membrane and developed. Sod1: Cu/Zn superoxide dismutase; Sod2: Mn superoxide dismutase; Trx3: thioredoxin 3; Tsa1: cytosolic thioredoxin peroxidase I; Zwf1: glucose-6-phosphate dehydrogenase; Ald: aldehyde dehydrogenase; Cpr1: cyclophilin A; Ssa: heat shock protein family 70; Hsp104: heat shock protein 104. Actin was detected for housekeeping control. A2: ATCC24858; K5: KNU5377; W3: W303-1A; S2: S288C (A). Relative intensity of immunoblot analyses was expressed relative to the expression levels of KNU5377 without MD (100%). Values are means with standard deviations from at least three independent experiments (B).

(heat shock protein 70 family), Hsp104, Cpr1 (cyclophilin), Ald (aldehyde dehydrogenase) and Zwf1 (glucose-6-phosphate dehydrogenase) (Fig. 4). All expression levels were

compared with that of KNU5377 without or with MD. The Sod1 expressions of W303-1A and S288C were higher than that of KNU5377 without MD however, down-regulated after MD treatment (Fig. 4A). As antioxidant molecules, Trx3 expression was significantly up-regulated over 3-fold resulting from MD treatment in KNU5377 and S288C. Moreover, the expressions of Tsa1, Cpr1 and Zwf1 were extremely up-regulated in KNU5377 after MD treatment. The differences of Ssa, Hsp104 and Cpr1 could not be observed between four strains without MD.

Discussion

S. cerevisiae has long been studied as a model for the research of the stress response because yeast takes the energy by aerobic respiratory process. A major stress faced to yeast cells during aerobic growth is oxidative stress that cause reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^\cdot$) [4]. These ROS in yeast may be generated by various extra-environmental conditions or during the fermentation process. The produced ROS causes cellular damage to various cellular components such as DNA fragmentation, protein or enzyme inactivation via protein carbonylation, and change of membrane fluidity by lipid peroxidation [8]. Menadione (MD) is a quinone extensively used in studies of cellular oxidative stress as well as a therapeutic agent displaying anticancer activity. The main postulated mechanism of its toxicity is oxidative stress caused by the process of redox cycling and yielding peroxide radicals [18].

The *S. cerevisiae* KNU5377 strain is capable of production of a high concentration of (bio)ethanol via fermentation at high temperature [15]. During this fermentation process, a variety of stressors could be provoked, which are mainly oxidative stresses. The stress tolerance of *S. cerevisiae* KNU5377 was evaluated by comparing with three other *S. cerevisiae* strains under MD stress. Among the *S. cerevisiae* strains analyzed, it was found that *S. cerevisiae* KNU5377 has the most stress tolerance against MD (Fig. 1). Some biochemical and physiological properties of the KNU5377 strain were therefore analyzed. As seen in Fig. 3 and 4, the KNU5377 strain represented a significant accumulation of trehalose and glycogen (Fig. 3), massive induction of antioxidant enzymes such as Trx3p (thioredoxin 3), Tsa1p (cytosolic thioredoxin peroxidase I), molecular chaperones and cyclophilin (Hsp104p, Ssap and Cpr1p) and G6PDH (glucose-6-phosphate dehydrogenase) (Fig. 4). These proteins and molecules are very important in the process of intracellular detoxification of the by-products produced by MD-induced oxidative stress. Sod protein has an antioxidant function by catalyzing the disproportionation of superoxide anion to hydrogen peroxide, whose activity requires redox active metal ions. Mutants or down-regulation of Sod1p exhibit stress-sensitive phenotypes [12,19]. Thioredoxins are small thiol oxidoreductases, which contain two conserved cysteine residues at the active site participating in protein thiol reduction. The thioredoxin system is completed by thoredoxin reductase, which reduces oxidized thioredoxin to the active thiol form by using NADPH and thioredoxin peroxidase. Thioredoxin peroxidase reduces peroxides and peroxinitrites, with thioredoxins acting usually as electron donors [4-8,12]. The cytosolic thioredoxin system of *S. cerevisiae* is required for the defense against exogenous hydroperoxides [12,19,26]. In addition, the protective role of the cytosolic thioredoxin system against reductive conditions operates through the chaperone activity of Tsa1, which would prevent the aggregation of mis-folded ribosomal proteins occurring under reductive stress [26]. NADPH is an important cofactor in many biosynthesis pathways and the regeneration of reduced thioredoxin, critically important in cellular defense against oxidative damage. It is mainly produced by glucose 6-phosphate dehydrogenase (G6PDH). The role of G6PDH in the cell response to oxidative stress is well established in yeast, human erythrocytes and the mouse embryonic stem cells [12,17]. Aldehyde dehydrogenase (ALD) catalyzes the oxidation of aldehydes to their corresponding carboxylic acids by using NAD or NADP as a cofactor [31] and is known to involve in stress tolerance [19].

Secondly, the nonreducing disaccharide, trehalose (α-D-glucopyranonyl α-D-glucopyranoside) is widespread in nature. For a long time it had been assigned a role of only as a storage compound, but more recently its stress-protection properties have been elucidated [26]. In industrial yeasts, for example, improved stress tolerance is often correlated with cellular trehalose levels as a function of protective roles. Further evidence for stress-protective roles of trehalose has been provided by yeast strains genetically engineered in trehalose metabolism, revealing a clear link between trehalose levels and tolerance against different stress types such as freeze, heat shock, dehydration, ethanol, osmotic and oxidative stress [26,27]. This observation has also been extended to other yeast species such as *Schizosaccharomyces pombe* [25],

Candida albicans [1] *Zygosaccharomyces rouxii* [16] and *Hansenula polymorpha* [24]. How trehalose provides protective roles to cells is not entirely clear.

Finally, denaturation of proteins is a major cellular damage following stress and, not surprisingly, the action of molecular chaperones conserved microbes and man is a major stress tolerance mechanism in yeast cells. Molecular chaperone proteins such as heat shock proteins (Hsps) grouped by their molecular weight and high degree of amino acid homology can stabilize macromolecules to prevent them from aggregating. They recognize, selectively bind and reassemble proteins with an aberrant structure [2,26]. Among Hsps, Hsp104, Hsp70 (Ssa) and cyclophilin A (Cpr1) are important in maintaining tolerance, whose expression is induced by various stresses and is involved in protein folding [3]. Hsp104 facilitates disaggregation and reactivates aggregated proteins with the assistance of Hsp70 (Ssa1) and Hsp40 (Ydj1) in *S. cerevisiae* [10]. Although the direct interactions between Hsp104 and Ssa1 were not elucidated, the up-regulated of the two Hsps were observed in KNU5377 (Fig. 4) that might help to maintain a low degree of protein denaturation and reassemble damaged proteins during and after the imposition of the stress leading to the tolerance against MD. In addition, there is increasing evidence that trehalose and molecular chaperones act synergically as stress protectants [9]. During heat shock, trehalose has been shown to suppress the aggregation of denaturated proteins in yeast and can be activated by molecular chaperones [26,28-30].

In this paper, it has been shown that adaptive response of yeast *S. cerevisiae* KNU5377 induces multiple components of cellular processes to oxidative stress. A higher activation of ROS-scavenging systems in KNU5377 as compared with control strains probably resulted in a decreased level of oxidative stress biomarkers such as hydroperoxide level, carbonyl content and MDA level. Although the molecular basis of the greater stress resistance of KNU5377 could not be clearly understood, it may be, at least partially, explained in this paper.

Acknowledgements

This work was supported by Korea Research Foundation Grant (KRF-2004-005-F00063), and a grant (20070401-034-001) from BioGreen 21 Program, Rural Development Administration, Republic of Korea.

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초록 : 산화 스트레스 대한 *Saccharomyces cerevisiae* KNU5377의 항산화 활성의 증가

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산화적 스트레스는 정상적인 대사 과정뿐만 아니라 외부적인 환경에 노출 되었을 때 일어나는 것으로 잘 알려져 있다. 이러한 스트레스를 극복하기 위해 생물체들은 각자의 시스템에 맞게 다양한 항산화 시스템을 진화 발전시켜 왔다. *Saccharomyces cerevisiae* KNU5377 균주는 고온뿐만 아니라 다양한 스트레스에 대해 내성을 가짐을 확인하였다. 대부분의 스트레스는 궁극적으로는 산화적 스트레스로 귀결된다. 이러한 측면에서 본 연구는 KNU5377 균주가 어떠한 시스템에 의해서 다른 균주보다 스트레스 내성을 가지는지를 밝히기 위해 접근하였다. 수행된 연구결과에서 KNU5377 균주는 항산화 시스템과 밀접하게 관련된 단백질(superoxide dismutase, thioredoxin system, heat shock proteins)과 항산화 관련 물질(trehalose)을 과발현함을 확인하였다. 그러나 이러한 단백질들이 어떠한 조절 시스템에 의해서 균주 특이적인 발현 양상을 보이는지는 현재까지 확인되지 않고 있다. 본 연구는 KNU5377 균주 그 자체의 중요성과 함께 균주 내의 스트레스 내성과 관련된 유용한 유전자를 탐색하여 더욱 우수한 유전자원을 발굴하는데 기여 할 것으로 보인다.