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Expression of Ovotransferrin Enhances Tolerance of Yeast Cells toward Oxidative Stress

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ABSTRACT: Recently, we found that ovotransferrin (OTf) undergoes distinct self-cleavage in a redox-dependent process and exhibited *in vitro* superoxide dismutase (SOD)-like activity. In this study, we explore that the expression of OTf confers high tolerance to oxidative stress in yeast cells. The OTf gene was cloned into the vector pPICZB and was successfully expressed in methylotrophic yeast, *Pichia pastoris* KM71H. There was no growth difference between the non-transformed strain and recombinant strains harboring a mock vector (pPICZB) or the OTf gene carrying a vector (OTf-pPICZB). Intracellularly expressed OTf was found to undergo self-cleavage, producing a major fragment of 15 kDa, which corresponded to the disulfide kringle domain of the N-terminal lobe. The yeast OTf transformants exhibited strong tolerance to oxidative stress induced by either hydrogen peroxide (H_2O_2) or diethyl maleate (DEM). Further, OTf transformants showed higher intracellular reducing capacity and enhanced cytosolic reductase activity. This study is the first to describe the ability of OTf to confer *in vivo* antioxidative stress function within a complicated milieu of eukaryotic cells and provide novel insights for the potential of the OTf gene for molecular breeding of industrial yeast strains with high tolerance to oxidative stress.

KEYWORDS: *Ovotransferrin, Pichia expression, oxidative stress, intracellular redox regulation, self-cleavage, industrial yeast, metabolic engineering*

INTRODUCTION

Ovotransferrin (OTf) is a 686 residue glycoprotein with a molecular mass of 78 kDa, containing 15 disulfide cross-links and no free sulfhydryl groups.[1] It belongs to the transferrin (Tf) family, which are two lobe proteins (N- and C-terminal lobes) with a strong site for iron-binding located in each lobe. Three proteins typify the Tf family: serum transferrin (Tf), milk lactoferrin (Lf), and avian OTf. The global structure of OTf and transferrins are identical but differ in the nature of their attached glycan chain and their pI.[1] Besides the implication in the transport of iron to the target cells, Tfs are proposed to be involved in a large number of cellular events, where they act as event triggers or receptor activators. These physiological processes include the stimulation of cell proliferation[2] and tumoral processes and the promotion of endothelial cell migration and invasion.[3] The OTf expression level in chicken serum increases in the acute phase of inflammation and infection[4] and was found to induce intracellular oxidative response in chicken macrophages and neutrophil granulocytes.[5] The accumulating evidence indicates that OTf, as well as Lf, always seems associated with processes involving redox-linked signals and oxidative stress. For instance, expression of Lf has been shown to increase in line with antioxidant enzymes in oxidatively stressed mouse brain[6] and during embryogenesis of avian eggs, whereas diversified protein structures interconvert via a chain of stages, involving redox modulation.[7]

In our recent studies, we observed that OTf exposed to reducing conditions undergoes conformational changes in the vicinity of its two kringle regions and is subsequently self-cleaved,[8] whereas the self-cleaved OTf was found to possess superoxide dismutase (SOD)-like activity[9] and specific anticancer activity.[10] SOD is an important antioxidant enzyme that has recently been found to inhibit cancer cells.[11] Antioxidant-defense protein activation processes are often triggered by redox modification of the cysteine side chain, which is known to play a major role in redox sensing and response of SOD.[12] These observations suggest that OTf, with highly reactive cysteines holding the two redox-sensitive disulfide kringle domains, may respond to changes in the complicated cellular redox milieu and serve to protect eukaryotic cells against oxidative stress *in vivo*.

It is the purpose of this study to examine the role of OTf in oxidatively stressed eukaryotic cells to explore its *in vivo* biological function, which has yet to be unraveled. For this, the OTf gene was integrated into the genome under a methanol-inducible promoter and expressed intracellularly in methylotrophic yeast *Pichia pastoris* cells. The expression of OTf, within the complicated milieu of the yeast cell, would be expected to explore its *in vivo* antioxidative function toward an induced oxidative stress. From a biotechnological perspective, the generation of yeast with enhanced tolerance to oxidative stress by expressing OTf, a food protein, could be promising for breeding novel yeast strains for improved tolerance and fermentation.

MATERIALS AND METHODS

Materials. OTf was purchased from Wako Pure Chemicals (Osaka, Japan). Sephadex G-50 was a product of Amersham-Pharmacia Biotech (Tokyo, Japan). Nitroblue tetrazolium (NBT), hydrogen peroxide (H_2O_2), α-lipoic acid (α-LpA), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), diethyl maleate (DEM), and lyticase were from Sigma Chemicals Co. (St. Louis, MO). TA cloning vector pT7Blue

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(Novagen, Japan) was used for the cloning of polymerase chain reaction (PCR) products. *Escherichia coli* Top10 F′ (Invitrogen, Japan) was used for subcloning and routine plasmid amplification. The expression vector pPICZB (Invitrogen, Japan) was used for intracellular expression of OTf. *P. pastoris* KM71H the host for pPICZB was obtained with the EasySelect *P. pastoris* expression kit (Invitrogen, Japan). All restriction enzymes, T4 DNA ligase, and pfu DNA polymerase were purchased from Takara Bio (Japan). BioYeastar RNA isolation kit was from Zymo Research (Irvine, CA). The AccuPower RT-PreMix and AccuPower PCR PreMix were from Bioneer (Japan). The yeast extract peptone dextrose medium (YPD) and buffered glycerol-medium yeast extract (BMGY) were used for propagation, and the buffered methanol-medium yeast extract (BMMY) was used for induced expression of *P. pastoris*. Peptone and yeast extract were purchased from Difco Co., Ltd. (Detroit, MI). Unless otherwise stated, all other chemicals were analytical-grade.

Cloning Procedures. Generation of OTf cDNA from chick oviduct. Total RNA was extracted from chicken oviduct tissue using AquaPure RNA extraction kit (Bio-Rad, Japan). The first strand cDNA, produced from RT-PCR, was used as a template with specific OTf primers (forward 5′-CGGTACCCAGAGGGGACGGTCTGTG-3′ and reverse 5′-GTCTAGAGATTTTATTTCTTGCTGT-3′) based on the non-coding regions of the OTf gene. The primers contained the 5′-end restriction sites for *Kpn*I and *Xba*I (underlined). Amplification was performed using proofreading polymerase (Invitrogen, Inc., Carlsbad, CA) by PCR in a thermal cycler (Bio-Rad, iCycler).

Construction of the OTf-pPICZB Expression Vector. The PCR product was cloned into *Kpn*I/*Xba*I-digested pPICZB vector to generate the construct of the OTf-pPICZB vector. The vector was linearized with *Pme*I and transformed into *P. pastoris* KM71H by electroporation (MicroPulser, Bio-Rad). The cells were incubated at 30 °C for 1 h and then spread on YPD medium plus 1.0 M sorbitol (YPDS) plates using Zeocin (100 μg/mL) as a selective marker. The resulting cells were cultured at 30 °C for 2−3 days. The positive colonies were confirmed by colony PCR with the specific OTf primers. Then, 5 μL of each PCR-amplified product was analyzed on 1% agarose electrophoresis. These colonies were further screened for multicopies recombinant by spreading the PCR-selected colonies over YPD plates with 5−20-fold concentrations (500−2000 μg/mL) of Zeocin. *Pichia* transformants with a high Zeocin resistance were selected for OTf expression.

Confirmation of OTf Transcription. After induction of OTf gene expression (with methanol) in a selected clone, total RNA was extracted from recombinant *Pichia* using Yeastar RNA isolation kit according to the instructions of the manufacturer. The first-strand cDNA was synthesized with AccuPower RT-PreMix (Bioneer-Japan) and Oligo(dT)20-M4 adaptor primer. PCR was performed with the OTf specific primers or AOX1 promoter (forward 5′-GACTGGTTC-CAATTGACAAGC-3′) and AOX1 terminator (reverse 5′-GCAAATGGCATTCTGACATCC-3′) primers.

Electrophoresis and Western Blotting. A high level expressing *P. pastoris* clone (OTf-pPICZB) or a negative control transformant with an empty pPICZB vector was cultured in BMGY medium for approximately 18 h at 28 °C. Cells were induced in BMMY medium for a further 48 h, harvested, and suspended in breaking buffer (50 mM Tris at pH 7.4, 150 mM NaCl, and 0.01% Triton X-100). The suspension was treated with lyticase at 30 °C for 30 min and then lysed by freeze−thawing (3 cycles) and glass beads beating. The protein contents of the intracellular extracts were quantified by the Bradford method. The proteins (4 μg) were applied to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (12.5%) and either visualized by Coomassie Brilliant Blue (CBB) R-250 staining, or electroblotted onto a polyvinylidene difluoride (PVDF) membrane by a semi-dry unit. The polyvinylidene fluoride (PVDF) blot was loaded to SNAP i.d. protein detection system (Millipore-Japan) blocked with Blocking One agent (Nacalai-Japan) and then reacted with rabbit anti-OTf antibody. An alkaline phosphatase-conjugated anti-rabbit antibody (Sigma, St. Louis, MO) was reacted and developed with BCIP-NBT kit (Nacalai-Japan). Protein fragments immobilized on PVDF membrane were also subjected to direct N-terminal microsequencing using Applied Biosystems Procise sequencer (model 610A), equipped with a "blot cartridge".

Tolerance to Induced Oxidative Stress. Two OTf (OTf1 and OTf10) and control empty vector (empty1) KM71H clones were cultured in BMGY broth for 24 h at 28 °C. Cells were collected and resuspended in BMMY broth to a final OD_{600} of 3.0. An oxidizing agent (H_2O_2 or DEM) was added to a final concentration of 1 or 2.5 mM for H_2O_2 or 2 or 3 mM for DEM and cultured for 48 h at 28 °C in a rotary shaker (250 rpm). The culture was supplemented daily with methanol. A total of 50 μL of the culture were taken daily, serially diluted in Milli-Q water containing the same concentration of the oxidizing agent. Viable cells [colony forming units (CFU)] were counted on YPD agar, and a portion of the diluted culture was spotted onto oxidizing MGY agar, containing the oxidizing agent. Plates were incubated for 72 h at 28 °C, and CFU/mL count was calculated. Photos of the growing colonies on the oxidizing MGY agar were taken by a digital camera.

Intracellular Reducing Capacity. The assay is based on α-LpA, a disulfide compound that rapidly enters cells and is then reduced by the pyridine nucleotide-dependent disulfide reductases to a form that can be quantified with DTNB over time without disrupting the cells. Recombinant *P. pastoris* was induced with methanol in BMMY at a final OD_{600} of 1.0 for 24 h at 28 °C in a rotary shaker. Cells was pelleted, rinsed, and suspended in Krebs-Ringer HEPES (KRH) buffer. Suspensions were dispensed in 24-well plates and incubated at 37 °C with gentle mixing in 500 μL of KRH containing 0.6 mM α-LpA and 0.1 mM DTNB. Every 30 min, a 50 μL aliquot of the supernatant was transferred to a microcuvette, and the absorbance at 412 nm was measured with a Bio-Rad SmartSpec3000 spectrophotometer. Results were corrected for absorbance of a blank containing the amounts of α-LpA and DTNB used in the experiment without cells. In parallel, the absorbance at 600 nm was monitored for cell growth.

Cellular NADPH-Dependent Oxidoreductase Activity. Recombinant clones were induced with methanol in BMMY at a final OD_{600} of 1.0 at 28 °C in a rotary shaker. When cultures reached an OD_{600} of about 6.0, cells were harvested and washed and about 3 g of cell pellet was suspended in breaking buffer and lysed with lyticase and glass beads. DNaseI and RNase were added to the cell lysate to final concentrations of 5 and 20 μg/mL, respectively, and incubated for 30 min in an ice bath. The intracellular soluble extracts and membranes were separated by centrifugation for 60 min at 15 000 rpm. For cytosolic NADPH-dependent reductases, extracts (150 μg/mL proteins) were incubated at 37 °C with gentle mixing in 200 μL of 100 mM HEPES buffer at pH 7.2 containing 200 μM NADPH in 96-well black fluoroplates. Fluorescence of NADPH was recorded kinetically from four parallel wells per sample at excitation and emission of 355 and 460 nm, respectively, in a microplate fluorometer, Fluoroskan Ascent F (Labsystems, Helsinki, Finland), with on-board software. After subtracting controls and blank values from each sample, fluorescence read-outs (RFU), of two independent experiments, were represented as delta-RFU obtained by subtracting the reading at zero time of reaction. Decreased RFU of the reaction mixture indicates consumption of NADPH by reductases while reducing the thiol compounds, such as glutathione and thioredoxin.

For membrane-bound NADPH oxidase, membranes (80 μg/mL proteins) were incubated at 37 °C in 200 μL of 50 mM HEPES buffer at pH 7.2 containing 200 μM NADPH and 100 μM NBT in 96-well plates. The absorbance at 562 nm was measured kinetically for 20 min in a Biotrak II microplate reader (Amersham-Biosciences), and the blank value was subtracted from each sample. Increased absorbance indicates the reduction of NBT to formazan by superoxide anions generated by NADPH oxidase. In parallel, alkaline phosphatase (APase) activity was measured, to check the vitality of cells, using a colorimetric kit (Funakoshi-Japan).

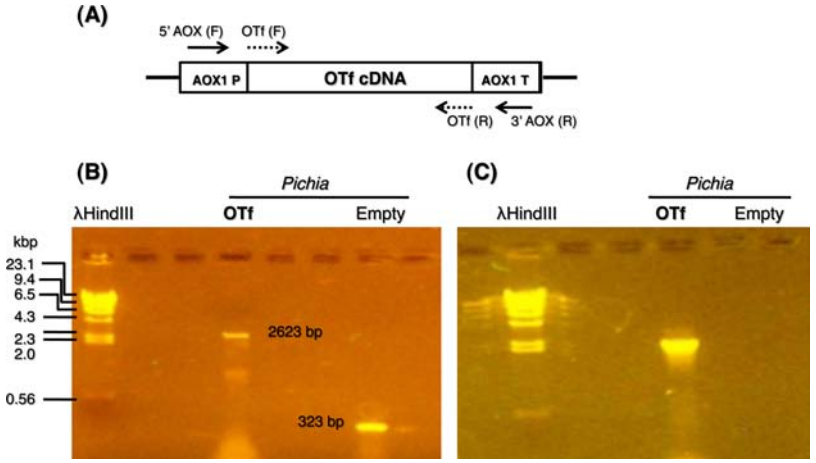

Figure 1. Schematic representation of the OTf-pPICZB construct and genomic integration into the yeast *P. pastoris* KM71H. (A) Sequence region of OTf cDNA, 2623 bp, downstream of the AOX1 promoter and upstream of the AOX1 terminator, is shown. (B) Confirmation of the OTf genomic integration by colony PCR with primers, AOX1 (F) and AOX1 (R) that anneal in the AOX1 region, amplifying a fragment of 323 bp (empty), in addition to the 2300 bp of the OTf gene (OTf) in the pPICZB vector. Lane OTf, amplicon obtained from the KM71H/OTf-pPICZB recombinant (2300 + 323 = 2623 bp); lane empty, a 323 bp amplicon obtained from the mock KM71H/pPICZB recombinant (empty vector, negative control) of *P. pastoris* from its Mut+ phenotype. (C) Expression of OTf mRNA by RT-PCR of clones with primers specific to the OTf gene, OTf (F) and OTf (R). On all gels, we used the molecular weight marker lambda *Hind*III digest (*λ-Hind*III).

RESULTS

Construction of Recombinant *P. pastoris* Expressing OTf. According to the reported sequence of OTf cDNA, a 2374 bp DNA product was synthesized, which was ligated into pPICZB vector, resulting in the recombinant vector OTf-pPICZB (Figure 1A). Then, the empty pPICZB and OTf-pPICZB vectors were transformed into *P. pastoris* KM71H to obtain recombinant strains KM71H/pPICZB and KM71H/OTf-pPICZB. The transformants were confirmed by PCR amplification from genomic DNA, using a colony PCR technique with primers specific for AOX1 promoter and terminator, generating 323 and 2623 bp DNA fragments for empty and OTf-loaded strains, respectively (Figure 1B). Transcription of OTf into intact mRNA (2374 bp) was confirmed by RT-PCR with primers specific for OTf of methanol-induced KM71H/OTf-pPICZB but not in the empty vector transformed strain KM71H/pPICZB (Figure 1C).

SDS−PAGE revealed that the molecular mass of the intracellularly expressed OTf in the recombinant strain KM71H/OTf-pPICZB was about 78 kDa (Figure 2A), which is consistent with the theoretical molecular weight of OTf. The expressed OTf showed similar mobility to that of the control protein, cOTf (Figure 2A). Western blot analysis revealed that OTf was correctly expressed but found to undergo self-cleavage, producing a major fragment of 15 kDa (Figure 2B). The N-terminal sequence of the 15 kDa fragment was TGLGRSAG with a molecular mass of 15 089.39, as revealed from microsequencing and matrix-assisted laser desorption ionization−time of flight (MALDI−TOF) analysis. The mass and N-terminus sequence allow for the prediction of a 15 kDa self-cleaved peptide of 140 amino acid residues corresponding to the 117−247 sequence of reduced autocleaved OTf (racOTf). Interestingly, this major self-cleaved peptide is the disulfide kringle domain in the N-terminal lobe of OTf, which is released from racOTf as reported previously.[8]

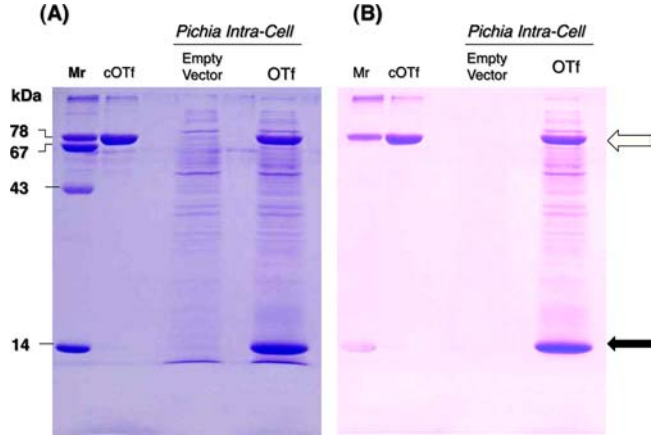

Figure 2. SDS−PAGE and western blots confirm the intracellular protein expression of OTf. Recombinant strains KM71H/OTf-pPICZB and KM71H/pPICZB were induced by MeOH, and intracellular proteins were isolated. Portions of the intracellular proteins were applied to SDS−PAGE and (A) stained with Coomassie Blue or (B) electroblotted onto a PVDF membrane, and the western blots were developed using specific anti-OTf antibody. Mr, protein markers; cOTf, standard OTf protein. The intact OTf band (open arrow) and its 15 kDa self-cleaved fragment (solid arrow) are indicated.

Tolerance of Recombinant *P. pastoris* against Oxidative Stress. The *in vitro* SOD-like activity of OTf and its ability to self-cleave in a redox-dependent manner led us to anticipate that OTf could contribute to the cellular antioxidant defense system to protect the model eukaryotic cells against oxidative stress *in vivo*. The ability of *Pichia* cells to tolerate H_2O_2-induced oxidative stress was assessed by inducing the intracellular expression of OTf with methanol in two high expressing colonies (OTf1 and OTf10), followed by spotting the serially diluted (arrow direction) cells on agar plates containing mild (1 mM) and strong (2.5 mM) concentrations of H_2O_2 (Figure 3). At a mild H_2O_2 concentration, both of the OTf-expressing cells showed a progressive increase in the number and bigger colonies than the empty vector-harboring cells (Figure 3A). However, at extensive H_2O_2 stress conditions, the OTf-expressing colonies produced a considerable number of big colonies, while the empty vector-

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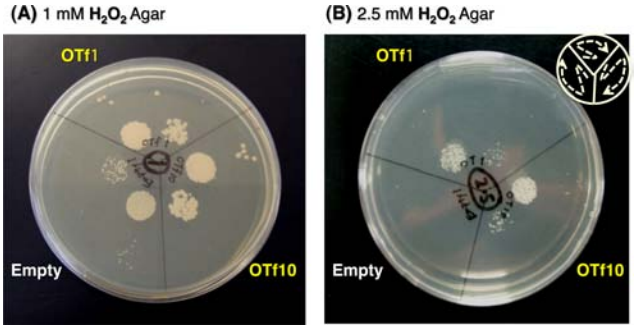

Figure 3. Tolerance to H_2O_2-induced oxidative stress of the recombinant *Pichia* yeast cells. Cells were precultured to the exponential growth phase in BMMY broth. H_2O_2 was added in BMMY to a final concentration of 1 or 2.5 mM and cultured for 72 h at 28 °C. Cultures were adjusted to an A_{600} of 1.0. Serial dilutions of 10^1−10^5 (arrow direction) in water containing the same concentration of H_2O_2 were spotted onto MGY agar containing (A) 1 mM or (B) 2.5 mM H_2O_2 plus methanol and then incubated at 28 °C for 48 h. Two randomly chosen colonies expressing OTf (OTf1 and OTf10) and one control colony (empty) were used for the spotting test.

harboring cells were unable to form any colony (Figure 3B). The results indicate that OTf appears to be redox-sensing and act as an intracellular antioxidant defense protein.

An experiment with a highly oxidative stress inducer (DEM), a thiol-binding agent with specificity to deplete intracellular glutathione (GSH), was conducted (Figure 4). DEM is

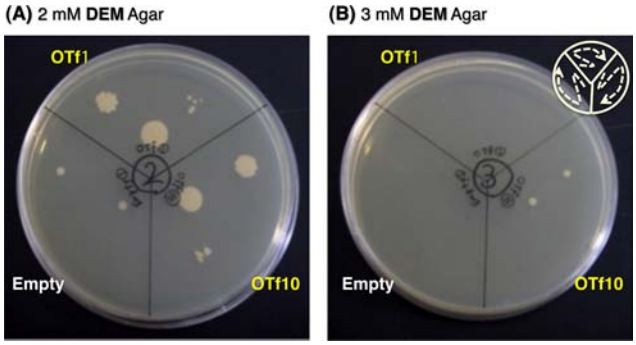

Figure 4. Tolerance to DEM-induced oxidative stress of the recombinant *Pichia* yeast cells. Cells were precultured to the exponential growth phase in BMMY broth. DEM was added to a final concentration of 2 or 3 mM and cultured for 72 h at 28 °C. Cultures of strains were adjusted to an A_{600} of 1.0. Serial dilutions of 10^2−10^4 (arrow direction) in water containing the same concentration of DEM were spotted onto MGY agar containing (A) 2 mM or (B) 3 mM DEM plus methanol and then incubated at 28 °C for 48 h.

commonly used to induce oxidative stress in cells at a concentration of 0.5−2 mM.[13] At 2 mM DEM, both of the OTf-expressing recombinant cells showed a large number of colonies even at higher dilutions compared to the very few colonies of the empty vector-harboring cells (Figure 4A). Interestingly, at extreme conditions of DEM-induced oxidative stress (3 mM), the OTf10-recombinant cells showed superior colony-forming potency (Figure 4B). Quantitation of CFU of *Pichia* cells under H_2O_2-induced (Figure 5A) and DEM-induced (Figure 5B) oxidative stress as a function of initial cell densities indicated complete death of the empty vector-harboring cells, while the OTf-expressing cells retained a high survival rate under both oxidative conditions. These results allow for the conclusion that OTf acts as an antioxidant protein likely through modulation of the intracellular reduction−oxidation (redox) state toward more reducing conditions.

Intracellular Reducing Capacity of Recombinant *P. pastoris*. The intracellular redox environment is an important determinant of cell survival and contributes to regulation of diverse processes within cells, including resistance to oxidative stress.[14,15] Protein thiols contained in cysteines contribute to the cellular redox environment, especially when they form the active sites of crucial enzymes, such as in the pyridine nucleotide−disulfide oxidoreductases, glutathione reductase, and thioredoxin reductase. Regeneration of GSH and protein thiols from disulfides by these enzymes reflects both the redox environment and the redox capacity of the cells. The ability of OTf to enhance tolerance to oxidative stress in *Pichia* implies its role as a direct modulation of the intracellular thiol redox state of cells. An approach to assess the thiol-reducing capacity of cells is the use of α-LpA, a disulfide compound that rapidly enters cells and then is reduced by the enzymes to a form that can be quantified kinetically over time without disrupting the cells.[16] Because each of the pyridine nucleotide-dependent reductases (disulfide oxidoreductases, glutathione reductase, and thioredoxin reductase) can reduce α-LpA[17] and neither GSH nor L-cysteine reacts with α-LpA,[18] thus, its reduction will reflect the activity of pyridine nucleotide−disulfide oxidoreductases and not simply the cell content of GSH or protein thiols. Figure 6 shows the kinetics of α-LpA reduction in *Pichia* cells harboring the OTf1 clone or empty vector (empty) compared to the kinetic curve of growth (inset). Both recombinant cells exhibited similar growth curves with a 18 h lag phase and a 20 h lag phase before reaching the stationary phase (inset in Figure 6). When the reducing capacity of cells at 24 h of growth was measured using the α-LpA/DTNB assay, the reduction of α-LpA increased during the first 30 min and reached a maximum at 60 min for empty cells and at 120 min for OTf-expressing cells, represented as absorbance changes of DTNB (Figure 6). The intracellular reductive capacity of cells expressing OTf was greatly enhanced (2-fold) compared to the empty cells. This suggest that the ability of OTf to enhance tolerance to oxidative stress in *Pichia* would be attributed to its direct modulation of the thiol-reducing state of cells, specifically pyridine nucleotide−disulfide oxidoreductases.

To further support this hypothesis, the cellular NADPH-dependent oxidoreductase activities were measured (Figure 7). The OTf-expressing cells exhibited much higher activity of NADPH-dependent reductases in the cytosol compared to that in the empty cells, with activity monitored by the NADPH consumption (Figure 7A). However, both strains showed similar activities of the membrane-bound NADPH oxidases (Figure 7B). When intracellular APase activity was measured, the OTf-expressing and empty cell strains showed comparable activity, indicating similar vitality, and excluded any lethal effect of OTf (Figure 7C). Taken together, the impressive intracellular antioxidative defense mechanism of OTf seems operating through participation with or activation of the cytosolic NADPH-dependent disulfide oxidoreductases (redox regulatory antioxidant enzymes), comprising thioredoxin (Trx), thioredoxin reductase (TrxR), and glutathione reductase (GR).

DISCUSSION

Recently, we reported evidence supporting self-cleavage of OTf through thiol rearrangement under reducing conditions or by the thioredoxin (Trx/TrxR/NADPH) system,[8] thus generating

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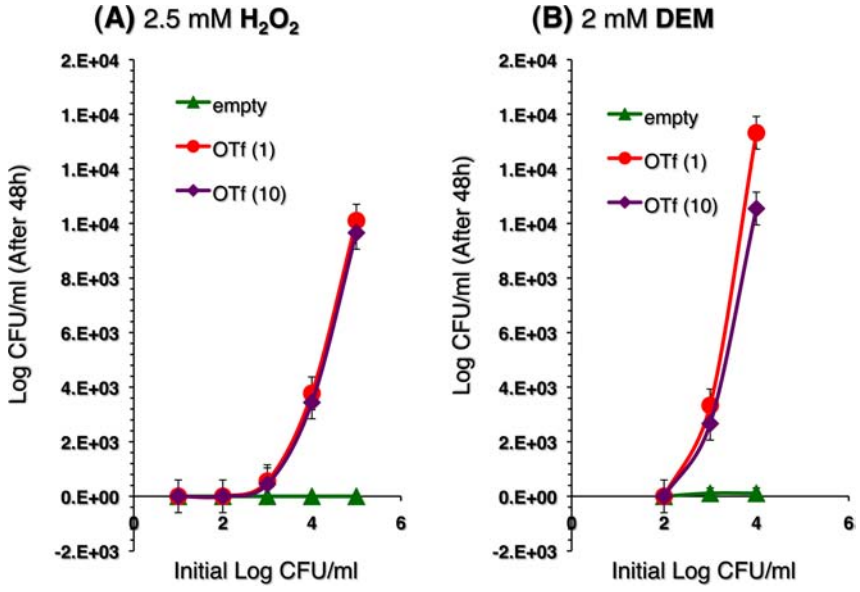

Figure 5. OTf expression confers resistance toward (A) H_2O_2 and (B) DEM. Two OTf expressing colonies (OTf1 and OTf10) and a control colony (empty) were grown in BMMY broth overnight at 28 °C. Cells were seeded in BMMY broth, containing the indicated amount of H_2O_2 or DEM, starting with 10^5 cells in steps of 10-fold dilutions and incubated at 28 °C for 6 h. Cultures were spotted onto MGY agar plates, containing the same amount of the respective oxidizing agent, and cultivated at 28 °C for 2 days before scoring growth as CFU/mL.

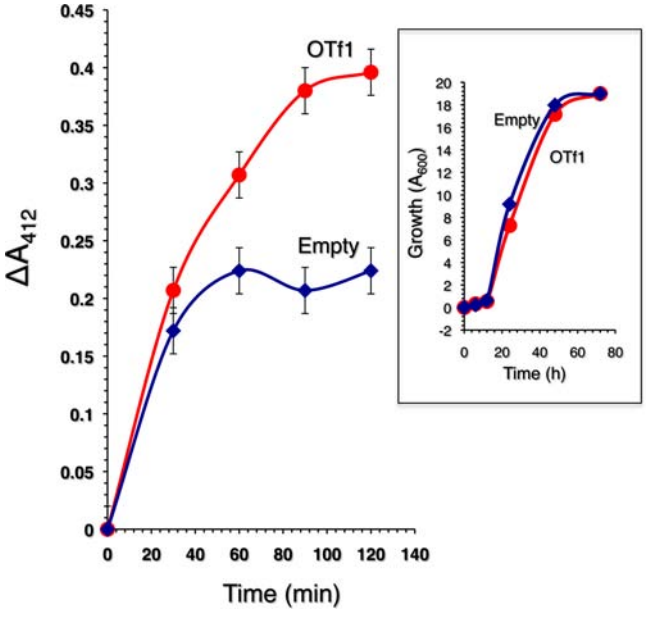

Figure 6. Intracellular thiol-reducing capacity of the recombinant *Pichia* yeast cells. Cells were seeded in BMMY broth at 10^7 cells/mL and incubated at 28 °C for 24 h. (Inset) Cell growth of the recombinant cells was monitored for 3 days by measuring the absorbance at A_{600}. Rinsed cells, from the 24 h culture, were incubated at 37 °C in buffer containing DTNB and α-LpA. Every 30 min, the absorbance (412 nm) of the supernatant was measured and represented as changes in absorbance. Results are an average from four experiments, with data in OTf-harboring cells (OTf1) fit to a hyperbolic model and non-saturable disulfide reducing capacity up to 2 h.

peptides with SOD-like activity[9] and specifically killing cancer cells through induction of apoptosis.[10] On the basis of our earlier observations, we have suggested that the redox-sensing force of OTf may be crucial for its physiological function *in vivo* as a defense molecule against oxidative stress. This study describes an *in vivo* antioxidative stress function of OTf in protection of eukaryotic cells. The intracellular expression in yeast cell *P. pastoris* was employed to explore the *in vivo* function of OTf to act as a defense molecule against oxidative stress. Cells expressing OTf exhibited significant tolerance toward oxidative stress induced with either H_2O_2 or the glutathione-depleting agent DEM. It is worth noting that the mechanisms of oxidative stress induced by H_2O_2 and DEM are different. H_2O_2-induced oxidative stress *in vivo* is attributed to increased superoxide anions ($O_2^{\bullet -}$) generated by NADPH oxidase.[19] On the other hand, DEM contributes to oxidative stress by depleting the intracellular pool of reduced glutathione (GSH), has been shown to inhibit nuclear factor (NF)-κB activation, and increases the oxidative free radicals.[20] We found that OTf expression rendered the cells resistant to DEM-induced oxidative stress more efficiently than to H_2O_2-induced conditions (Figures 3−5). The tolerance imparted by OTf expression was particularly significant because the concentration of the oxidizing agent used in this study (2.5 mM H_2O_2 and 2 mM DEM) was considerably higher than the doses commonly used to induce cellular oxidative stress (60−500 μM).[19−21]

Aerobic life of eukaryotic yeast requires the maintenance of an appropriate intracellular redox environment that minimizes the generation of reactive oxygen species (ROS) that damage nucleic acids, proteins, and lipids and yet allows for essential metabolic functions.[22] To preserve this crucial reducing milieu, aerobes have evolved efficient redox-sensing antioxidant systems.[23] The redox state of a cell is mediated by the ratios of reduced and oxidized pyridine nucleotides [NADPH/$NADP^+$] and thiols, such as glutathione/glutathione disulfide (GSH/GSSG) and thioredoxin/thioredoxin disulfide [Trx-$(SH)_2$/Trx$(S)_2$]. These ratios are intimately related to the cellular levels of ROS because the activities of many enzymes involved in antioxidant functions depend upon the redox state of the pyridine nucleotide pools and the redox state of cellular thiols, such as glutathione and thioredoxin.[24] For instance,

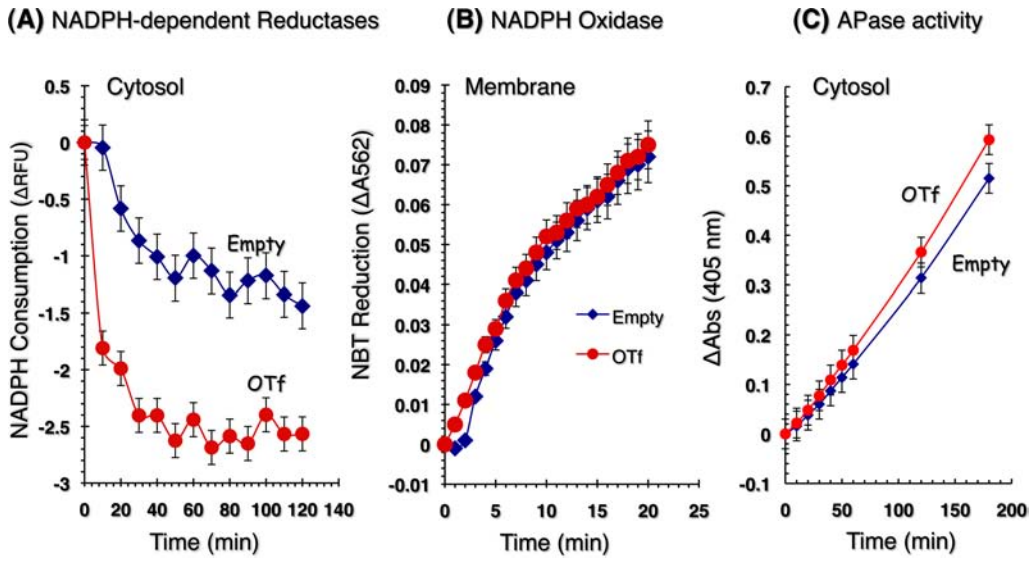

Figure 7. Activities of NADPH-dependent oxidoreductases of the recombinant *Pichia* cells. (A) NADPH-dependent reductases in the intracellular extract of methanol-induced recombinant strains KM71H/OTf-pPICZB (OTf) and KM71H/pPICZB (empty) were measured by monitoring the decrease in fluorescence of NADPH, because of consumption by oxidoreductases. (B) Activity of membrane-bound NADPH oxidase in the isolated cell membranes, represented as the rate of NBT reduction. (C) Activity of APase in the cellular extracts using the colorimetric *p*-nitrophenyl phosphate (pNPP) as a substrate, which turns yellow (λ_{max} = 405 nm) when dephosphorylated by APase. The values are the means of results from three independent experiments.

hydrogen peroxide generated by SOD is reduced to water and oxygen by a variety of peroxidases, including catalase, glutathione-dependent peroxidases (GSH-Px), and thioredoxin-dependent peroxiredoxins (Trx-Px).[23,25] GSH-Px and Trx-Px obtain their reducing equivalents from two distinct redox systems, the glutathione and the thioredoxin redox systems.[26] Both systems comprise a cascade of redox-active proteins and peptides that transfer reducing equivalents from NADPH to acceptor molecules. We found that the intracellular disulfide reducing capacity (Figure 6) and cytosolic NADPH consumption (Figure 7A) but not membrane-bound NADPH oxidases (Figure 7B) are enhanced in OTf-expressing cells, suggesting a progressive recycling of reductases. The results suggest that OTf and likely its 15 kDa disulfide kringle fragment (Figure 2) are directly involved in molecular activation of intracellular antioxidant defense enzymes, which are redox-active proteins that transfer reducing equivalents from NADPH to acceptor molecules.

Judging by the relatively high NADPH consumption in the cytosol of OTf-expressing cells compared to the empty vector-loaded cells (Figure 7A) while maintaining equal vitality (Figure 7C), OTf appears to enhance activities of intracellular enzymes involved in antioxidant functions that depend upon the pyridine nucleotide and the redox state of cellular thiols, such as glutathione, thioredoxin, and their reductases. However, a study has shown that GSH-deficient yeast are still able to induce an adaptive stress response to H_2O_2, suggesting that GSH is not important as a sensor molecule in this stress response.[27] Antioxidant-defense activation processes are often triggered by oxidative modification of cysteine, which is known to play a major role in redox sensing and the antioxidant response.[28] OTf-expressing cells showed much greater intracellular disulfide reducing capacity than the control empty cells (Figure 6) while maintaining equal growth ability (inset of Figure 6). OTf contains two Trx-sensitive disulfide kringles between Cys160/Cys174 and Cys171/Cys182 in the 15 kDa fragment (117−247 sequence of OTf). Trx can preferentially reduce the disulfides, although the physiological electron donor that supports the radical-scavenging activity of OTf remains to be identified. During oxidative stress, one cysteine sulfenic acid (Cys-SOH) is attacked by the other cysteine (Cys-SH), resulting in the formation of a stable intramolecular disulfide bond, which can be reduced by Trx or other electron donors. The reaction mechanisms by which two cysteines remove oxidative radicals, such as peroxides, are well-characterized.[29] Further, OTf itself has been found as a superoxide scavenger in the presence of copper or manganese ions.[9] Thus, the results provide evidence that the potency of OTf to enhance cellular antioxidant response is perhaps attributed to oxidation of its cysteine acid side chain, which directly creates a chemical reducing species. This hypothesis may be supported by the observation of self-cleavage of OTf in the cytosol of *Pichia* (Figure 2). It is worth noting that an antioxidant peptide ($W^{125}NIP^{128}$) from OTf has recently been reported,[30] whereas this peptide is located within the sequence of the intracellularly released 15 kDa fragment (residues 117−247) found in this study. However, the possibility that OTf or its 15 kDa peptide act as a regulator of transcription of adaptive stress response and/or reductase genes remains to be answered and would merit further investigation.

In conclusion, our findings presented here have a number of implications. They clearly show, for the first time, that OTf is a defense protein against oxidative stress *in vivo*, whereas it enhances the intracellular reducing capacity of the cells through activation of NADPH-dependent reductases. The results highlight a novel function for OTf during embryogenesis of avian eggs, which involve redox modulation. Our results not only explore the novel *in vivo* defensive role of OTf against oxidative stress but also provide novel insights for the potential of the OTf gene as an element in molecular breeding of industrial yeast strains. It suggests that the OTf gene could be an important element for the rational development of unicellular eukaryotic yeasts for industrial applications. The rationale behind this strategy is that yeast is an important

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microorganism in basic science as a model for higher eukaryotes and a useful microbe in the fermentation industry for the production of breads and bioethanol. During fermentation, yeast cells are exposed to various stresses, including ethanol, high temperature, desiccation, and osmotic pressure. Such stresses induce ROS generation and lead to growth inhibition or defected fermentation. In terms of industrial application, oxidative stress tolerance is the key for yeast cells. Our results indicate that expression of OTf in yeast strains confers oxidative stress tolerance by enhancing the cellular reducing capacity in the presence of oxidants, suggesting that these strains are capable of adapting rapidly to various compounds that inhibit fermentation by inducing ROS accumulation. Given the fact that OTf and its kringle peptides exhibit potent antimicrobial activity,[31] the OTf gene represents a challenge to design new bakery yeast strains with resistance to spoilage microorganisms while conferring high tolerance to oxidative stress. Eventually, it would be rewarding to ascertain whether the transferrins within the various species also have the ability to function as defense proteins as part of a comprehensive antioxidant system *in vivo*.

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Notes

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ABBREVIATIONS USED

OTf, ovotransferrin; DEM, diethyl maleate; α-LpA, α-lipoic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NBT, nitro-blue tetrazolium; CBB, Coomassie Brilliant Blue; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ROS, reactive oxygen species; SOD, superoxide dismutase; GSH, reduced glutathione; GSSG, oxidized glutathione; Trx, thioredoxin; TrxR, thioredoxin reductase

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