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Staszczak, Magdalena
Department of Biochemistry, Maria Curie-Sklodowska University

Sajewicz, Joannna

Ohga, Shoji
Laboratory of Forest Resources Management, Division of Forest Environment Sciences, Department of Agro-Environment Sciences, Faculty of Agriculture, Kyushu University

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Effect of Endoplasmic Reticulum Stress on Laccase Production and the 26S Proteasome Activity in the White Rot Fungus *Trametes versicolor*

Magdalena STASZCZAK[1], Joanna SAJEWICZ[1] and Shoji OHGA*

Laboratory of Forest Resources Management, Division of Forest Environmental Sciences,
Department of Agro–environmental Sciences, Faculty of Agriculture,
Kyushu University, Sasaguri, Fukuoka 811–2415, Japan
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The ubiquitin–proteasome pathway is a major system for degrading intracellular proteins in eukaryotes. It degrades many important proteins involved in signal transduction, cell cycle progression, and in general metabolism, including key metabolic enzymes and transcription factors. In addition, the ubiquitin–proteasome system is responsible for endoplasmic reticulum–associated degradation. Our previous studies have demonstrated the presence of proteasomes in white rot Basidiomycetes and indicated that ubiquitin–proteasome–mediated pathway in *Trametes versicolor* is involved in the regulation of laccase, a main ligninolytic enzyme of this fungus, upon nutrient starvation as well as in response to cadmium exposure. Fungal laccases are useful biocatalysts in a wide range of biotechnological applications. Here, we studied the effects of tunicamycin–induced endoplasmic reticulum stress on laccase activity and the 26S proteasome–mediated proteolysis in nitrogen–sufficient and nitrogen–deprived cultures of *T. versicolor*. We found that short–term ER stress (6 h) leads to inhibition of both laccase activity and the 26S proteasome activity. This effect was more pronounced in the nitrogen–deprived cultures. However, after prolonged ER stress (24 h) laccase activity in tunicamycin–treated cultures recovered to levels comparable to those of untreated cultures. In contrast to what was observed after short–term stress, a 24–h tunicamycin treatment resulted in a significant increase in the 26S proteasome activity detected in mycelia from nitrogen–sufficient and nitrogen–deprived cultures (approximately 1.5–fold and 2–fold increase, respectively). Moreover, we found that blocking of proteasome function in *T. versicolor* subjected to prolonged ER stress resulted in a decrease of laccase activity. These findings suggest a potential role of the proteasome–mediated degradation as an important mechanism by which laccase is regulated under prolonged ER stress.

INTRODUCTION

White rot Basidiomycetes play an essential role in the carbon cycle by degrading all major components of wood including cellulose, hemicellulose and lignin (Watkinson, 2006). White–rot fungi produce four types of extracellular oxidative enzymes for the degradation of lignin: laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2), lignin peroxidase (LiP; EC 1.11.1.14), manganese–dependent peroxidase (manganese peroxidase, MnP; EC 1.11.1.13), and versatile peroxidase (VP; EC 1.11.1.16) (reviewed by Cullen and Kersten, 2004; Kersten and Cullen, 2007; Kirk and Cullen, 1998; Hatakka, 1994). Ligninolytic enzymes are mainly produced during secondary metabolism triggered in the white rot fungi by nutrient deprivation and typically under conditions of limited nitrogen (Hammel, 1997; Jeffries *et al*., 1981; Keyser *et al*., 1978).

Trametes versicolor is an efficient and highly selective lignin degrading Basidiomycete. This fungus produces laccase, a multiple copper–containing glycosylated protein, as the main ligninolytic enzyme. Fungal laccase has been investigated for widespread technological applications ranging from bioremediation of industrial effluents (Han *et al*., 2004; Paszczynski and Crawford, 2000; Shah and Nerud, 2002) to the use in paper production (Kenealy and Jeffries, 2003; Widsten and Kandelbauer, 2008) and for biosensors construction (Barton *et al*., 2002). These varied uses of laccase can be ascribed to its ability to oxidize a wide range of phenolic and non–phenolic compounds.

To date, relatively little research has been devoted to specific mechanisms of laccase secretion. It is well established that secretion of glycoproteins can be influenced by tunicamycin, an endoplasmic reticulum (ER) stress inducer (Elbein, 1987; Schröder and Kaufman, 2005). Tunicamycin, a nucleoside antibiotic produced by *Streptomyces lysosuperficus*, inhibits N–linked glycosylation and blocks the formation of N–glycosidic protein–carbohydrate linkages (Elbein, 1987). During the past years it has been recognized that secretory proteins that ultimately fail to fold properly are removed from the ER and degraded in the cytosol by the ubiquitin–proteasome pathway in a process referred to as ER associated degradation (ERAD) (Hirsch *et al*., 2009; McCracken and Brodsky, 2003).

The ubiquitin–proteasome pathway is the major non–lysosomal system for degrading proteins in eukaryotic cells; it is responsible for the degradation of 80% to 90% of all cellular proteins. The importance of the ubiquitin–proteasome pathway to cellular regulation in eukaryotes was acknowledged with the 2004 Nobel Prize in Chemistry to Aaron Ciechanover, Avram Hershko and Irwin Rose for their pioneering work on the pathway. This highly selective proteolytic system requires ATP and the 26S proteasome, a large ~2.5 MDa multisubunit complex which degrades protein targets marked by a covalently attached polyubiquitin chain (Ciechanover,

[1] Department of Biochemistry, Maria Curie–Sklodowska University, 20–033 Lublin, Poland
* Corresponding author (E–mail: ohga@forest.kyushu–u.ac.jp)

2005; Glickman and Ciechanover, 2002; Pickart and Cohen, 2004). Ubiquitin is a small (76–residue) evolutionary conserved protein (Pickart and Eddins, 2004). The ubiquitin targeting system consists of the ubiquitin activating enzyme (E1), ubiquitin conjugating enzymes (E2s), and ubiquitin ligases (E3s). The 26S proteasome is composed of a 20S proteolytic core and one or two 19S regulatory complexes. The 20S barrel–shaped core is built from 28 subunits arranged as an $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ stack of four rings. The two external rings are composed of non–proteolytic α–type subunits, while the two inner rings of β–type subunits are responsible for the proteolytic activity. Each β–ring contains three active centres of distinct peptidase specificities, namely chymotrypsin–like (cleaves after C–terminal of hydrophobic residues), trypsin–like (cleaves after basic residues), and caspase–like (cleaves after acidic residues). The 19S regulatory complex confers both ubiquitin and ATP dependence. As the principal system for degrading cellular proteins, the ubiquitin–proteasome pathway has two primary functions: a quality control function (i.e. degradation of damaged, misfolded, and functionally incompetent proteins) and a major regulatory function (i.e. the targeted degradation of many important proteins involved in cell cycle control, signalling pathway, and in general metabolism, including transcription factors and key metabolic enzymes).

Earlier studies from our laboratory have demonstrated for the first time the presence of proteasomes in fungi other then yeast, namely white rot Basidiomycetes *Trametes versicolor* (Staszczak and Jarosz–Wilkołazka, 2005) and *Phlebia radiata* (Staszczak, 2007). Our previous studies have indicated that proteasomal degradation of intracellular proteins is involved in the regulation of laccase activity in *T. versicolor* upon nitrogen and carbon starvation as well as in response to cadmium exposure (Staszczak, 2002; Staszczak and Jarosz–Wilkołazka, 2005). Moreover, the very recent studies performed in our laboratory have demonstrated the important role of the ubiquitin–proteasome system in the response of *T. versicolor* to nitrogen deprivation, providing new insights into nitrogen starvation stress responses in wood–degrading fungi (Staszczak, 2008).

In this paper, we describe the effects of short–term (6 h) and long–term (24 h) ER stress induced by tunicamycin treatment on the 26S proteasome–mediated proteolysis and laccase activity in nitrogen–sufficient and nitrogen–deprived cultures of *T. versicolor*. Our results suggest a potential role of the proteasomal degradation of proteins as an important mechanism by which laccase is regulated under prolonged ER stress.

MATERIALS AND METHODS

Materials

7–amino–4–methylcoumarin (AMC), bovine albumin, dimethyl sulfoxide (DMSO), Suc–Leu–Leu–Val–Tyr–7–amido–4–methylcoumarin (Suc–LLVY–AMC), tunicamycin, syringaldazine [N,N'–bis(3,5–dimethoxy–4–hydroxybenzylidene hydrazine)], and Z–Leu–Leu–Leu–al (MG132) were obtained from Sigma (St. Louis). L–asparagine was purchased from Merck (Darmstadt). SDS was provided by Pierce (Rockford). All aqueous solutions were prepared using water purified with Milli–Q system (Millipore, Milford, CT, USA). All other chemicals were of analytical grade.

Microorganism and culture conditions

Mycelia of *Trametes versicolor* (ATCC 44308) were grown for 7 days as surface cultures, without shaking, at 26 °C on nutrient–rich media containing L–asparagine (2.5 g/l) as a nitrogen source and glucose (20 g/l) as a carbon source (Fahraeus and Reinhammar, 1967).

Conditions of nitrogen starvation

Nitrogen starvation was performed essentially as described previously (Staszczak, 2008). Briefly, after the 7–day cultivation period, mycelia were transferred from the nutrient–rich media to either nitrogen–deprived media (without L–asparagine) or to nitrogen–sufficient media, and the incubation at 26 °C continued. The nitrogen–sufficient media contained L–asparagine and glucose of the same concentrations as those measured after 7 days of fungal growth (40% and 57% of their initial values, respectively). Other conditions of transfer experiments were the same as those described previously (Staszczak *et al.*, 2000). Mycelia were sub–cultured for 6 h and 24 h on the nitrogen–deprived or nitrogen–sufficient media, harvested, and frozen until used.

Induction of ER stress

For experiments involving induction of ER stress response, mycelia sub–cultured on the nitrogen–deprived or nitrogen–sufficient media were separately treated for 6 h or 24 h with tunicamycin (to a final concentration of 5 μg/ml). Tunicamycin was added from a stock solution in dimethyl sulfoxide (DMSO) at the time of the transfer of mycelia to the media described above. Mycelia treated with vehicle (DMSO) were used as a control. DMSO concentration was never higher than 0.1%.

In vivo proteasome inhibition

For inhibitor studies, mycelia sub–cultured on the nitrogen–deprived or nitrogen–sufficient media were treated for 24 h with tunicamycin (to a final concentration of 5 μg/ml) in combination with the proteasome inhibitor MG132 (to a final concentration of 40 μM). Drugs were added from stock solutions in DMSO. Controls were set–up with equivalent amounts of the solvent used.

Preparation of the extracellular culture fluid

Extracellular samples were collected by separating culture fluids from mycelia by filtration through Miracloth (Calbiochem). The filtrates were desalted and concentrated in Microcon YM–3 0.5–ml centrifugal filter devices (NMWL 3000 Da, Millipore). The Microcon units were spun at 14000 × g for 30 min at 4 °C and recovered protein aliquots were kept frozen at −20 °C until further analysis.

Preparation of crude mycelial extracts

Crude mycelial extracts were prepared essentially as described previously (Staszczak, 2008). Briefly, mycelia were homogenized in an ice–chilled motor–driven Potter's homogenizer, in 50 mM Tris–HCl buffer, pH 7.3, containing 5 mM $MgCl_2$. The homogenates were then centrifuged at 10000 × g for 10 min, at 4 °C. The supernatant fractions were collected, aliquoted, and kept frozen at −20 °C for later use.

Determination of laccase (benzenediol:oxygen oxidoreductase; EC 1.10.3.2) activity

Laccase activity was determined in a 1–ml reaction mixture by measuring the oxidation of 0.025 mM syringaldazine in 0.1 M citrate–NaOH buffer at pH 5.0, essentially according to Leonowicz and Grzywnowicz (1981). The oxidation of syringaldazine to the coloured *tetra*–methoxy–azo–*bis*–methylene quinone (Harkin *et al.*, 1974) was monitored by the increase in the A_{525} at 25 °C. The specific activity of laccase was expressed in nkat/mg using a molar absorption coefficient of $6.5 \times 10^4 M^{-1}cm^{-1}$ at 525 nm. Absorbance measurements were made using a Varian Cary–3E UV/visible spectrophotometer.

Measurement of proteasome activity

For the measurement of the 26S proteasome activity, the fluorogenic peptide substrate Suc–LLVY–AMC for the chymotrypsin–like activity in a final concentration of 100 μM was used. The modified stopped procedure (Skoda and Malek, 1992; Tanaka *et al.*, 1989) was performed essentially as described previously (Staszczak, 2008). Reaction mixtures (100 μl) contained 100 mM Tris–HCl buffer (pH 8.0), 2 mM ATP, 5 mM $MgCl_2$, and the mycelial extract; incubations were done at 37 °C for 30 and 60 min. The reaction was quenched by the addition of 100 μl of 10% SDS (w/v) and 2.0 ml of 100 mM Tris–HCl buffer, pH 9.0. Fluorescence of the proteolytically released 7–amino–4–methylcoumarin (AMC) was determined at 360 nm excitation and 440 nm emission using a FluoroMax–2 spectrofluorometer (Instruments S.A., Inc., JOBIN YVON/SPEX Division, USA). The specific activity was expressed in nmoles AMC per mg of protein.

Determination of protein

Protein concentrations were determined using Bradford reagent and bovine serum albumin as a standard (Bradford, 1976).

RESULTS

Effect of ER stress on laccase activity

Tunicamycin (TM), an inhibitor of N–linked protein glycosylation, was used in the current study as an inducer of the ER stress. After the 7–day cultivation period on the nutrient–rich medium (Fahraeus and Reinhammar, 1967), mycelia of *T. versicolor* were transferred to either nitrogen–sufficient or nitrogen–deprived medium. Mycelia were sub–cultured for 6 h or 24 h on the media described above. Tunicamycin (5 μg/ml) was added from a stock solution at the time of transfer of mycelia to the nitrogen–sufficient or nitrogen–deprived media. Mycelia treated with solvent (DMSO) were used as a control. Dimethyl sulfoxide concentration was never higher than 0.1% and did not alter laccase activity at this concentration (Staszczak and Jarosz–Wilkołazka, 2005). Figure 1A shows that a 6–h tunicamycin treatment resulted in a significant decrease in specific activity of extracellular laccase both in the nitrogen–sufficient and nitrogen–deprived cultures (by about 60% and 70%, respectively). Intracellular laccase activity was also markedly reduced (90%) by the tunicamycin treatment for 6 h (Fig. 2A).

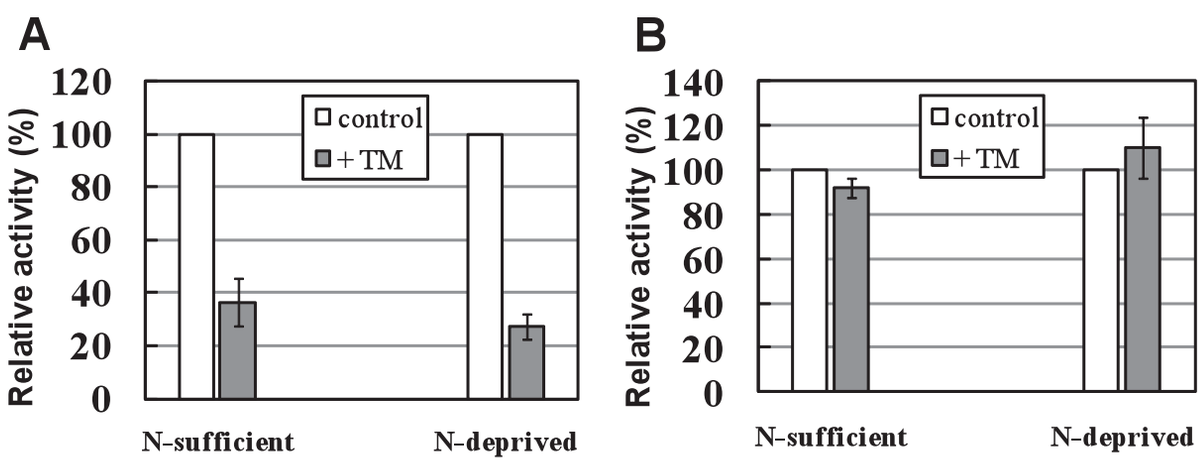

Fig. 1. Effect of ER stress on extracellular laccase activity in *T. versicolor* cultures. Mycelia grown on a nutrient–rich medium were transferred for 6 h **(A)** or 24 h **(B)** to either nitrogen–deprived or to nitrogen–sufficient media (for details see *Materials and methods).* Tunicamycin (TM), an ER–stressor, was added (to a final concentration of 5 μg/ml) at the time of transfer of mycelia to the nitrogen–sufficient or nitrogen–deprived media. Controls were set–up with equivalent amounts of the solvent used (DMSO). Laccase activity was assayed with syringaldazine as the chromogenic substrate. The specific activity (per mg of protein) of samples from the control culture (nitrogen–sufficient or nitrogen–deprived) without tunicamycin was taken to be 100%. Bars represent means ± SD of three independent experiments performed in duplicate.

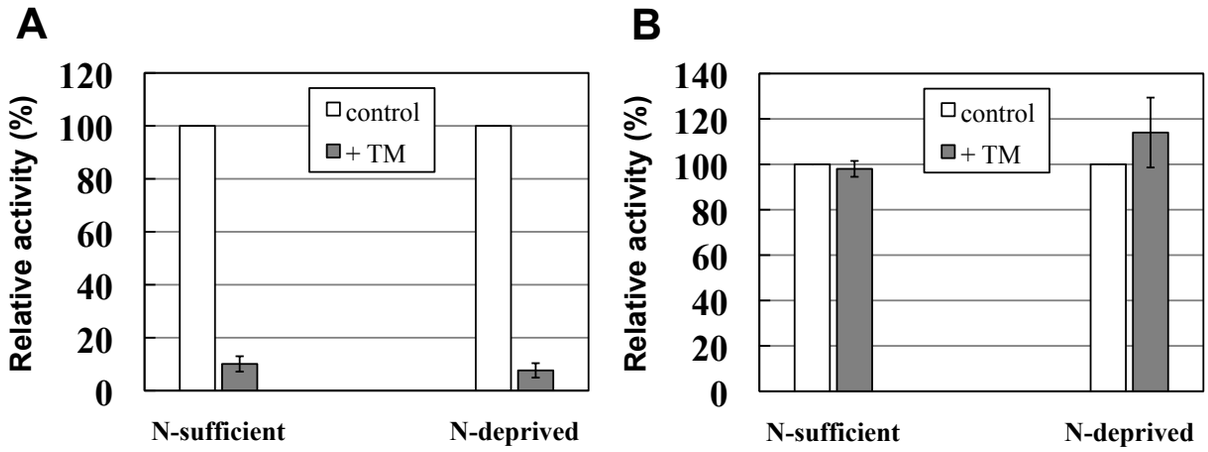

Fig. 2. Effect of ER stress on intracellular laccase activity of *T. versicolor*. Mycelia grown on a nutrient–rich medium were transferred for 6 h **(A)** or 24 h **(B)** to either nitrogen–deprived or to nitrogen–sufficient media (for details see *Materials and methods*). Tunicamycin (TM), an ER–stressor, was added (to a final concentration of 5 μg/ml) at the time of transfer of mycelia to the nitrogen–sufficient or nitrogen–deprived media. Controls were set–up with equivalent amounts of the solvent used (DMSO). Laccase activity was assayed with syringaldazine as the chromogenic substrate. The specific activity (per mg of protein) of samples from the control culture (nitrogen–sufficient or nitrogen–deprived) without tunicamycin was taken to be 100%. Bars represent means ± SD of three independent experiments performed in duplicate.

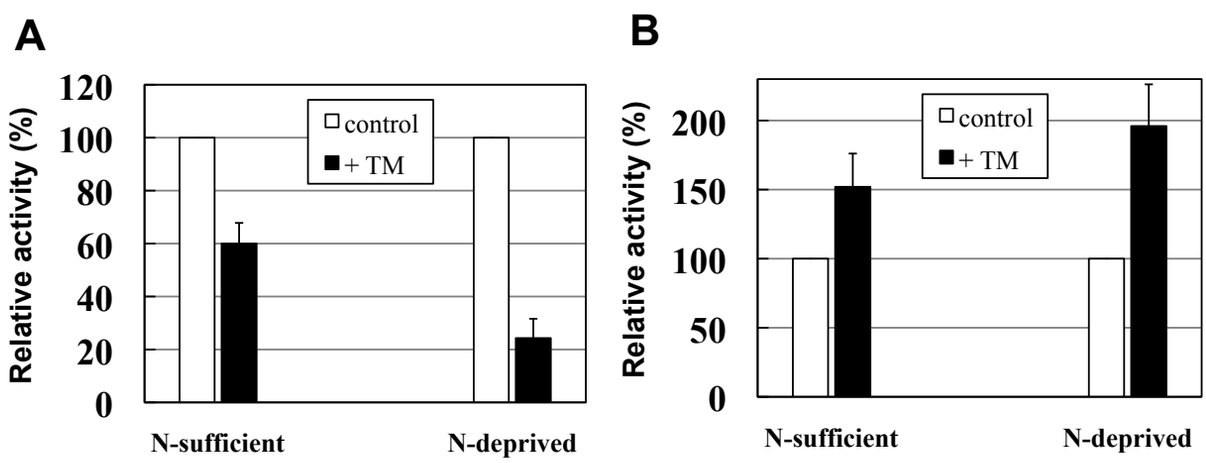

Fig. 3. Effect of ER stress on the 26S proteasome activity of *T. versicolor*. Mycelia grown on a nutrient–rich medium were transferred for 6 h **(A)** or 24 h **(B)** to either nitrogen–deprived or to nitrogen–sufficient media (for details see *Materials and methods*). Tunicamycin (TM), an ER–stressor, was added (to a final concentration of 5 μg/ml) at the time of transfer of mycelia to the nitrogen–sufficient or nitrogen–deprived media. Controls were set–up with equivalent amounts of the solvent used (DMSO). The 26S proteasome activity was assayed in mycelial extracts by monitoring cleavage of the fluorogenic peptide substrate Suc–LLVY–AMC. The specific activity (nmoles AMC per mg of protein) of samples from the control culture (nitrogen–sufficient or nitrogen–deprived) without tunicamycin was taken to be 100%. Bars represent means ± SD of three independent experiments performed in duplicate.

On the other hand, in contrast to what was observed in the case of short–term ER stress, where a decrease in laccase activity was found, after prolonged ER stress (24 h) laccase activity in tunicamycin–treated cultures recovered to levels comparable to those of untreated cultures (Fig. 1B and 2B).

Effect of ER stress on the 26S proteasome activity

In this study, a potential influence of tunicamycin–induced ER stress on the 26S proteasome–mediated proteolysis in *T. versicolor* was examined. For experiments involving induction of ER stress response, mycelia sub–cultured on the nitrogen–deprived or nitrogen–sufficient media were treated for 6 h or 24 h with tunicamycin (5 μg/ml). We found that short–term ER stress (6 h) leads to inhibition of specific activity of the 26S proteasome (Fig. 3A). Mycelia from the nitrogen–sufficient cultures retained approximately 60% of the proteasome activity, whereas about 25% of the proteasome activity remained in mycelia from nitrogen–deprived cultures after a 6–h exposure to the ER stressor.

Figure 3B shows that in contrast to what was observed after short–term stress, a 24–h tunicamycin treatment resulted in a significant increase in the 26S proteasome activity detected in mycelia from nitrogen–sufficient and nitrogen–deprived cultures (approximately 1.5–fold and 2–fold increase, respectively).

Effect of in vivo proteasome inhibition

Until very recently, our understanding of the physiological role of the ubiquitin–proteasome pathway has been limited by the lack of selective inhibitors of this proteolytic system. Several substrate–related peptide aldehydes that can block the activities of proteasomes have been identified (Kisselev and Goldberg, 2001; Lee and Goldberg, 1998). These agents can readily enter cultured cells and inhibit the proteasomes, without being acutely toxic, at least for many hours. One of the more potent of these peptide aldehyde inhibitors MG132 (CbzLLLal) has proven particularly useful as an inhibitor of the ubiquitin–proteasome pathway in mammalian cells. We have previously shown that MG132 can also efficiently inhibit proteasome activities in the white rot fungus *T. versicolor* (Staszczak, 2002; Staszczak and Jarosz–Wilkołazka, 2005).

The present study revealed differences in the response of *T. versicolor* to short–term ER stress and to prolonged ER stress induced by tunicamycin (compare Figs. 1A, 2A, and 3A with Figs. 1B, 2B, and 3B, respectively). In order to examine a potential relationship between laccase levels and activity of the 26S proteasomes under conditions of prolonged ER stress, we studied the effects of proteasome inhibition under these conditions. Mycelia of *T. versicolor* were sub–cultured for 24 h on the nitrogen–sufficient or nitrogen–deprived media (see the legend to Table 1 for details) in the presence or absence of the ER stressor tunicamycin and proteasome inhibitor MG132. Tunicamycin was added (to a final concentration of 5 μg/ml) in combination with MG132 (40 μM) at the time of transfer of the seven–day–old mycelia to the nitrogen–sufficient or nitrogen–deprived media. Mycelia treated with solvent (DMSO) were used as a control. We found that blocking of proteasome function in cultures subjected to prolonged ER stress resulted in a decrease of specific activity of extracellular laccase (Table 1). Intracellular laccase activity was also significantly reduced under conditions of prolonged ER stress by the presence of the proteasome inhibitor MG132 (Table 2). The effect of proteasome inhibition was more pronounced in the nitrogen–deprived cultures. The results suggest a potential role of the proteasome–mediated degradation as an important mechanism by which laccase is regulated under prolonged ER stress.

DISCUSSION

Perturbations in the ER environment have an impact on the ability of proteins to reach their native conformation. Quality control mechanisms ensure that only cor-

Table 1. Effect of *in vivo* proteasome inhibition on extracellular laccase activity in *T. versicolor* cultures subjected to prolonged ER stress

Treatment	Relative activity (% of control) [a]	
	Nitrogen–sufficient culture	Nitrogen–deprived culture
None	100	100
Tunicamycin + MG 132	57.5 ± 3.8	38.9 ± 6.7

[a] Mycelia grown on a nutrient–rich medium were transferred for 24 h to either nitrogen–deprived or nitrogen–sufficient media. Tunicamycin, an ER–stressor, was added (to a final concentration of 5 μg/ml) in combination with the proteasome inhibitor MG132 (to a final concentration of 40 μM) at the time of transfer of mycelia to the nitrogen–sufficient or nitrogen–deprived media. Controls were set–up with equivalent amounts of the solvent used (DMSO). Laccase activity was assayed with syringaldazine as the chromogenic substrate. The specific activity (per mg of protein) of samples from the control culture (nitrogen–sufficient or nitrogen–deprived) without drugs was taken to be 100%. Data represent means ± SD of three independent experiments performed in duplicate.

Table 2. Effect of *in vivo* proteasome inhibition on intracellular laccase activity of *T. versicolor* under conditions of prolonged ER stress

Treatment	Relative activity (% of control) [a]	
	Nitrogen–sufficient culture	Nitrogen–deprived culture
None	100	100
Tunicamycin + MG 132	32.8 ± 4.6	23.1 ± 5.3

[a] Mycelia grown on a nutrient–rich medium were transferred for 24 h to either nitrogen–deprived or nitrogen–sufficient media. Tunicamycin, an ER–stressor, was added (to a final concentration of 5 μg/ml) in combination with the proteasome inhibitor MG132 (to a final concentration of 40 μM) at the time of transfer of mycelia to the nitrogen–sufficient or nitrogen–deprived media. Controls were set–up with equivalent amounts of the solvent used (DMSO). Laccase activity was assayed with syringaldazine as the chromogenic substrate. The specific activity (per mg of protein) of samples from the control culture (nitrogen–sufficient or nitrogen–deprived) without drugs was taken to be 100%. Data represent means ± SD of three independent experiments performed in duplicate.

rectly folded and processed glycoproteins exit the endoplasmic reticulum. Misfolded ER proteins are removed from the ER lumen by retrograde translocation to the cytosol, where ubiquitin–conjugating enzymes target them for degradation by the 26S proteasome. The ER–associated protein degradation (ERAD; McCracken and Brodsky, 2003) is a component of a coordinated cellular response to ER stress, termed the unfolded protein response (UPR; Schröder and Kaufman, 2005). The UPR is induced in response to accumulation of unfolded and misfolded proteins inside the ER lumen, following exposure to ER stressors such as tunicamycin, dithiothreitol or genetic mutations. The UPR is an intracellular signaling pathway that is responsible for: (1) upregulation of the folding capacity of the ER through increased expression of ER–resident chaperones and (2) down–regulation of a load of misfolded proteins in the ER through reduced protein synthesis on a transcriptional and translational level and enhanced degradation of proteins that cannot be refolded (Schröder and Kaufman, 2005).

The ER–associated degradation of proteins is a very complex process. Upregulation of ERAD components, such as ubiquitination enzymes, appears to be an essential mechanism by which ER stress enhances ERAD (Travers *et al*., 2000). Moreover, it has been recently suggested that the differential regulations of ERAD ubiquitin ligases and their substrates by ER stress may represent a novel mechanism by which ER stress enhances ERAD in mammalian cells (Shen *et al*., 2007).

Functional and genomic analyses of UPR targets engaged in ER–associated protein degradation have revealed an essential coordination between the unfolded protein response and ERAD: efficient ERAD requires an intact UPR, and UPR induction increases ERAD capacity (Travers *et al*., 2000). The UPR is known to activate components of proteasomal degradation and ERAD in its late stages (Schröder and Kaufman, 2005). It has been previously shown that perturbations in the ER caused by the addition of compounds causing the UPR, such as tunicamycin and 2–mercaptoethanol, induced the transcription of the *prs12* gene from the filamentous fungus *Trichoderma reesei* which encodes a homologue of the mouse and Drosophila regulatory subunit 12 of the 26S proteasome (Goller *et al*., 1998). On the other hand, it has been found that in its early stages the UPR leads to a transient inhibition of proteasomal disposal of cytosolic substrates and those targeted to ERAD (Shenkman *et al*., 2007). The transient arrest in ubiquitin/proteasomal degradation caused by inhibition of translation during the early stages of the UPR may be an important mechanism to prevent depletion of essential short–lived proteins while the cell undergoes a temporary block in protein synthesis. The transient arrest in the proteasomal degradation is followed by activation of the degradation in the later stages of the UPR by relief of the protein synthesis block and increased expression of participating genes (Travers *et al*., 2000). Several lines of evidence suggest that the 26S proteasome may participate in earlier steps of ERAD upstream to its final role in proteolysis at the end step of the ubiquitin–dependent proteolytic pathway. The two sub–complexes of the 26S proteasome, the 20S catalytic core and the APTase–containing 19S regulatory particle, were implicated in the dislocation of ERAD substrates (Jarosch *et al*., 2002; Lipson *et al*., 2008).

The UPR is a highly conserved pathway in eukaryotic cells, however there appear to be some differences between mammals and yeast in the way they react to ER stress. In contrast to what was observed in mammalian cells (Shen *et al*., 2007), brief ER stress (1h) induced by tunicamycin completely compromised ERAD in yeast (Travers *et al*., 2000). It has been recently reported that prolonged ER stress (17 h) inhibited ERAD in mammalian cells (Menéndez–Benito *et al*., 2005), suggesting that these cells are better prepared to cope with ER–stress induced accumulation of misfolded proteins. In the present study we observed differences in the response of the wood–decaying fungus *T. versicolor* to short–term ER stress (6 h) and to prolonged ER stress (24 h) induced by tunicamycin.

Proteasome inhibitors have been shown to disrupt the unfolded protein response in myeloma cells (Lee *et al*., 2003). As expected, the authors have found that tunicamycin–mediated inhibition of the N–linked glycosylation resulted in the induction of expression of UPR target genes. Treatment of myeloma cells with the proteasome inhibitor MG132 blocked rather than further augmented the tunicamycin–induced stress response, raising the possibility that proteasome inhibitors might also suppress the UPR. Another study (Liao and Chan, 2001), using HepG2 cells, also demonstrated that the effect of tunicamycin was partly reversed by inhibition of the proteasome. Our data are consistent with these reports.

In conclusion, the present study revealed differences in the response of the ligninolytic fungus *T. versicolor* to short–term ER stress (6 h) and to prolonged ER stress (24 h) induced by tunicamycin treatment. This effect was more pronounced in the nitrogen–deprived cultures. Moreover, the results of our study suggest a potential role of the proteasome–mediated proteolysis as an important mechanism by which laccase is regulated under conditions of prolonged ER stress.

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