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Effect of Inhibition of Proteasome-Mediated Proteolysis on Ligninolytic Activities of White-Rot Fungi

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It has recently been established that most short- and long-lived cellular proteins (80–90%) are degraded by a highly selective non-lysosomal pathway that requires ATP and a large (~2.5 MDa) multisubunit, multicatalytic proteinase complex known as the 26S proteasome. It degrades many important proteins involved in signaling pathway, in cell cycle control, and in general metabolism, including transcription factors and key metabolic enzymes. Here, we demonstrated all distinct proteasome activities: chymotrypsin-like, trypsin-like, and caspase-like (peptidylglutamyl-peptide hydrolyzing) in mycelial extracts of the white-rot fungi *Trametes versicolor* and *Phlebia radiata* by monitoring cleavage of three different fluorogenic peptide substrates: Suc-LLVY-MCA, Z-GGR-MCA, Z-LLE-βNA, respectively. We also found that this cleavage was ATP-dependent. Reagents that inhibit proteasome-mediated protein degradation in intact cells have recently become available, including substrate-related peptide aldehydes. These inhibitors are useful tools to demonstrate that a process exhibits proteasome-dependent biochemical regulation. In the present study, we report that *in vivo* Cbz-LLal treatment strongly inhibited all tested proteasome activities and affected ligninolytic activities in nutrient deprived cultures of both fungi.

INTRODUCTION

For many years proteolysis has been considered to be a nonspecific process mainly involved in basal protein turnover. Our knowledge of intracellular protein degradation advanced considerably during the past two decades. Intracellular proteolysis is the most recently discovered regulatory system of cellular physiology. It is clear now that degradation of cellular proteins is a highly complex, temporally controlled, and tightly regulated process. In eukaryotic cells protein degradation occurs via two pathways: a lysosomal (vacuolar) pathway and a non-lysosomal (non-vacuolar) pathway. The latter includes: proteasomes, calpains and organellar proteases.

It has recently been established that most short- and long-lived cellular proteins (80–90%) are degraded by a highly selective non-lysosomal pathway that requires ATP and a large (~2.5 MDa) multisubunit, multicatalytic proteinase complex known as the 26S proteasome (Coux *et al.*, 1996; Goldberg *et al.*, 1997; Rechsteiner, 1998; Voges *et al.*, 1999; Glickman and Ciechanover, 2002; Pickart *et al.*, 2004). The 26S proteasome consists of a barrel-shaped proteolytically active core (20S proteasome) and one or two 19S regulatory complexes attached to the outer surface of the core particle. These com-

plexes associate together in an ATP-dependent manner. The 20S proteasome is arranged as four axially stacked rings with two central rings of seven distinct β -subunits and two distal rings composed of seven distinct α -type subunits (α_{1-7} β_{1-7} α_{1-7}).

The proteasome's multiple catalytic sites are located within the internal cavity of the β -subunits. These catalytic sites are classified based on their specificity toward short synthetic peptides. Two termed 'chymotrypsin-like' cut preferably after hydrophobic residues and are located on the β_5 subunits. Two 'trypsin-like' sites, located on the β_2 subunits, cleave after basic amino acids. The two remaining sites, located on the β_1 subunits, are referred to as peptidylglutamyl-peptide hydrolyzing ('post-acidic', 'caspase-like') (Kisselev *et al.*, 1999). These names are used to indicate their general similarities to the substrate specificities of 'classical' proteases, though they do not imply any similarity in catalytic mechanisms or physiological functions. Unlike all other known proteases, the proteasome uses nucleophilic N-terminal threonine residues in catalysis (Seemüller *et al.*, 1995).

The 20S proteasome is a unique multicatalytic enzyme whose multiple catalytic sites function only as an integral part of this particle and degrade proteins in a processive fashion without the release of polypeptide intermediates into the cytoplasm. The proteolytic chamber of the 20S proteasome is accessible only to unfolded protein substrates through a narrow channel leading from the surface of the α -rings. The majority of substrates targeted for degradation by the proteasome are marked by covalent attachment of chains containing of at least four molecules of G76–K48 isopeptide-linked ubiquitin (Ub) (Thrower *et al.*, 2000), a small (76 residue) evolutionarily conserved protein found in all

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eukaryotic cells (Pickart, 1998). Ubiquitination involves the sequential action of the Ub activating enzyme (E1), a Ub conjugating enzyme (E2), and a Ub-protein ligase (E3). Ubiquitin and ATP dependence to proteolysis is conferred by the 19S regulatory complex comprising of at least 18 different subunits that include 6 ATP-ases, polyubiquitin binding sites, and isopeptidases. After binding to the proteasome, polyubiquitinated protein conjugates are unfolded, translocated into the proteolytic core, deubiquitinated, and degraded into small peptides.

The presence of the proteasome pathway has been documented in all eukaryotes examined for it, including mammals, plants, fish, parasites, and yeast. During the past decade, the 26S proteasome found both in the cytosol and nucleus, was shown to play vital regulatory functions. It degrades many important proteins involved in signaling pathway, in cell cycle control, and in general metabolism, including transcription factors and key metabolic enzymes.

White-rot fungi are mostly known as excellent degraders of lignin. These microorganisms are able to degrade lignin by producing extracellular enzymes, the best characterized of which are laccase, lignin peroxidases, and manganese peroxidases (Eriksson, 1990). Lignin-modifying enzymes of white-rot fungi are mainly expressed during secondary metabolism (*i.e.* idiophase) in response to environmental triggers such as C- or nitrogen starvation (Keyser *et al.*, 1978; Jeffries *et al.*, 1981). Recent studies revealed that intracellular proteolysis plays an essential role in response to stress conditions such as nutrient deprivation or high temperatures (Hilt and Wolf, 1992). It has been demonstrated for many eukaryotic organisms, including yeasts, that both the lysosomal (vacuolar) and non-lysosomal (non-vacuolar) proteolytic systems are activated by nutrient starvation (Hilt and Wolf, 1992; Cuervo and Dice, 1998).

While lignin degradation by the white-rot fungi long has been studied, very little has yet been done on proteolytic systems of these organisms. Studies on proteases of the white-rot fungi have been confined mainly to extracellular enzymes (Eriksson and Pettersson, 1988; Dosoretz *et al.*, 1990; Dey *et al.*, 1991; Datta, 1992; Dass *et al.*, 1995; Staszczak *et al.*, 1996; Cabaleiro *et al.*, 2002), intracellular proteolysis is less well explored (Staszczak *et al.*, 1996; Lilly *et al.*, 1994; Wadekar *et al.*, 1995). The presence of multiple intracellular and extracellular proteolytic activities in cultures of the white-rot fungi *Trametes versicolor* and *Phlebia radiata* have been previously demonstrated in our laboratory (Staszczak *et al.*, 1996). Native PAGE with copolymerized denatured hemoglobin as a substrate revealed changes in patterns of secreted and mycelial proteases upon carbon or nitrogen starvation. We have subsequently investigated the possibility of proteinases, intracellular and extracellular, being involved in the regulation of ligninolitic activities of *Trametes versicolor* (Staszczak *et al.*, 2000). On the basis of our previous findings on the ability of mycelial extracts of *Trametes versicolor* and *Phlebia radiata* to hydrolyze Suc-Leu-

Leu-Val-Tyr-4-methylcoumaryl-7-amide (SucLLLVY-MCA), a well known substrate used to detect chymotrypsin-like activity of the proteasome (Staszczak, 2002), it was deemed important to study whether all distinct proteasome activities: chymotrypsin-like, trypsin-like, and caspase-like, are simultaneously detectable in these fungi. Here, we demonstrated these activities by monitoring cleavage of three different fluorogenic peptide substrates and found that this cleavage was ATP-dependent. Recently, reagents that inhibit proteasome-mediated protein degradation in intact cells have become available, including substrate-related peptide aldehydes. In the present study, we report that *in vivo* Cbz-LLLaI treatment strongly inhibited all tested proteasome activities and affected ligninolitic activities in nutrient deprived cultures of both fungi.

MATERIALS AND METHODS

Culture condition

Culture conditions were essentially those described previously (Staszczak *et al.*, 2000). Mycelia of the white-rot fungi *Trametes versicolor* (ATCC 44308) and *Phlebia radiata* (ATCC 64658) were maintained, through periodic (every 7 days) inoculation with floating discs of mycelium (5 mm of the diameter) as surface cultures at 26 °C, in scintillation flasks containing 10 ml of nutrient-rich growth medium with glucose (20 g/l) as a carbon (C) source and L-asparagine (19 mM) as a nitrogen(N) source.

After the 7-day cultivation period, mycelia were transferred to flasks containing 2 ml of the fresh growth media deprived of glucose (C-starvation) or L-asparagine (N- starvation), and the incubation at 26 °C continued for 6 hours. The mycelia were washed twice with 5 ml portions of the relevant medium, before being transferred to the media deprived either of glucose or L-asparagine. The trophophasic media (non-starved cultures) in transfer experiments contained glucose and asparagine of the same concentrations as those measured after seven days of fungal growth. The other components were the same as in the nutrient-deprived media; washing was also applied. The concentration of glucose in nitrogen deprived media as well as the concentration of L-asparagine in C-depleted media were the same as corresponding concentrations measured after 7-day cultivation period. Three independent experiments were performed in duplicate.

In vivo MG 132 treatment

The proteasome inhibitor carbobenzoxy-Leu-Leu-leucinal (MG 132) in dimethyl sulfoxide (DMSO) was added to a final concentration of 40 μ M at the time of transfer of mycelia to the nutrient-deprived or trophophasic media. An equivalent amount of solvent used (DMSO) was added to parallel sets of cultures (nutrient-deprived and trophophasic). At 6 hours after exposure to the inhibitor, enzymatic activities were assayed by the methods described below. The results are expressed as a percentage of remaining activity. For each determina-

tion, the specific activity (per mg of protein) of samples from the parallel culture C-deprived, N-deprived and trophophasic) without the inhibitor was taken to be 100%.

Preparation of extracellular culture fluid

Extracellular samples were collected by separating culture fluid from mycelium by filtration. The filtrates were desalted through a Sephadex G-25 column. The elution was performed with 0.001 M Tris, pH 7.0.

Preparation of mycelial extracts

Mycelia were harvested and homogenized in an ice-chilled motor-driven Potter's homogenizer, in 3 ml of 0.05 M Tris-HCl buffer, pH 7.3, containing 0.005 M $MgCl_2$. The homogenates were then centrifuged for 10 min at $10000\times g$, at 4 °C. The supernatants were desalted through a Sephadex G-25 column as described above.

Proteasome assay

Activities of the 26S proteasome were detected in mycelial extracts by monitoring cleavage of three different fluorogenic peptide substrates: Suc-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Suc-LLVY-AMC), Z-Gly-Gly-Arg-7-amido-4-methylcoumarin (Z-GGR-AMC) and Z-Leu-Leu-Glu-2-naphthylamide (Z-LLE-βNA) for chymotrypsin-like, trypsin-like, and caspase-like (peptidylglutamyl-peptide hydrolyzing) activity, respectively. The modified stopped procedure was performed essentially as described previously. Briefly, 100 μl assay mixtures containing 10–25 μl of the mycelial extract (7–14 μg of protein), 100 μM substrate (in DMSO), 100 mM Tris-HCl buffer (pH 8.0), 2 mM ATP, 5 mM $MgCl_2$, were incubated at 37 °C for 30 and 60 min. Reactions were stopped by addition of 100 μl of 10% SDS (w/v) and 2.0 ml of 0.1 M Tris-HCl buffer, pH 9.0. Blanks were prepared without the addition of enzyme. The fluorescent proteolysis products were quantified in a spectrofluorometer (FluoroMax-2, Instruments S.A., Inc., JOBIN YVON/SPEX Division, USA), with an excitation wavelength of 360 nm and an emission wavelength of 440 nm for MCA-substrates or an excitation wavelength of 335 nm and an emission wavelength of 410 nm for NA-substrate.

Determination of laccase activity (EC 1.10.3.2)

Activity of laccase (benzenediol : oxygen oxidoreductase) was determined in desalted samples of culture filtrates (Leonowicz and Grzywnowicz, 1981) using syringaldazine as a substrate. 0.1 M citrate-NaOH buffer, pH 5.0 was used for determinations. Specific activity was calculated with the extinction coefficient of $65,000\text{ M}^{-1}\text{cm}^{-1}$ and expressed in nanokatals per milligram of protein.

Determination of peroxidase activity (EC 1.11.1.7)

Peroxidase activity was assayed in desalted samples of culture filtrates (Claiborne and Fridovich, 1979). 0.003% H_2O_2 , 0.01% *o*-dianisidine, and 0.1 M citrate-NaOH buffer, pH 5.0, were used for determinations.

Specific activity was expressed in nkat per milligram of protein; ϵ used for calculations was $29,300\text{ M}^{-1}\text{cm}^{-1}$.

Determination of protein

Protein concentration was measured according to the method described by Lowry and modified by Schacterle and Pollack (1973). Bovine serum albumin was used as standard.

RESULTS AND DISCUSSION

To date, proteasomes from other than animal or plant cells were studied only in yeast (Chen and Hochstrasser, 1995; Glickman *et al.*, 1998; Fujimuro *et al.*, 1998). Our research is, to our best knowledge, the first attempt to detect multiple proteolytic activities attributed to proteasomes in basidiomycete fungi. We demonstrated all distinct proteasome activities: chymotrypsin-like, trypsin-like, and caspase-like (peptidylglutamyl-peptide hydrolyzing) in mycelial extracts of the white-rot fungi *Trametes versicolor* and *Phlebia radiata* by monitoring cleavage of three different fluorogenic peptide substrates: Suc-LLVY-MCA, Z-GGR-MCA, Z-LLE-βNA, respectively. We also found that the cleavage of substrates used was significantly stimulated by MgATP. Thus, assay mixtures were supplemented with ATP and $MgCl_2$ (for details see Materials and Methods).

The lack of specific cell-permeable inhibitors of the proteasome pathway has long been a major factor limiting an understanding of its function *in vivo*. Substrate-related peptide aldehydes were the first proteasome inhibitors to be developed and are still the most widely used inhibitors (Lee and Goldberg, 1998; Kisselev and Goldberg, 2001). More specific inhibitors of the proteasome, such as lactacystin β-lactone or epoxomicin are too expensive for most *in vivo* experiments.

MG 132 (carbobenzoxy-Leu-Leu-leucinal, also termed Z-Leu-Leu-Leu-al or Cbz-LLL) is the most potent and selective of commercially available aldehydes. It is considered to be the first choice to study proteasome involvement in a process in intact cells (Kisselev and Goldberg, 2001). In the present study, we used MG 132 as the proteasome inhibitor in cultures of *Trametes versicolor* and *Phlebia radiata* and examined its effect on distinct proteasome activities: chymotrypsin-like, trypsin-like, and caspase-like under C- or N- starvation. We also tested whether the exposure of mycelia to this agent can lead to changes in activity of ligninolytic enzymes upon nutrient limitation. To assess its effect on fungal metabolism, MG 132 was added to cultures of the fungi at the time of transfer of mycelia to the nutrient-deprived or trophophasic media.

In this study, all three catalytic activities of the proteasome were inhibited by MG 132 treatment, but to different degrees (Table 1 and Table 3). MG 132 is known to act predominantly on the chymotrypsin-like activity but also has been found to have some, usually weaker, effects on the two other sites: trypsin-like and caspase-like. The three peptidase activities of *T. versicolor* were inhibited in a conditions dependent fashion (Table 1).

Table 1. Effect of *in vivo* proteasome inactivation on proteasome activities in mycelial extracts of *Trametes versicolor*

Culture ¹	Treatment	Relative activity (% of control) ²		
		Chymotrypsin– like (SucLLVY–AMC)	Trypsin– like (Z– GGR–MCA)	Caspase– like (Z– LLE– β NA)
Non– starved	None	100	100	100
	MG 132	65 $\pm$ 7	78.5 $\pm$ 6	81 $\pm$ 11
C– starved	None	100	100	100
	MG 132	31 $\pm$ 8	61 $\pm$ 9	48 $\pm$ 16
N– starved	None	100	100	100
	MG 132	36.5 $\pm$ 6	55 $\pm$ 7.5	60 $\pm$ 5

¹ After seven days of growth on nutrient– rich media mycelia were transferred to media deprived of glucose (C– starvation) or L– asparagine (N– starvation) and to trophophasic media (for details see “Materials and Methods”). Enzymatic activities were assayed after 6 hr of exposure to the proteasome inhibitor, MG 132 (Cbz– LLLal).

² The specific activity (per mg of protein) of samples from the parallel culture (C– deprived, N– deprived, or trophophasic) without the inhibitor was taken to be 100%.

Table 2. Effect of *in vivo* proteasome inactivation on ligninolytic activities in cultures of *Trametes versicolor*

Culture ¹	Treatment	Relative activity (% of control) ²	
		Laccase	Peroxidase
Non– starved	None	100	100
	MG 132	109 $\pm$ 12	106 $\pm$ 16
C– starved	None	100	100
	MG 132	25 $\pm$ 6	30 $\pm$ 11
N– starved	None	100	100
	MG 132	670 $\pm$ 107	428 $\pm$ 130

¹ After seven days of growth on nutrient– rich media mycelia were transferred to media deprived of glucose (C– starvation) or L– asparagine (N– starvation) and to trophophasic media (for details see “Materials and Methods”). Enzymatic activities were assayed after 6 hr of exposure to the proteasome inhibitor, MG 132 (Cbz– LLLal).

² The specific activity (per mg of protein) of samples from the parallel culture (C– deprived, N– deprived, or trophophasic) without the inhibitor was taken to be 100%.

The chymotrypsin–like activity was most strongly inactivated both in starved and non–starved mycelia of this fungus. Mycelia from the non–starved cultures of *T. versicolor* retained 65% of the chymotrypsin–like activity, 78.5% of the trypsin–like activity, and 81% of the caspase–like activity after exposure to 40 μ M MG 132 for 6 hours. More potent inhibition was reported for the nutrient–starved mycelia of *T. versicolor*. The chymotrypsin–like activity was found to be inhibited to 31% and 36.5% in C– and N–starved mycelia, respectively.

The results shown in Table 2 demonstrate that changes in ligninolytic activities of *T. versicolor* occurred as a result of proteasome inactivation. A significant decrease in these activities was observed in C–deprived cultures, with laccase inhibited to 25% of the control activity and peroxidase inhibited to 30% of the control activity. On the other hand, the inhibitor addition under nitrogen limitation resulted in an approximately 7–fold

Table 3. Effect of *in vivo* proteasome inactivation on proteasome activities in mycelial extracts of *Phlebia radiata*

Culture ¹	Treatment	Relative activity (% of control) ²		
		Chymotrypsin– like (SucLLVY– AMC)	Trypsin– like (Z– GGR– MCA)	Caspase– like (Z– LLE– β NA)
Non– starved	None	100	100	100
	MG 132	46 $\pm$ 9	32 $\pm$ 5	78 $\pm$ 7
C– starved	None	100	100	100
	MG 132	67 $\pm$ 6	28 $\pm$ 8	61 $\pm$ 11
N– starved	None	100	100	100
	MG 132	38 $\pm$ 9	39 $\pm$ 7	52 $\pm$ 8

¹ After seven days of growth on nutrient– rich media mycelia were transferred to media deprived of glucose (C– starvation) or L– asparagine (N– starvation) and to trophophasic media (for details see “Materials and Methods”). Enzymatic activities were assayed after 6 hr of exposure to the proteasome inhibitor, MG 132 (Cbz– LLLal).

² The specific activity (per mg of protein) of samples from the parallel culture (C– deprived, N– deprived, or trophophasic) without the inhibitor was taken to be 100%.

Table 4. Effect of *in vivo* proteasome inactivation on ligninolytic activities in cultures of *Phlebia radiata*

Culture ¹	Treatment	Relative activity (% of control) ²	
		Laccase	Peroxidase
Non- starved	None	100	100
	MG 132	123±36	132±20
C- starved	None	100	100
	MG 132	54±17	85±18
N- starved	None	100	100
	MG 132	233±24	222±47

¹ After seven days of growth on nutrient- rich media mycelia were transferred to media deprived of glucose (C- starvation) or L- asparagine (N- starvation) and to trophophasic media (for details see "Materials and Methods"). Enzymatic activities were assayed after 6 hr of exposure to the proteasome inhibitor, MG 132 (Cbz- LLLal).

² The specific activity (per mg of protein) of samples from the parallel culture (C- deprived, N- deprived, or trophophasic) without the inhibitor was taken to be 100%.

and 5-fold increase in laccase and peroxidase activities, respectively. No effect could be detected for non-starved cultures of *T. versicolor* after exposure to the proteasome inhibitor.

Table 3 summarizes the effects of proteasome inactivation on peptidase activities in mycelial extracts of *Phlebia radiata*. All three proteasome activities: chymotrypsin-like, trypsin-like, and caspase-like were inhibited after *in vivo* treatment of the fungus by MG 132. In contrast to *T. versicolor*, most potent inhibition was reported for the trypsin-like activity. From Table 4 it can be seen that MG 132 addition to cultures of *P. radiata* affected activity of extracellular laccase and peroxidase upon nutrient starvation. The effect of proteasome inactivation was less notable than in the case of *T. versicolor*. In C-deprived cultures, laccase and peroxidase activities were inactivated to 50% and 80% of the control activity, respectively. On the other hand, the inhibitor addition to N-starved cultures led to an approximately twofold increase in laccase and peroxidase activities. Treatment of the non-starved mycelia with MG 132 did not appreciably affect activity of these enzymes.

CONCLUSIONS

The present study showed that all distinct proteolytic activities: chymotrypsin-like, trypsin-like, and caspase-like were simultaneously detectable in mycelial extracts of the white-rot fungi *Trametes versicolor* and *Phlebia radiata*. The involvement of the 26S proteasome in the cleavage of fluorogenic substrates used was strongly supported by the fact that degradation of these substrates was MgATP-stimulable. All three proteasome activities were inhibited after *in vivo* treatment of the fungi by MG 132.

Because of the importance of the proteasome pathway in many aspects of cellular function, its inhibition leads to a large number of changes in the cell. Here, we observed an increase in extracellular laccase and peroxi-

dase activities in N-starved cultures as a result of inhibiting proteasome-mediated proteolysis by MG132 treatment. In C-starved cultures, addition of MG 132 decreased laccase and peroxidase activities.

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