Oxidative stress response of *Inonotus obliquus* induced by hydrogen peroxide

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While the medicinal fungus *Inonotus obliquus* produces polyphenols as one of its main metabolites in natural habitats, it accumulates less polyphenols under laboratory conditions. In this study we found that the continuous addition of 1mM H$_2$O$_2$ at a rate of 1.6 ml/h into a submerged culture of the fungus enhanced its production of mycelia, melanins, flavonoids and hispidin analogs (HA). Simultaneous exposure of the fungus to both H$_2$O$_2$ and arbutin resulted in reduced production of mycelia, glycosylated flavonoids (GF) and HA, and inhibition of melanogenesis. However, superoxide dismutases (SOD) and catalase (CAT) activity were enhanced following the addition of H$_2$O$_2$ or H$_2$O$_2$ plus arbutin. The maximum levels of SOD and CAT activities reached 355.2 U/mg protein and 39.8 U/mg protein respectively in H$_2$O$_2$-added medium, and 264 U/mg protein and 35.9 U/mg protein respectively in H$_2$O$_2$ plus arbutin medium. Thus, detoxification of H$_2$O$_2$ is conducted mainly by polyphenols under normal physiological conditions, and by both polyphenols and antioxidant enzymes under oxidative stress when melanogenesis is inhibited. Although enhanced HA production occurred after melanogenesis inactivation, total extracellular polyphenol levels were reduced. These findings suggest that enzymatic activities convert superoxide to H$_2$O$_2$, and non-enzymatic mechanisms are largely responsible for detoxifying H$_2$O$_2$. Enhanced production of melanins is the most important non-enzymatic response of this fungus against oxidative stress.

**Keywords** Oxidative stress response, *Inonotus obliquus*, flavonoids, hispidin analogs, superoxide dismutases, catalase

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Introduction

In its natural habitats the medicinal fungus *Inonotus obliquus* produces melanins and hispidin analogs as its primary polyphenols [1,2] via DOPA [3] and phenylpropanoid pathway [2,4], respectively. These polyphenols possess substantial antioxidant potential and their synthesis is thought to be part of the mechanism used by this fungus to resist environmental stress [5]. Accordingly, the polyphenols have been adopted by humans for use as pharmaceuticals to reduce the incidence of several oxidative stress-induced human diseases [6] including cancer [7], hypertension [8], neurodegenerative (Alzheimer’s and Parkinson’s diseases) [9] and autoimmune diseases [10]. In addition, they have been reported to inhibit inflammation [11], HIV replication [12] and tumor cell proliferation [13], as well as protect human skin from UV irradiation damage [14] and low intensity ionizing radiation [15].

It is known that aerobic organisms use molecular oxygen (O$_2$) as their electron acceptor, and H$_2$O$_2$ is an unavoidable by-product of this process of oxidative phosphorylation for energy production [16]. The mitochondrion is the main site of H$_2$O$_2$ production where continuous production of superoxide caused by electron leakage takes place and incomplete reduction of superoxide is responsible for H$_2$O$_2$ generation [17]. For *I. obliquus* living in very cold habitats (45–50°N latitude), the freezing temperatures and UV irradiation are regular seasonal environmental stresses to which this fungus is exposed [18,19]. Furthermore, invasion of pathogenic microbes also poses a threat [20]. Thus, the strategies for surviving oxidative stress by the fungus
I. obliquus has been used as a folk health remedy in Russia and Eastern Europe for more than four centuries, where its powerful effects on treating several human diseases, in the absence of any unacceptable toxicity are well established [23]. In natural habitats this fungus accumulates polyphenols including hispidin analogs and melanins which possess high pharmacological activity [2,24]. However, its restricted location in cold habitats means this fungus grows very slowly and consequently, naturally occurring I. obliquus is not a reliable source for pharmaceuticals.

Recent attempts to grow this fungus aseptically in a continuously stirring tank reactor (CSTR) resulted in an increased accumulation of melanins in the presence of H\textsubscript{2}O\textsubscript{2}, but hispidin analogs were found to be minor components [3], and immuno-stimulating effects only reached about 50% of those of organism’s mycelia in nature [25]. In addition, it is unclear whether SOD and CAT in I. obliquus are involved in protection against reactive oxygen species (ROS) and how this fungus responds to oxidative stress in terms of polyphenol metabolism and expression of antioxidant enzymes. In an attempt to obtain under laboratory conditions I. obliquus metabolites containing polyphenols similar to those when the fungus is grown in nature we conducted a series of studies involving the growth of the fungus in submerged cultures under oxidative stresses. Batch culture experiments were undertaken in which I. obliquus was grown in a CSTR in media with or without addition of H\textsubscript{2}O\textsubscript{2}. Attempts were made to resolve how this fungus reacts to oxidative stress in terms of accumulation of biomass and polyphenols, and changes in the activities of SOD and CAT. In addition we wish to ascertain the conditions under which the fungus preferably produces polyphenols with potential pharmaceutical importance.

Materials and method

Organism, inoculum preparation and culture conditions

I. obliquus (Fr.) Pilat [KLBMP04005] was collected in Mudanjiang, Changbai Mountain, Northeast China. The voucher specimen, identified by Russian mycologist Prof. Pondertserva M A, was grown on potato dextrose agar (PDA) and preserved in liquid nitrogen in the fungal collection center of Key Laboratory for Biotechnology on Medicinal Plants of Jiangsu Province. To prepare a standard inoculum, the fungus was grown in Petri dishes containing PDA agar and incubated at 26°C for 7 d. This was followed by shake-flask culture for another 7 d using a 500 ml-Erlenmeyer flask containing 200 ml of medium consisting of glucose (2%), peptone (0.3%), yeast extract (0.3%), KH\textsubscript{2}PO\textsubscript{4} (0.01%) and MgSO\textsubscript{4}·7H\textsubscript{2}O (0.05%) (pH 5.5). The mycelium pellets from these cultures were transferred aseptically at a concentration of 1 g/l (dry weight) into a CSTR (Eastbio, Zhengjiang China) consisting of a 10 l vessel containing 7 l of culture medium with a composition identical to that used for the shake-flask cultures as detailed previously [3]. The CSTR was fitted with three 6.5 cm double-bladed Rushton turbines, evenly spaced on the drive shaft, and six baffles. Cultures were aerated at a rate of 0.3vvm and agitated at 200 rpm. Any foaming was detected by a foam sensor and vegetable salad oil was added when needed. A constant pH of 5.5 and temperature of 26°C was used in all experiments. Culture pO\textsubscript{2} and pH were monitored with Ingold polarographic pO\textsubscript{2} and pH probes (Hamilton) respectively. All incubations were repeated in triplicate.

Imposition of oxidative stress on I. obliquus cultures

For imposing oxidative stress to these cultures, H\textsubscript{2}O\textsubscript{2} was pumped continuously into the tank reactor from a 1 mM stock solution at a constant rate of 1.6, 2.4 and 3.2 ml/h, respectively, starting in the early exponential phase (72 h when cell dry weight was = 4.7 g/l). For inhibiting melanin synthesis, arbutin (Sigma) was added at 96 h at a final concentration of 30 mg/l [26] to the medium supplemented with H\textsubscript{2}O\textsubscript{2} at a rate of 1.6 ml/h. Controls consisted of cultures grown in the medium without supplementation and those in the medium supplemented with arbutin at a concentration of 30 mg/l (unless otherwise stated specifically, the control means the culture with no medium supplementation in the following text). Samples (40 ml) were withdrawn from the tank reactor at 24 h intervals up to 336 h, and analyzed as described below.

Analytical methods

Mycelial biomass was estimated by dry weight. Samples (10 ml) were filtered through pre-weighed GC-50 filter papers (1.2 μm, Toyo Roshi Kaisha, Japan). Retained mycelia were washed with 2×10 ml RO water, dried in a microwave oven on the defrost setting for 10 min, and weighed. To prepare mycelial phenolic compounds, samples of mycelia were washed three times with RO water,
and then disrupted by ultrasonication (JY92-2D, Scientz, China) in an ice bath for 5 min at 9 s intervals in 10-vol of 70% aqueous acetone (v/v). Aliquots (1 ml) of the homogenates were filtered through GC-50 filter paper to determine dry weights of homogenized mycelia and the remainder extracted three times with 70% aqueous acetone at room temperature for 24 h followed by centrifugation for 10 min. (4500 g at room temperature). Supernatants were evaporated under N₂ to dryness and reconstituted to a volume (4500 ml) of the extract was reconstituted to 10 ml and stored at –20°C until assayed. For preparation of extracellular phenolic compounds, 20 ml aliquots of cell-free culture filtrate were extracted with ethyl acetate 3 times and the volume of the extract was reconstituted to 10 ml and stored at –20°C until assayed. The content of melanin in culture filtrates was determined as detailed previously [27], and melanin productivity by mycelia was defined as the amount of melanin (gram) produced by one gram of dry mycelia (g/g). In this study, polyphenol analysis was conducted with the controls, cultures to which H₂O₂ was added at a rate of 1.6 ml/h and cultures supplement with H₂O₂ at a rate of 1.6 ml/h and arbutin.

**HPLC analysis of polyphenols**

Phenolic compounds were analyzed by HPLC on a Shimadzu Class-VP HPLC with computer-controlled upgraded Class-VP 5.03 software and a SCL-10A VP System controller. Accessories consisted of a Shimadzu GT-104 degasser, a FCV-10AL mixer, two LC-10AD Shimadzu liquid chromatography pumps, a SIL-10a XL autoinjector, a CTO-10A column oven, and a SPD-M10A VP diode array detector. The composition of phenolic compounds was analyzed with a 5 μm Ultimate ODS 250×4.6 mm reversed phase column. Absorbance data were collected between 220 and 600 nm, and chromatograms monitored at 280 and 310 nm. The temperature of the column oven was 30°C. Mobile phase consisted of acetonitrile (A) and ultra-pure water containing 0.1% H₃PO₄ (B). A linear gradient was programmed from 0 to 25% A over 15 min and maintained at 25% for 10 min, from 25–100% A over 40 min, and then returned to 0% A for 5 min. The flow rate was 1 ml/min. An autoinjector was used to inject 20 μl of test solution or standards into the HPLC system. Identification of phenolic compounds was performed as previously detailed [28].

**Standards**

Flavonoid standards rhoifolin,isorhoifolin, naringin, isorhamnetin-δ-rutinoside, narirutin, eriocitrin, didymin, hesperidin, narirutin-4’-glucoside, naringin-4’-glucoside, neoeocitrin, neohesperidin and poncirin, kaempferol, quercetin, isohamnetin, luteolin, narigenin fortuneletin, apiginin, diosmetin, catechin gallate, epicatechin gallate, epigallocatechin, epigallocatechin gallate, gallocatechin and gallocatechin gallate were purchased from National Institute for the Control of Pharmaceutical and Biological Products, China. Hispidin was purchased from Sigma Chemical Co., St. Louis, MO. Other standards were isolated from Chaga in our laboratory with purities greater than 97%. These included inoscavins A and B, phelligridins D, F, G, which were prepared following the procedures described below.

Well-powdered Chaga (1.0 kg) was extracted five times with 1.5 l of 80% aqueous methanol at room temperature (8 h each). Extracts were combined and concentrated under vacuum to afford 35.73 g extract, which was then partitioned between ethyl acetate and H₂O to give an ethyl acetate soluble fraction (15.70 g) and H₂O soluble fraction (9.87 g). The ethyl acetate soluble fraction was applied to a Sephadex LH-20 column (4 cm×100 cm), and compounds eluted with methanol at a flow rate of 6 drops/min. The resulting elutants were combined and separated by TLC (Silica gel 60 F254; Merck), which revealed four distinct fractions (F₁-₄). Fraction F₁ was applied to a reverse C-18 preparative column (20×250 mm, Inertsil®, JL Sciences Inc., Japan) on HPLC system (Waters 600E) and eluted at a flow rate of 5 ml min⁻¹ with a mobile phase consisting of acetonitrile (A) and RO water containing 0.01% acetic acid (B) in the following gradients: 0–15% A over 10 min and maintained 10 min; from 15–55% A over 30 min, which resulted in the isolation of phelligridin D (18.1 mg), phelligridin F (21.9 mg), phelligridin G (15.9 mg), inoscavin A (27.6 mg) and inoscavin B (32.1 mg). The identification of these compounds was conducted by ¹H-NMR (400 MHz) and ¹³C-NMR (100MHz) Bruker KPX-400 spectrometer in DMSO-d₆. Their purities were determined by HPLC (Shimadzu, Japan) using conditions identical to those used for their preparation as described above.

**Calculation of total glycosylated flavonoids, flavonoid aglycones and hispidin analogs**

Polyphenols involved in this study were GF, flavonoid aglycones (FAG) and HA, expressed in their total amount summed up by the amount of each individual compound belonging to respective groups. The levels of these polyphenols are indicated as mg per one liter culture medium for extracellular and mg per one gram dry biomass for intracellular concentrations. GF identified in this study included rhoifolin, isorhoifolin, naringin, isorhamnetin, rutin, narirutin, FAG epicatechin gallate, epigallocatechin gallate, luteolin, narigenin, fortuneletin, kaempferol, quercetin, isorhamnetin and apigenin, and HA inoscavin B, phelligridin G, davallialactone and phelligridin F.
Assays for antioxidant enzyme activities

Mycelial samples were washed three times with RO water and disrupted by ultrasonication as described above in 10-vol of acetic acid-sodium acetate buffer (50 mM, pH 5.5). Aliquots (5 ml) of each homogenate were centrifuged (4500 g for 10 min), and supernatants assayed for their SOD and CAT activities with assay kits for superoxide dismutases and catalase (Jiancheng, Nanjing China). One unit of SOD was defined as the amount of enzyme required to inhibit the rate of nitroblue tetrazolium chloride (NBT, Sigma, Chemical Co., St. Louis, MO) photoreduction by 50%, and one unit of CAT activity is defined as the amount of enzyme required to convert 1μM H₂O₂ into O₂ and H₂O in one minute. Levels of both SOD and CAT are expressed as the number of Units per milligram of protein [29]. Total protein content of supernatants was measured with Comassie Blue [30].

Statistics

Experiments for submerged cultures and determination of polyphenols were conducted in triplicates. Results from representative experiments are expressed as means ± standard deviation. Data of all experiments were analyzed by T test (SPSS 11.0). The assumptions of analysis of variance were considered to be statistically significant at P<0.05.

Results

Stress-induced response in biomass accumulation

An exponential increase in mycelial biomass production occurred in the absence of H₂O₂ between 24 h and 120 h, which was followed by a slight increase resulting in yields reaching 11.1 g/l by the end of the culture period. Similar incubation profiles were seen when *I. obliquus* was exposed to H₂O₂ delivered at different rates. However, the final biomass yields were higher (14.5 g/l) between 144 h and 336 h in the medium to which H₂O₂ was added at a rate of 1.6 ml/h (*P*=0.001) and exponential growth continued longer (144 h). The mycelia biomass also increased between 216 h and 240 h when H₂O₂ was delivered at a rate of 2.4 ml/h (*P*=0.03). In contrast, biomass production was inhibited after 96 h in the medium when H₂O₂ was introduced at a rate of 3.2 ml/h and only reached 8.31g/l by the end of incubation, remarkably less than that seen in control (*P*=0.002) (Fig. 1a). With addition of both H₂O₂ and arbutin, mycelial exponential growth was prolonged to 240 h when maximum biomass yields of 11.8 g/l were achieved. However, they fell after 264 h to 9.6 g/l by the end of incubation. Addition of arbutin had no effect on biomass production with the incubation profiles nearly identical to that of control (Fig. 2b).

Accumulation of extracellular melanins

The productivity of melanin by *I. obliquus* mycelia, defined as amount (gram) produced by one gram of dry mycelia, were different in media to which only H₂O₂ was added as compare to when both H₂O₂ and arbutin were added. In control medium, melanin productivity fell to 0.25 g/g after 120 h, which was followed by an increase, reaching to nearly 0.37 g/g (Fig. 2a), and a yield of 4.7 g/l (Fig. 2b) by the end of incubation. The addition of H₂O₂ into the medium markedly enhanced melanin productivity compared to that observed in control medium after 120 h (*P*=0.002), and reaching a productivity of about 0.49 g/g (Fig. 2a) and a yield of 7.01 g/l by the end of incubation. Different incubation profiles were observed when arbutin was added to the media: in control medium, melanin productivity was increased to 0.49 g/g after 120 h, reaching a productivity of 0.57 g/g and a yield of 5.6 g/l by the end of incubation. The addition of H₂O₂ into the medium markedly enhanced melanin productivity compared to that observed in control medium after 120 h (*P*=0.002), and reaching a productivity of about 0.49 g/g (Fig. 2a) and a yield of 7.01 g/l by the end of incubation.

![Fig. 1 Profiles of mycelial biomass accumulation of *Inonotus obliquus* grown in the media with different H₂O₂ addition (a) and in H₂O₂-added, H₂O₂-arbutin-added and arbutin-added medium. Results are the mean of three independent experiments. Error bars indicates standard deviation.](image)
incubation (Fig. 2b). As expected, the presence of arbutin in H₂O₂-added medium reduced melanin productivity to no more than 0.08 g/g (Fig. 2a) and a yield of 1.64 g/l by the end of incubation (Fig. 2b).

Accumulation of mycelial flavonoids and hispidin analogs

A total of 15 flavonoids and 4 HAs were identified in *I. obliquus* mycelia grown under different culture conditions. These include GF consisting of rhoifolin (1), isorhoifolin (2), naringin (3), isorhamnetin (4), rutin (5) and narirutin (6), and FAG epicatechin gallate (ECG, 7), epigallocatechin gallate (EGCG, 8), luteolin (9) narigenin (11), fortuneletin (12), kaempferol (13), quercetin (15), isorhamnetin (16), apigenin (18), and HA inoscavin B (10), phelligridin G (14), davallialactone (17) and phelligridin F (19) (Fig. 3a, b and c). These polyphenols were almost all produced in mycelia grown under the three culture conditions, except that compounds 10, 12, 17 and 19 disappeared from the control medium after 312 h (Fig. 3a). In this study, accumulation of these polyphenols at each sampling time point is expressed as the sum of the amount of each of GF, FAG or HA. The accumulation of the three groups of polyphenols differed in mycelia grown under the three culture conditions. Enhanced GF was determined between 120 h and 216 h in H₂O₂-arbutin-supplemented medium (P=0.001) (Fig. 4a), and increased FAG (0.001–0.004) (Fig. 4b) and HA (Fig. 4c) from 120 h to the end of incubation in H₂O₂-supplemented (P=0.02–0.03) and H₂O₂-arbutin-supplemented media (P=0.01–0.04).

Accumulation of exocellular flavonoids and hispidin analogs

The accumulation of GF, FAG and HA were also affected by the supplementation of the media, in that reduced GF was observed both in H₂O₂- and H₂O₂-arbutin-supplemented media (Fig. 5a). There is no evident difference in FAG accumulation from 264 h onwards under the three culture conditions, but marked increase of FAG was detected in H₂O₂-supplemented medium after 288 h (P=0.03) (Fig. 5b). The accumulation of HA suggested a gradual increase and reached its maximum level at 288 h. The addition of H₂O₂ increased HA’s rate of accumulation to 33.5 mg/l at 216 h, after which it fell to lower levels. The simultaneous introduction of H₂O₂ and arbutin reduced HA production to almost zero after 168 h. However, it increased with culture age to 49.8 mg/l at 288 h, but then decreased finally to 33.5 mg/l.

Changes in activities of protective enzyme activities

SOD and CAT activity detected in *I. obliquus* mycelia were both affected by the addition of H₂O₂ and arbutin to the culture medium (Fig. 6). In the control medium, SOD activity increased with incubation time reaching 257 U/mg protein after 96 h, but then fell to 87 U/mg protein at 120 h. A slight increase was then observed after 168 h to an eventual maximum of 355 U/mg protein at 312 h (Fig. 6a). CAT activity increased up to 48 h after inoculation, but then fell to 3.45 U/mg protein at 120 h, after which time a slight increase to 9 U/mg protein was reached by the end of incubation.

The addition of H₂O₂ led to a detectable increase in SOD activity between 144 h and 192 h, although by 216 h activity had decreased to 257 U/mg protein. It then gradually increased to 355 U/mg protein at 312 h and then fell to 322 U/mg protein (P=0.01–0.02) (Fig. 6a). A similar trend in CAT activity was observed following the addition
However, in our study, $H_2O_2$ addition at a rate of 1.6 ml/h to batch cultures of *I. obliquus* resulted in an increased production of mycelial biomass ($P=0.001$), which contradicts other published data [21,32]. This difference in results probably reflects the low concentration of $H_2O_2$ to which the fungal mycelia was exposed, and after interaction with extracellular polyphenols, the remainder might act as a stimulus for mycelial growth in a way similar to oxidative stress responses shown by some bacteria and yeast [33]. In addition, overproduction of mycelial biomass will lower the effective $H_2O_2$ concentration per unit mass of mycelia and thereby the oxidative stress of the mycelia. Biomass increases may also be attributed to the production of melanins and a subsequent efficient detoxification of $H_2O_2$, which would protect the mycelia from oxidative damage [34]. This concept was supported by mycelial growth in $H_2O_2$-arbutin-added medium, where reduced biomass yields coincided with inhibition of melanogenesis by arbutin, and is in agreement with data recently reported in the literature [3]. However, excessive levels of $H_2O_2$ lowered the increase of mycelial biomass (i.e., 2.4 ml/h) or inhibited (i.e., 3.2 ml/h) mycelial growth, suggesting that exposure of the fungal mycelia to higher levels of $H_2O_2$ may also result in hindrance of mycelial growth or even autolysis.

**Discussion**

Data presented here suggest that addition of $H_2O_2$ at a rate of 1.6 ml/h to culture media enhances accumulation of mycelial biomass, melanins, flavonoids and HA in batch cultures of *I. obliquus*. Simultaneous exposure of mycelia to both $H_2O_2$ and arbutin resulted in a reduced accumulation of mycelial biomass, inhibition of melanin synthesis and enhanced production of HA and mycelial GF. Furthermore, SOD and CAT activity were both enhanced by the addition of $H_2O_2$ or $H_2O_2$ and arbutin to the culture medium. Maximum levels of 355.2 U/mg protein for SOD and 39.8 U/mg protein for CAT were reached in $H_2O_2$-added medium, and 264 U/mg protein for SOD and 35.9 U/mg protein for CAT in $H_2O_2$-arbutin-added media.

It is recognized that $H_2O_2$ is one of ROS and is highly damaging to cell constituents including DNA, lipids and proteins [31]. It can also act as a signal transduction molecule able to inhibit spore germination and mycelial growth at higher concentrations [21,32]. However, in our study, $H_2O_2$ addition at a rate of 1.6 ml/h to batch cultures of *I. obliquus* resulted in an increased production of mycelial biomass ($P=0.001$), which contradicts other published data [21,32]. This difference in results probably reflects the low concentration of $H_2O_2$ to which the fungal mycelia was exposed, and after interaction with extracellular polyphenols, the remainder might act as a stimulus for mycelial growth in a way similar to oxidative stress responses shown by some bacteria and yeast [33]. In addition, overproduction of mycelial biomass will lower the effective $H_2O_2$ concentration per unit mass of mycelia and thereby the oxidative stress of the mycelia. Biomass increases may also be attributed to the production of melanins and a subsequent efficient detoxification of $H_2O_2$, which would protect the mycelia from oxidative damage [34]. This concept was supported by mycelial growth in $H_2O_2$-arbutin-added medium, where reduced biomass yields coincided with inhibition of melanogenesis by arbutin, and is in agreement with data recently reported in the literature [3]. However, excessive levels of $H_2O_2$ lowered the increase of mycelial biomass (i.e., 2.4 ml/h) or inhibited (i.e., 3.2 ml/h) mycelial growth, suggesting that exposure of the fungal mycelia to higher levels of $H_2O_2$ may also result in hindrance of mycelial growth or even autolysis.

The results presented here also suggest that mycelial growth of the fungus is not affected by inactivation of melanogenesis under physiological conditions, but is inhibited in the presence of $H_2O_2$. This would seem to
Flavonoids and styrylpyrone derivatives (hispidin analogs) are potential antioxidant polyphenols in several plants and fungi, by acting as ROS scavenger to protect organisms from oxidative damage [36, 37]. In our study, the addition of $\text{H}_2\text{O}_2$ at a rate of 1.6 ml/h at 72 h substantially enhanced mycelial FAG between 96 h and 168 h ($P=0.001$), and exocellular FAG at 312 h ($P=0.001$), reaching a maximum of 8.1 mg/g (Fig. 4b) and 230 mg/l (Fig. 5b), respectively.

Fig. 4 Production profiles of GF (a), FAG (b) and HA (c) in mycelia of Inonotus obliquus grown in the control, $\text{H}_2\text{O}_2$-added and $\text{H}_2\text{O}_2$-arbutin-added medium. Results are the mean of three independent experiments. Error bars indicates standard deviation. GF, glycosylated flavonoids; FAG, flavonoids aglycones; HA, hispidin analogs. $p1$, $\text{H}_2\text{O}_2$-arbutin-added culture versus control between 144 h and 192 h; $p2$ and $p3$, $\text{H}_2\text{O}_2$-added and $\text{H}_2\text{O}_2$-arbutin-added culture versus control respectively between 120 h and 336 h; $p4$ and $p5$, $\text{H}_2\text{O}_2$-added and $\text{H}_2\text{O}_2$-arbutin-added culture versus control respectively between 120 h and 336 h.

Fig. 5 Production profiles of GF (a), FAG (b) and HA (c) produced in medium by Inonotus obliquus grown in the control, $\text{H}_2\text{O}_2$-added and $\text{H}_2\text{O}_2$-arbutin-added medium. Results are the mean of three independent experiments. Error bars indicates standard deviation. Others are detailed in the text. GF, glycosylated flavonoids; FAG, flavonoids aglycones; HA, hispidin analogs. $p1$, control versus $\text{H}_2\text{O}_2$- and $\text{H}_2\text{O}_2$-arbutin-added culture between 144 h and 192 h; $p2$, $\text{H}_2\text{O}_2$-added culture versus control and $\text{H}_2\text{O}_2$-arbutin-added culture between 312 h and 336 h.

Indicate that melanins are part of an important defense response by I. obliquus against oxidative stress, and any inhibition of melanin synthesis renders it more susceptible to attack by free radicals. This is of particular importance during its stationary phase of growth, where mycelia are faced with nutrient limitation leading to autolysis [35].
Oxidative stress response of *Inonotus obliquus*

of polyphenols produced by filamentous fungi including *Gymnopulis* spp., *Cortinarius* spp., *Pholiota* spp. and *Hypholoma* spp., and the basidiomycete fungi *Inonotus* spp. and *Phellinus* spp. [2]. Our data indicate that HA was hardly detectable in control cultures and though oxidative stress resulted in increased HA production, its levels were still markedly less than those of the flavonoids in batch cultures of *I. obliquus*. These results are similar to those found in *Inonotus xeranticus* and *Phellinus linteus* [37]. Our data is also in contrast to those found in the fungus in its natural habitats, where HA are the primary polyphenols and flavonoids are not detected [2,25].

In ascomyceteous fungi, SOD and CAT are both actively involved in the removal of ROS, and their enhanced expression is one of the most important responses to oxidative stress [21]. In this study, oxidative stress imposed in batch cultures of *I. obliquus* also led to enhanced activities of SOD and CAT, supporting their involvement in its defense responses against attack by free radicals. It should be noted that inhibition of melanin synthesis coincided with reduced activities of both SOD and CAT, possibly resulting from the antioxidant activity of arbutin. Its addition would also serve to scavenge free radicals at the concentration used (30 mg/l) [39], and consequently reduce any likelihood of high oxidative stress.

It has been reported that phenolic compounds produced by *I. obliquus* in batch cultures possess high capacities for scavenging free radicals [3]. Thus, its defense systems against oxidative stress would seem to consist of both non-enzymatic and enzymatic based mechanisms. Removal of free radicals is conducted mainly by phenolic compounds, particularly polyphenols under normal physiological conditions, and jointly by phenolic compounds and defense enzymes under oxidative stress. Our data also showed that SOD activity is nearly 9–15 times higher than that of CAT, and thereby the produced H₂O₂ from dismutation of superoxide by SOD would be largely removed by oxidization of polyphenols [40]. Although enhanced production of melanins was achieved after inhibition of melanin synthesis, accumulation of total polyphenols was reduced, which seems to indicate that more HA and flavonoids had been consumed for detoxifying H₂O₂ in the absence of melanins.

Simultaneous addition of H₂O₂ and arbutin resulted in a peak level of GF at 120 h and of HA between 120–168 h, with a yield reaching 12 mg/g, and 5.5 mg/g, respectively. GF is known to possess greater *in vivo* antioxidant activity than the corresponding aglycones, and FAG is more effective at scavenging free radicals *in vitro* [38]. Thus, their enhanced production by this fungus exposed simultaneously to H₂O₂ and arbutin might be an effective method for removing any ROS after melanogenesis is inhibited. Furthermore, suitable oxidative stress imposed in submerged cultures of *I. obliquus* might provide a possible method to improve production of polyphenols of pharmaceutical importance.

In natural habitats with permanent risk of exposure to biotic and abiotic stresses, HA are one of the major groups of polyphenols produced by filamentous fungi including *Gymnopulis* spp., *Cortinarius* spp., *Pholiota* spp. and *Hypholoma* spp., and the basidiomycete fungi *Inonotus* spp. and *Phellinus* spp. [2]. Our data indicate that HA was hardly detectable in control cultures and though oxidative stress resulted in increased HA production, its levels were still markedly less than those of the flavonoids in batch cultures of *I. obliquus*. These results are similar to those found in *Inonotus xeranticus* and *Phellinus linteus* [37]. Our data is also in contrast to those found in the fungus in its natural habitats, where HA are the primary polyphenols and flavonoids are not detected [2,25].
for mycelial growth. Thus, melanins and HA are the polyphenols produced by *I. obliquus* from interaction with environmental factors. Under laboratory conditions, addition of H$_2$O$_2$ also triggered overproduction of melanins, similar to what occurs in nature. However, in laboratory cultures the polyphenols produced by *I. obliquus* were predominantly flavonoids even after the addition of H$_2$O$_2$ to the medium. Though increased production of HA in H$_2$O$_2$-arbutin-added medium was observed, its final yield was still much lower than those in mycelia grown under natural conditions. These results suggest that the oxidative stress responses of *I. obliquus* induced by multiple environmental stimuli differ from those induced by exposure to H$_2$O$_2$ by virtue of the nature of end products of phenylpropanoid metabolism. Under environmental conditions, the precursor phenylalanine (Phe) is streamed predominantly into the synthesis of styrylpyrone derivatives [2], but is used to synthesize flavonoids under laboratory conditions [41].

It should be noted that time-coursed changes of polyphenols in this study are only expressed in terms of total amounts of GF, FAG and HA. This is because flavonoids (FAG and GF) and HA are the end metabolites of *p*-coumaric acid via two different pathways [41], where the former are produced in the presence of chalcone synthase and the latter in the presence of *p*-coumaric acid hydroxylase and hispidin synthase [2]. Although accumulation of each single polyphenol varied in different culture conditions and with respect to culture age, its variation can not reflect the partitioning of *p*-coumaric acid, the intermediate of phenylpropanoid pathway, into the synthesis of flavonoids and HA. Detailed information on accumulation profile of each individual polyphenol is thereby not provided.

This study has examined responses of *I. obliquus* to oxidative stress imposed by H$_2$O$_2$ and its subsequent production of flavonoids and HA and activities of SOD and CAT. Further experiments are needed to determine whether exposure to other factors such as light, NO and oxylipins also affect the accumulation of these polyphenols by this fungus. Regardless, this study does provide some insights into the relationships between oxidative stress and accumulation of polyphenols, as well as the involvement of SOD and CAT, and provides information which may assist in efforts to obtain pharmaceutically important polyphenols by large scale incubation of *I. obliquus*.

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**References**


