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## Methionine oxidation of Carbohydrate-Active enZymes during white-rot wood decay

Lise Molinelli, Elodie Drula, Jean-Charles Gaillard, David Navarro, Jean Armengaud, Jean-Guy Berrin, Thierry Tron, Lionel Tarrago

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19 **Abstract**

20 White-rot fungi employ secreted carbohydrate-active enzymes (CAZymes) along with reactive oxygen  
21 species (ROS), like hydrogen peroxide ( $H_2O_2$ ), to degrade lignocellulose in wood.  $H_2O_2$  serves as a co-  
22 substrate for key oxidoreductases during the initial decay phase. While the degradation of  
23 lignocellulose by CAZymes is well-documented, the impact of ROS on the oxidation of the secreted  
24 proteins remains unclear and the identity of the oxidized proteins is largely unknown. Methionine  
25 (Met) can be oxidized to Met sulfoxide (MetO) or Met sulfone (MetO<sub>2</sub>) with potential deleterious,  
26 antioxidant, or regulatory effects. Other residues, like proline (Pro), can undergo carbonylation. Using  
27 the white-rot model *Pycnoporus cinnabarinus* grown on aspen wood, we analyzed the Met content of  
28 the secreted proteins and their susceptibility to oxidation combining labelled  $H_2^{18}O_2$  with proteomics.  
29 Strikingly, their overall Met content was significantly lower (1.4%) compared to intracellular proteins  
30 (2.1%), a feature conserved in fungi but not in other eukaryotes. We also evidenced that a catalase,  
31 widespread in white-rot fungi, protects the secreted proteins from oxidation. Our redox proteomics  
32 approach allowed identification of 49 oxidizable Met and 40 oxidizable Pro residues within few  
33 secreted proteins, mostly CAZymes. Interestingly, many of them had several oxidized residues localized  
34 in hotspots. Some Met, including those in GH7 cellobiohydrolases, were oxidized up to 47%, with a  
35 substantial percentage of sulfone (13%). These Met are conserved in fungal homologs, suggesting  
36 important functional roles. Our findings reveal that white-rot fungi safeguard their secreted proteins  
37 by minimizing their Met content and by scavenging ROS and pinpoint redox-active residues in  
38 CAZymes.

39

40 **Importance**

41 The study of lignocellulose degradation by fungi is critical for understanding the ecological and  
42 industrial implications of wood decay. While carbohydrate-active enzymes (CAZymes) play a well-  
43 established role in lignocellulose degradation, the impact of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) on secreted  
44 proteins remains unclear. This study aims at evaluating the effect of H<sub>2</sub>O<sub>2</sub> on secreted proteins,  
45 focusing on the oxidation of methionine (Met). Using the model white-rot model *Pycnoporus*  
46 *cinnabarinus* grown on aspen wood, we showed that fungi protect their secreted proteins from  
47 oxidation by reducing their Met content and utilizing a secreted catalase to scavenge exogenous H<sub>2</sub>O<sub>2</sub>.  
48 The research identified key oxidizable Met within secreted CAZymes. Importantly, some Met like those  
49 of GH7 cellobiohydrolases, undergone substantial oxidation levels suggesting important roles in  
50 lignocellulose degradation. These findings highlight the adaptive mechanisms employed by white-rot  
51 fungi to safeguard their secreted proteins during wood decay and emphasize the importance of these  
52 processes in lignocellulose breakdown.

53

## 54 **Introduction**

55 Filamentous fungi are crucial in the carbon cycle because of their ability to decompose organic  
56 matter and release carbon back into the ecosystem (1). Wood-decay fungi extract carbon from  
57 recalcitrant polymers like cellulose and hemicelluloses embedded in a lignin matrix, by secreting  
58 enzymes into the extracellular environment (2). The secreted proteins, commonly referred to as the  
59 "secretome", contains the carbohydrate-active enzymes (CAZymes), including large sets of glycoside  
60 hydrolases (GH) and auxiliary activity (AA) enzymes (3, 4). The GH class contains the endo- and exo-  
61 acting enzymes targeting cellulose and hemicelluloses. The AA class contains laccases (AA1) and class  
62 II peroxidases (AA2) involved in lignin degradation, and lytic polysaccharide monooxygenases (LPMO,  
63 AA9) proposed to play an important role in the early phase of wood degradation (5–7). Reactive oxygen  
64 species (ROS), particularly hydrogen peroxide ( $H_2O_2$ ), play a crucial role in the early phase of wood  
65 decay (8–10).  $H_2O_2$  is generated by glucose-methanol-choline oxidoreductases (AA3) and copper  
66 radical oxidases (AA5), that target carbohydrate or aliphatic/aromatic alcohols (11, 12). In white-rot  
67 fungi,  $H_2O_2$  acts as a co-substrate to fuels different types of LPMOs and AA2 peroxidases (13–15). While  
68 the roles of CAZymes and  $H_2O_2$  during the degradation of lignocellulose is well-documented, the impact  
69 of ROS on the oxidation of CAZymes remains unclear. Fenton-generated ROS were shown to decrease  
70 enzymatic activity of some GHs in white-rot secretomes, through the oxidation of the secreted  
71 proteins, and the most abundant form of oxidation found was the oxidation of Met residues (16). Met  
72 can be converted by  $H_2O_2$  into Met sulfoxide (MetO) or Met sulfone (MetO<sub>2</sub>) and this has been shown  
73 to play critical roles in many organisms (17–20). For instance, Met oxidation can lead either to a loss  
74 of function or act as a regulatory switch, to activate enzymes (21, 22). The presence of oxidizable Met  
75 in proteins can also be associated to an antioxidant role by serving as a protection to avoid the  
76 oxidation of residues critical for the protein function (23). MetO can be reduced back to Met by the  
77 methionine sulfoxide reductases, but, as these enzymes are most likely not secreted they cannot  
78 reduce oxidized Met of secreted proteins (24). Regarding the importance of  $H_2O_2$  during wood decay,  
79 our hypothesis is that some Met of secreted proteins can be oxidized and play antioxidant or regulatory

80 roles. Interestingly, the treatment of an  $\alpha$ -galactosidase (GH27) from *Trichoderma reesei* with H<sub>2</sub>O<sub>2</sub>  
81 strongly increased its activity due to the oxidation of a Met located near the active site, suggesting that  
82 Met oxidation of CAZymes can have a positive effect on their activity (25). To identify secreted proteins  
83 with oxidizable Met, we selected the white-rot *Pycnoporus cinnabarinus* grown on aspen wood as a  
84 model. *P. cinnabarinus* secretes H<sub>2</sub>O<sub>2</sub>-producing enzymes in the early phase of wood degradation, to  
85 provide co-substrate for the H<sub>2</sub>O<sub>2</sub>-consuming CAZymes (26), but also as potential oxidant of Met. We  
86 characterized the secreted proteins and investigated the sensitivity of their Met to oxidation by using  
87 a redox proteomics approach based on the use of <sup>18</sup>O-labeled hydrogen peroxide (H<sub>2</sub><sup>18</sup>O<sub>2</sub>) (27–30). Our  
88 investigations reveal the extend of Met oxidation in fungal secretomes and the precise localization of  
89 oxidizable residues in secreted proteins and CAZymes, suggesting potential antioxidant or regulatory  
90 roles.  
91

92 **Material and methods**

93 ***Culture of P. cinnabarinus on aspen wood.***

94 The dikaryotic strain *Pycnoporus cinnabarinus* CIRM-BRFM 145 was grown for 3, 5 or 7 days at  
95 as described (26). The media contained maltose (2.5 g.l<sup>-1</sup>) and *Populus tremuloides* aspen sawdust  
96 particles of size < 2 mm (15 g. l<sup>-1</sup>). Six replicates were mixed in pairs to obtain three replicate samples  
97 per time-point.

98 ***Preparation of P. cinnabarinus secretomes and protein oxidation with H<sub>2</sub><sup>18</sup>O<sub>2</sub>.***

99 The culture supernatants containing the secreted proteins were centrifugated (1 h, 30,000xg,  
100 10 °C) and sterilized by filtration (0.22 µm; Express Plus; Merck Millipore). The supernatants were  
101 equilibrated to pH 7 with 0.5 mM NaOH and the proteins were purified using two ion exchange  
102 chromatographies (**Fig. S1**): first, an anion exchange on DEAE Sephadex A-25 (Cytiva) and a cation  
103 exchange on CM Sephadex A-25 (Cytiva) for proteins not retained on the anion exchanger. Both resins  
104 were initially equilibrated with 20 mM sodium phosphate buffer (pH 7). Elution of the secreted  
105 proteins was performed with 1 M NaCl. Fractions from both anion and cation exchangers were pooled,  
106 desalted on Sephadex G-25 (PD-10 Desalting columns, Cytiva) in 50 mM sodium acetate (pH 5.2) and  
107 concentrated using Vivaspin polyethersulfone membrane (3 kDa cut-off, Sartorius). Protein  
108 concentrations were determined using the Bradford method (Bio-Rad) and the secretomes were used  
109 immediately. Secreted proteins (50 µg) were incubated 30 min with 1 mM hydroxylamine, desalted on  
110 Sephadex G-25 (PD SpinTrap, Cytiva), then incubated with 100 mM of H<sub>2</sub><sup>18</sup>O<sub>2</sub> for 1 h in the dark, then  
111 desalted. Protein concentrations were determined, and the samples were stored at -20°C.

112 ***Proteomics analysis.***

113 Proteins (15 µg) were precipitated trichloroacetic acid and processed for trypsin proteolysis as  
114 described (31). Peptides (5 µl) were injected into a nanoscale Easy-Spray™ PepMap™ Neo 2 µm C18  
115 (75 µm x 750 mm) capillary column (Thermo Scientific) operated with a Vanquish Neo UHPLC system  
116 (Thermo Scientific) and resolved with a 65-min gradient of CH<sub>3</sub>CN (5-40%), 0.1% formic acid, at a flow

117 rate of 0.25  $\mu$ L per min. Data-dependent acquisition analysis of the peptides was performed with an  
118 Orbitrap Exploris 480 tandem mass spectrometer (Thermo) as described (32). Each full scan of peptide  
119 ions in the high-field Orbitrap analyzer was acquired from m/z 350 to 1800 at a resolution of 120,000,  
120 selecting only 2+ and 3+ charged precursors for high-energy collisional dissociation and with a dynamic  
121 exclusion of 10 seconds. MS/MS spectra of fragmented precursors were acquired at a resolution of  
122 15,000 and assigned to peptide sequences by the MASCOT Server 2.5.1 search engine (Matrix Science)  
123 using the *P. cinnabarinus* annotated proteome, with fixed and variable modifications (**Datasets 3; 4**).  
124 Proteins were validated when at least two different peptides (p-value <0.01) were detected, resulting  
125 in a protein identification false discovery rate below 1%. Protein abundance was estimated using the  
126 Normalized Spectral Abundance Factor (NSAF) (33). The Met content of the proteins was calculated  
127 using the entire sequence. The Met content corrected by the NSAF was calculated using the following  
128 formula:

$$129 \frac{\sum(\%Met_{protein\ n} \times NSAF_{protein\ n})}{\sum NSAF_{protein\ n}}$$

130 With  $\%Met_{protein\ n}$  and  $NSAF_{protein\ n}$  corresponding to the percentage of Met and the NSAF value of the  
131 individual proteins identified, respectively. The percentage of modification of Met or Pro residues was  
132 calculated by dividing the number of spectral counts (SC) of the residue with the modification by the  
133 total number of SC for the residue and then multiplying the result by 100. The mass spectrometry  
134 proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (34) partner  
135 repository with the dataset identifier PXD046271 and 10.6019/PXD046271.

136

### 137 ***H<sub>2</sub>O<sub>2</sub>-consuming activity***

138 Secreted proteins (20  $\mu$ g) were incubated 1 h with H<sub>2</sub>O<sub>2</sub> (30 mM) in sodium acetate buffer, pH  
139 5.2. A control without proteins was done. Then, the samples were filtered using Vivaspin 3 kDa cut-off



140 and the remaining H<sub>2</sub>O<sub>2</sub> in filtrates was titrated by absorbance measurement at 240 nm and compared  
141 to H<sub>2</sub>O<sub>2</sub> concentration in controls.

142

### 143 ***Analysis of Met content in P. cinnabarinus, fungi, metazoans, and plants proteins.***

144 *P. cinnabarinus* proteins were predicted secreted if they fulfilled three conditions: (i) presence  
145 of a secretion signal peptide, (ii) absence of endoplasmic reticulum retention motif and (iii) absence of  
146 transmembrane helix outside the signal peptide, as described (26). Experimentally validated fungal  
147 secreted proteins were retrieved from proteomics analyses (7, 35–49), from Uniprot  
148 ([www.uniprot.org](http://www.uniprot.org)) and FunSecKB2 (50). Metazoans experimentally validated secreted were retrieved  
149 from Uniprot, MetazSecKB (51) and the Human Protein Atlas (52, 53). Experimentally validated plant  
150 secreted proteins were retrieved from Uniprot, PlantSecKB (54) and the SUBA database (55). The  
151 datasets of fungal, metazoans and plants non-secreted proteins were made using proteins retrieved  
152 from Uniprot, the Human Protein Atlas and the SUBA database. For all datasets, only manually curated  
153 proteins, and those found with least 5 SC in proteomics, were conserved. Membrane proteins, proteins  
154 shorter than 50 amino acids, or not starting with Met were discarded. Statistical analyses were  
155 unpaired t-test with Welch's correction.

156

### 157 ***Conservation of proteins and oxidized residues.***

158 A hundred and twenty fungal genomes were selected based on their public availability and  
159 their representativity in the 9 fungal phyla (56, 57). Targets of the oxidized proteins were searched  
160 using BLASTP (v 2.6.0+) (58). The 100 sequences with the highest e-values were aligned using MAFFT  
161 tool (version 6.864b) (59). The conservation of individual residues was made by counting its occurrence  
162 in all the sequences.

163

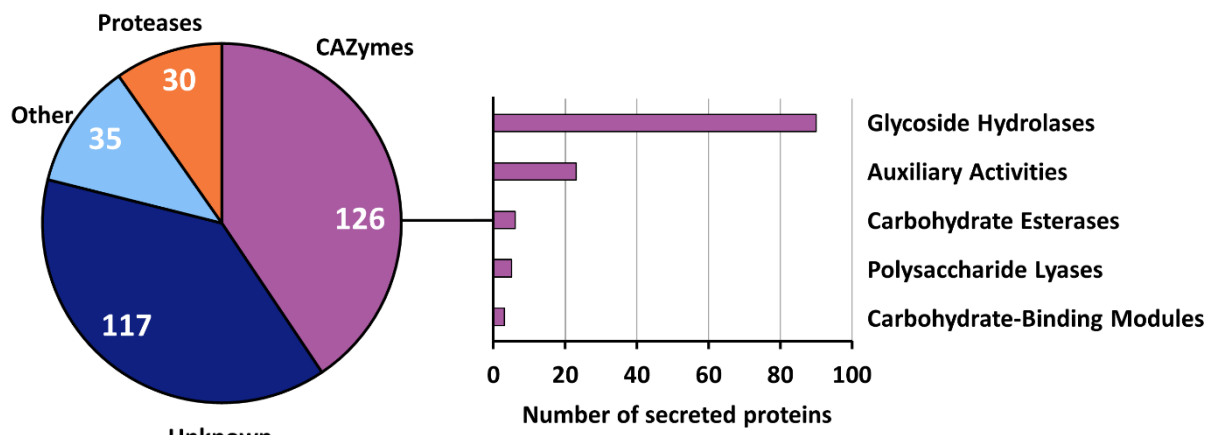
## 164 Results

### 165 ***Optimized methods allowed to obtain secreted proteins representative of early days decay.***

166 To identify oxidizable Met in the secreted proteins of *P. cinnabarinus*, we focused on the early  
167 days of wood decay, during which production and consumption of H<sub>2</sub>O<sub>2</sub> are likely to occur (42). We  
168 performed a time-course growth of the fungus on aspen wood and purified the proteins from the  
169 culture supernatants at days 3, 5 and 7 on successive anion and cation exchange chromatography to  
170 avoid artifactual oxidation. Then, we oxidized the secreted proteins with H<sub>2</sub><sup>18</sup>O<sub>2</sub> and identified them  
171 and their potential oxidative Met modifications using proteomics (**Fig. S1**). We identified 308 secreted  
172 proteins, among which CAZymes and proteases represented 41% and 10%, respectively (**Fig. 1; Table**  
173 **S1; Dataset 1**). Among the CAZymes, GH was by far the most represented class, with 89 proteins (71%)  
174 spread over 34 families (**Fig. 1; Table S1**). We identified most of the CAZymes expected during wood  
175 degradation, i.e. cellulases (GH3, GH5\_5, GH5\_9 and GH7, GH12, GH45), hemicellulases (GH10, GH43,  
176 GH51, CE15, CE16) (4), as well as pectinases (GH28, CE8) and also some CAZymes targeting fungal cell  
177 wall components (e.g. GH18 chitinases) (**Table S1**). Out of 23 identified AAs (18%), we found laccases  
178 (AA1) and class II peroxidases (AA2), LPMOs (AA9) and H<sub>2</sub>O<sub>2</sub>-generating enzymes (AA3, AA5). We  
179 estimated the abundance of the individual proteins using the Normalized Spectral Abundance Factor  
180 (NSAF) (**Dataset 1**). The mean %NSAF of all identified proteins was ~ 0.3% and 52 proteins were above  
181 this value. The most abundant protein categories were CAZymes (52%), proteins of unknown function  
182 (35%) and proteases (8%). Among the most abundant CAZymes, we found the three cellobiohydrolases  
183 (GH7), three chitinases (GH18) and two laccases (AA1). Interestingly, three H<sub>2</sub>O<sub>2</sub>-generating enzymes  
184 (two AA3\_2 and one AA5\_1) were among these most abundant CAZymes (**Dataset 1**). Overall, the  
185 identity of the secreted proteins and their abundance validated our approach, indicating that the  
186 growth conditions, the proteins preparation method, and the oxidative treatment used were suitable  
187 for studying Met sensitivity to oxidation.

188

189



190

191

192 **Figure 1. Distribution of the identified secreted proteins and CAZymes.** Values shown are the  
193 aggregate of all replicates for each day (Table S1; Dataset 1). Bar graphs represent the number of  
194 CAZymes per class.

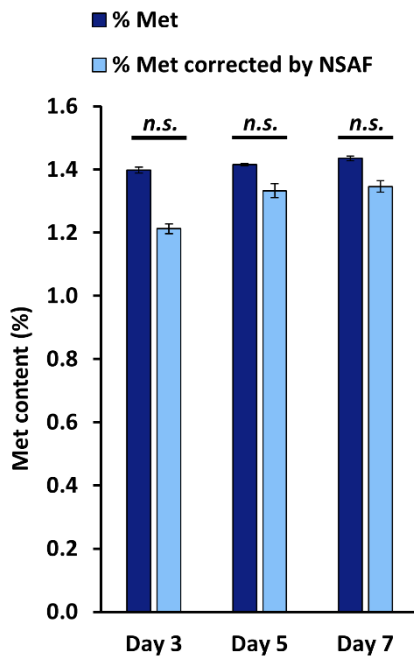
195

196 ***Met content is strongly reduced in fungal secreted proteins.***

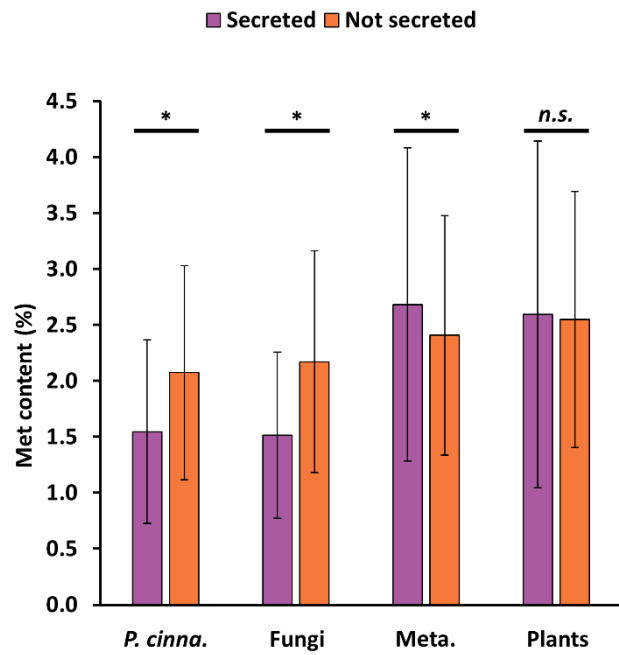
197 First, we evaluated the Met content of the secreted proteins, which was ~1.4% for each time-  
198 point (**Fig. 2A**). Then, to determine whether some abundant proteins with specific Met content could  
199 influence the overall content, we corrected the values by the proteins NSAF. The corrected Met  
200 contents were  $1.21 \pm 0.02$ ,  $1.33 \pm 0.02$ , and  $1.35 \pm 0.02$ , for days 3, 5, and 7, respectively (**Fig. 2A**).  
201 These results indicate that the Met content in the proteins secreted by *P. cinnabarinus* is comprised  
202 between 1.2 to 1.4%. Strikingly, this value is twice lower than the Met content of proteins in general,  
203 i.e. 2.4% (60). We investigated whether this characteristic may apply to all secreted proteins of *P.*  
204 *cinnabarinus*. The theoretical *P. cinnabarinus* secretome consists of 666 proteins with an average Met  
205 content of  $1.55 \pm 0.82\%$ , while the fungus non-secreted proteins have a higher mean of  $2.07 \pm 0.96\%$   
206 Met. (**Fig. 2B; Table S2; Dataset 2**). These results show that *P. cinnabarinus* secreted proteins contain  
207 significantly less Met than the non-secreted. Then, we compared the Met content of experimentally  
208 validated secreted proteins from fungi, metazoans, and plants with the Met content of intracellular  
209 proteins (**Fig. 2B; Table S2; Dataset 2**). Fungal secreted proteins have a mean Met content of  $1.52 \pm$   
210  $0.74\%$ , significantly lower than that of non-secreted proteins ( $2.17 \pm 0.99\%$ ). In contrast, the Met  
211 content of metazoans secreted proteins ( $2.68 \pm 1.40\%$ ) is significantly higher than that of the non-  
212 secreted proteins ( $2.41 \pm 1.07\%$ ). No statistical difference exists between the Met content in secreted  
213 ( $2.59 \pm 1.55\%$ ) and non-secreted ( $2.55 \pm 1.14\%$ ) proteins of plants (**Fig. 2B; Table S2; Dataset 2**).  
214 Altogether, these results show that the secreted proteins of fungi contain less Met than the  
215 intracellular proteins, and that this feature is not conserved in other eukaryotes.

216

A



B



217

218 **Figure 2. Met content of *P. cinnabarinus* secreted proteins and comparison with fungal, metazoan,**  
 219 **and plant secreted and intracellular proteins. A)** Met content of the identified *P. cinnabarinus*  
 220 secreted proteins calculated for each protein and averaged for the three secretomes at each time  
 221 point. Alternatively, the Met content for each protein was normalized by the protein NASF and  
 222 averaged. Values shown are the mean of the 3 replicates  $\pm$  SD. **B)** Met content of intracellular proteins  
 223 and of secreted in the *P. cinnabarinus* theoretical proteome versus the Met content of experimentally  
 224 validated intracellular and secreted proteins from fungi, metazoans, and plants (**Table S2; Dataset 2**).  
 225 Values shown are the mean of all proteins in each set  $\pm$  SD. Statistical analysis performed by unpaired  
 226 t-test with Welch's correction (\* $P \leq 0.001$ ; *n.s.*, not significant).

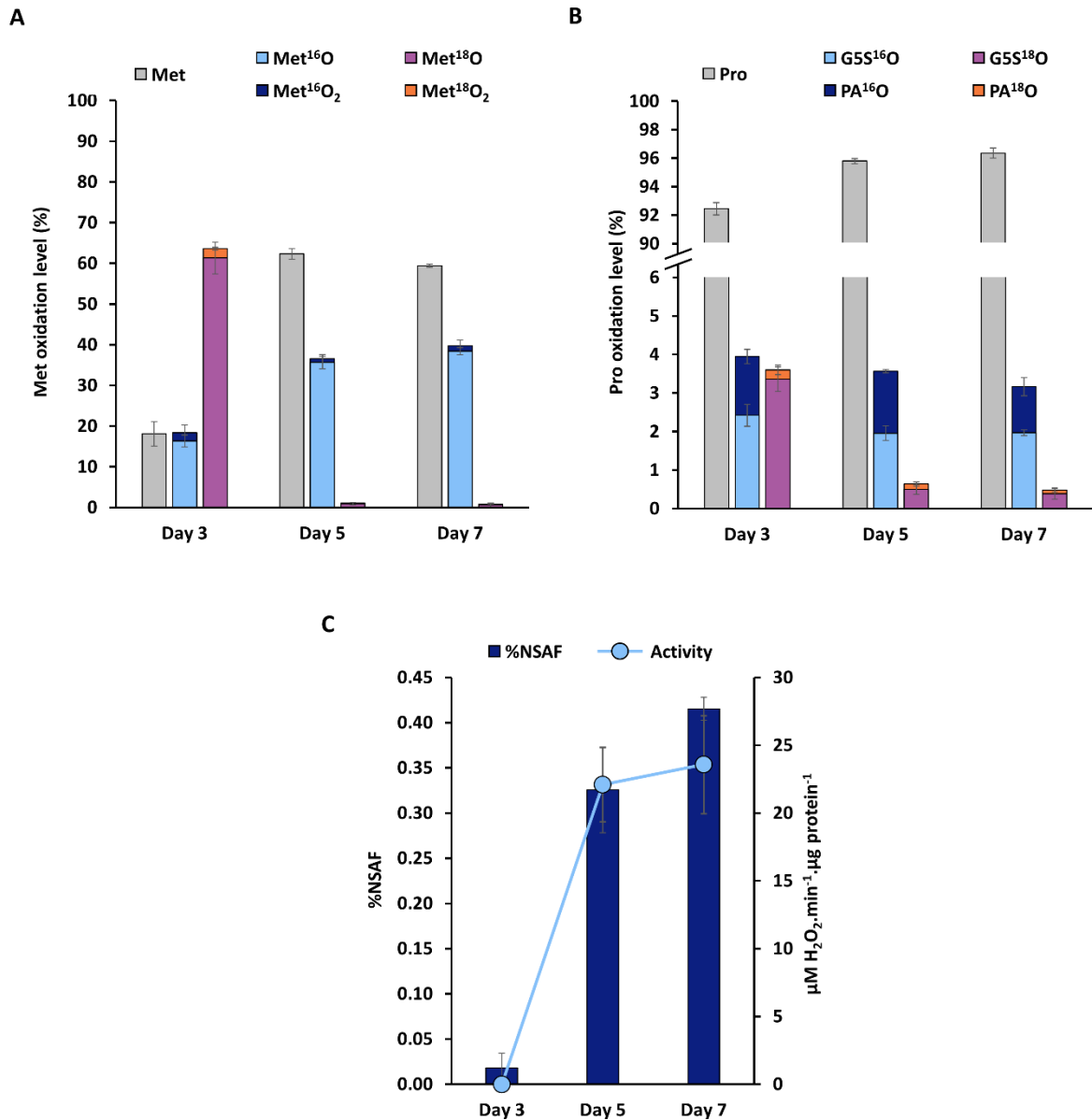
227

228 ***A catalase protects secreted proteins from oxidation.***

229           The identification of oxidizable Met in *P. cinnabarinus* secreted proteins required accurate  
230 quantification of their oxidation levels. H<sub>2</sub><sup>18</sup>O<sub>2</sub> was used to prevent artifactual Met oxidation potentially  
231 occurring during sample preparation by blocking the Met reduced in the samples and converting them  
232 to Met sulfoxide or sulfone containing <sup>18</sup>O atom (Met<sup>18</sup>O and Met<sup>18</sup>O<sub>2</sub>, respectively). The use of a  
233 concentrated H<sub>2</sub><sup>18</sup>O<sub>2</sub> solution (100 mM) should allow the oxidation of all the reduced Met and the  
234 reliable quantification of the initial levels of Met<sup>16</sup>O and Met<sup>16</sup>O<sub>2</sub> (28, 29). For the day 3 secretome, the  
235 levels of Met carrying <sup>18</sup>O or <sup>16</sup>O, either as sulfoxide or sulfone, were ~64% and ~18%, respectively.  
236 Although, ~18% of Met in reduced form were still present, this indicated that most Met from the  
237 identified proteins were oxidized after the treatment with H<sub>2</sub><sup>18</sup>O<sub>2</sub>. Of note, <sup>16</sup>O or <sup>18</sup>O sulfone levels  
238 reached ~2% (**Fig. 3A; Table S3; Dataset 3**). On the contrary, in day 5 and day 7 secretomes, the levels  
239 of MetO and MetO<sub>2</sub> carrying <sup>18</sup>O were extremely low (~1%), indicating that the blocking strategy failed  
240 for these samples (**Fig. 3A; Table S3; Dataset 3**). To evaluate whether this lack of oxidation by H<sub>2</sub><sup>18</sup>O<sub>2</sub>  
241 in secretomes of days 5 and 7 affected only Met residues, we reanalyzed our data by searching for two  
242 main forms of Pro carbonylation bearing <sup>18</sup>O. For the day 3 secretome, we found slightly less than 4%  
243 of Pro oxidized with <sup>18</sup>O. A similar content of <sup>16</sup>O was found, indicating that the large majority of Pro  
244 were not oxidized (~92%) (**Fig. 2B; Table S4; Dataset 4**). This low level of Pro oxidation was anticipated  
245 due to its low reactivity with H<sub>2</sub><sup>18</sup>O<sub>2</sub> (61). The percentage of <sup>18</sup>O oxidation decreased to ~0.5% in the  
246 day 5 and day 7 secretomes, while <sup>16</sup>O oxidation levels remained comparable to those observed for  
247 the day 3 secretome. Thus, the trend for Pro is the same as for Met indicating that the lack of oxidation  
248 observed for Met was not specific to this residue. Proteins secreted during the days 5 and 7 of decay  
249 were not oxidized by exogenous H<sub>2</sub><sup>18</sup>O<sub>2</sub>, unlike those secreted at day 3. These results prompted us to  
250 search for secreted enzymes that scavenge H<sub>2</sub>O<sub>2</sub>. A catalase stood out: almost absent at day 3 (0.02 ±  
251 0.02 %NSAF), but more abundant at days 5 and 7, with %NSAF values of 0.33 ± 0.05 and 0.42 ± 0.01,  
252 respectively (**Fig. 2C; Dataset 1**). When we measured the H<sub>2</sub>O<sub>2</sub>-consuming activity of the secretomes,  
253 we observed no activity at day 3, but strong activities at days 5 and 7 secretomes, in a perfect

254 consistency with the catalase abundance (**Fig. 2C**). Although few potential peroxidases and LPMOs  
255 were present, there was no difference in their abundance during the time course (**Dataset 1**). These  
256 results indicated that this catalase was very likely responsible for most, if not all, of the H<sub>2</sub>O<sub>2</sub>-consuming  
257 activity. Altogether, these results show that, under our experimental conditions, *P. cinnabarinus*  
258 secreted a catalase at days 5 and 7, which, by consuming potential exogenous H<sub>2</sub>O<sub>2</sub>, protected the  
259 secreted proteins from oxidation. On the contrary, at day 3, the secreted proteins are left without any  
260 protection against exogenous oxidant. On the other hand, this highlight that the strategy consisting of  
261 using high concentrations of H<sub>2</sub><sup>18</sup>O<sub>2</sub> to block reduced Met can hardly be applied to the secretomes  
262 collected at days 5 and 7, at least in conditions where hydroxylamine was not an efficient inhibitor of  
263 H<sub>2</sub>O<sub>2</sub>-consuming activity. Nonetheless, since Met oxidized with <sup>18</sup>O were detected, we analyzed the  
264 nature of the oxidized proteins to identify Met residues with potential antioxidant or regulatory roles.

265



266

267 **Figure 3. Levels of Met and Pro oxidation in the *P. cinnabarinus* secretomes and H<sub>2</sub>O<sub>2</sub>-consuming**  
 268 **activity. A)** Total levels of Met oxidation as sulfoxide and sulfone, either <sup>16</sup>O or <sup>18</sup>O calculated using the  
 269 spectral counts of all Met-containing peptides and compared to the level of non-oxidized Met for each  
 270 time point (**Table S3; Dataset 3**). **B)** Total levels of Pro oxidation as glutamic 5-semialdehyde (G5S) and  
 271 pyroglutamic acid (PA), either <sup>16</sup>O or <sup>18</sup>O calculated using the spectral counts of all Pro-containing  
 272 peptides and compared to the level of non-oxidized Pro for each day (**Table S4; Dataset 4**). **C)** %NSAF  
 273 of the catalase A0A060STN2 (**Dataset 2**) and total H<sub>2</sub>O<sub>2</sub>-consuming activity at each time point.

274

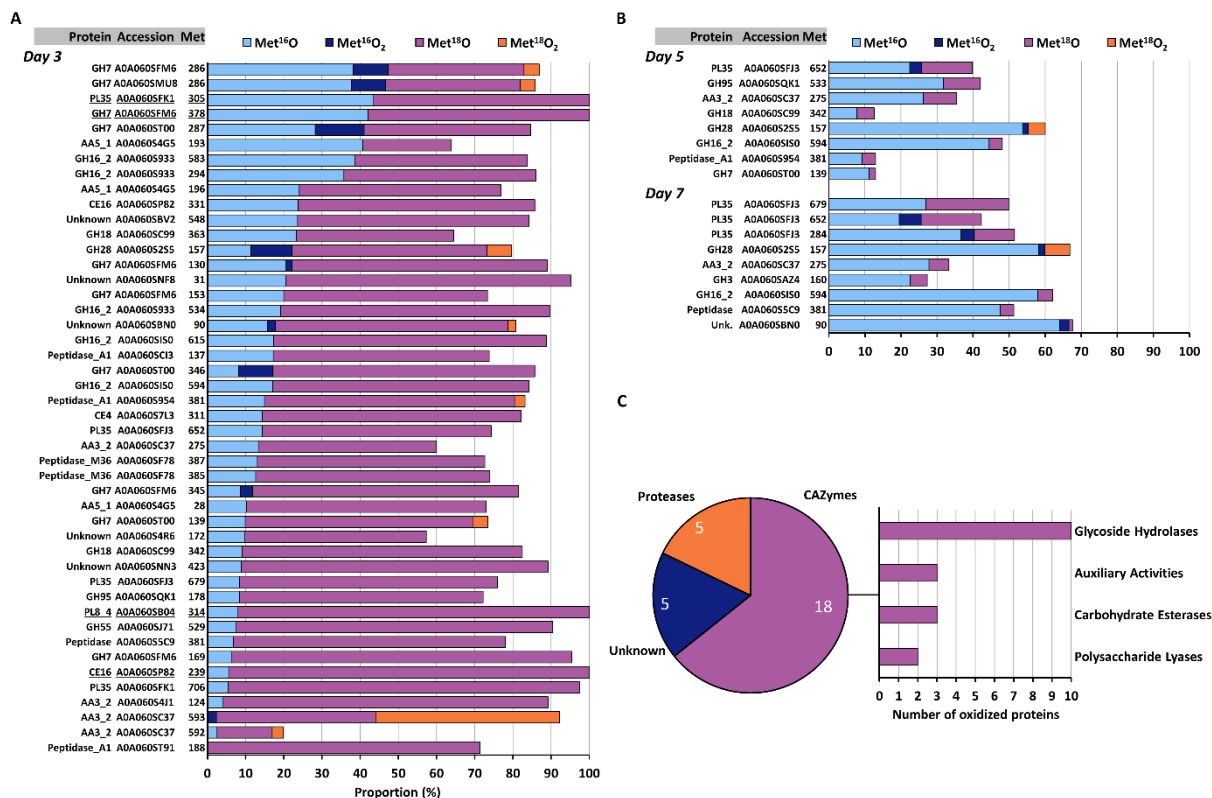


275 ***Most of the oxidations occur on a few secreted CAZymes.***

276           Among the 308 secreted proteins identified, we detected 265 Met belonging to 118 proteins  
277 **(Table S3; Dataset 3)**. We selected only the Met-containing peptides present in all replicates for each  
278 secretome, with at least five spectral counts, in at least two out of the three replicates. At day 3, all  
279 selected Met were found oxidized with  $^{18}\text{O}$  **(Fig. 4A; Dataset 3)**. The total oxidation ( $^{16}\text{O}$  and  $^{18}\text{O}$ ) ranged  
280 from ~20% to 100% for the 46 identified Met **(Fig. 4A)**. Four Met, all from CAZymes, were fully oxidized  
281 and thus saturated with  $^{18}\text{O}$ : Met305 of a PL35, Met378 of a GH7, Met314 of a PL8, and Met239 of a  
282 CE16 **(Fig. 4A; Dataset 3)**. Twenty-four proteins had oxidation levels ranging from ~81% to 97% and  
283 17 from 57% to ~80%. Only the Met592 from an AA3\_2 displayed a much lower value of total oxidation  
284 (~20%) **(Fig. 4A; Dataset 3)**. The quantification of the  $^{16}\text{O}$ -oxidation levels appeared clear for the four  
285  $^{18}\text{O}$ -saturated Met and should correspond to the levels "naturally" found during the wood decay.  
286 However, for all other Met with an oxidation value less than 100%, the saturation with  $^{18}\text{O}$  was  
287 incomplete and some artifactual oxidation may have occurred during the sample preparation, and thus  
288 the level of  $^{16}\text{O}$  might be overestimated. For these proteins, we assumed that the percentage of  $^{16}\text{O}$   
289 found during wood decay was necessarily equal to or lower than the measured one. The percentage  
290 of oxidation as  $^{16}\text{O}$  ranged from 0% to 47% with a mean of 18% **(Fig. 4A; Dataset 3)**. This value is  
291 consistent with the mean percentage of Met $^{16}\text{O}$  oxidation calculated considering all Met containing-  
292 peptides found **(see Fig. 3A)** and indicated that most of the oxidation was concentrated on these few  
293 secreted proteins. Concerning day 5 and day 7 secretomes, the very low levels of  $^{18}\text{O}$  oxidation  
294 precluded the use of the blocking strategy. However, to find proteins with Met $^{18}\text{O}$  may allow to  
295 consider  $\text{H}_2^{18}\text{O}_2$  as a probe for highly reactive residues, because of the low  $\text{H}_2^{18}\text{O}_2$  concentration they  
296 were exposed to, due to the catalase activity. We found eight and nine Met bearing  $^{18}\text{O}$  in the proteins  
297 secreted at day 5 and day 7, respectively, and five were common to both samples **(Fig. 4B; Dataset 3)**.  
298 The percentages of  $^{18}\text{O}$  ranged from ~2% to ~14%, and from ~1% to ~23%, for the secreted proteins  
299 of day 5 and day 7, respectively. Among the 11 oxidized Met, only three were uniquely found at day 5  
300 or day 7: Met533 of a GH95 at day 5, and Met284 and Met160 belonging to a PL35 and a GH3,

301 respectively, at day 7 (**Fig. 4B; Dataset 3**). This indicates that most of the proteins found oxidized with  
302  $^{18}\text{O}$  at day 5 or 7 were also oxidized at day 3, highlighting their high reactivity to  $\text{H}_2\text{O}_2$ . We found a total  
303 number of 49 oxidizable Met belonging to 28 proteins, representing ~24% and ~19% of all the  
304 identified Met and proteins from the secretome, respectively (**Fig. 4C; Dataset 3**). Interestingly, the 18  
305 identified CAZymes represented most of them (64%), while proteases and proteins of unknown  
306 function both represented 18% with five identified proteins. The most represented CAZymes were GHs  
307 with 10 identified proteins. These results indicates that the proportion of oxidized CAZymes was  
308 enriched compared to the different types of proteins present in the secretomes (**compare Fig. 4C with**  
309 **Fig. 1**).

310



312

313 **Figure 4. Identity of oxidized Met and their levels of oxidation in *P. cinnabarinus* secretomes. A)**  
 314 **Proportion of the different forms of oxidation for the Met identified in the secreted proteins at day 3.**  
 315 **Proteins are ranked based on the percentage of <sup>16</sup>O (MetO and MetO<sub>2</sub>). The four Met fully oxidized are**  
 316 **underlined. Values shown are the mean of oxidation without standard deviation, omitted for clarity.**  
 317 **All values are in Dataset 3. B) Proportion of the different forms of oxidation for the Met identified in**  
 318 **the secreted proteins at day 5 and day 7. Proteins are ranked based on the percentage of <sup>18</sup>O (MetO**  
 319 **and MetO<sub>2</sub>). All values are in Dataset 3. C) Distribution of the identified secreted proteins and CAZymes**  
 320 **with oxidized Met. Values shown are the aggregate of all replicates for each time point (Dataset 3).**  
 321 **Bar graphs represent the number of CAZymes per class.**

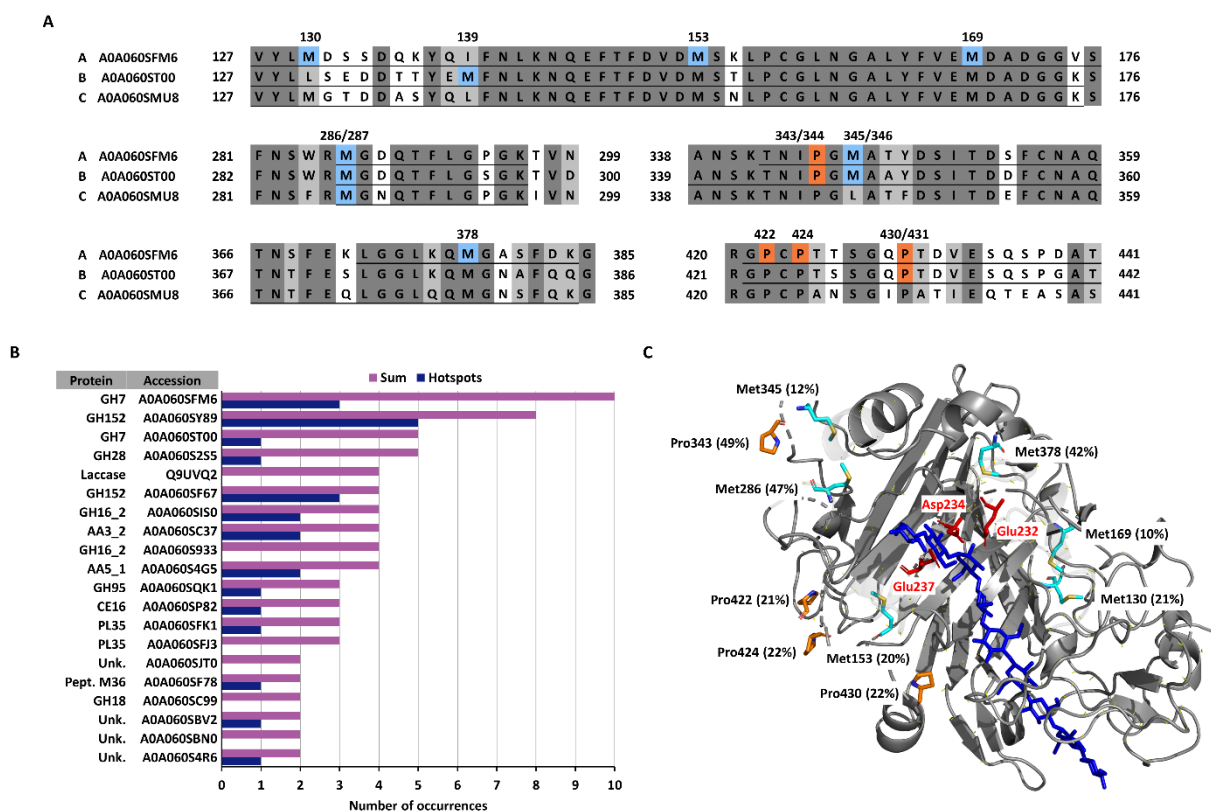
322

323 ***Oxidation occurs in multiple sites and hotspots of homologous proteins.***

324 Interestingly, among the five most oxidized, three Met belong to the three GH7 (A0A060SFM6,  
325 A0A060SMU8, A0A060ST00, named A, B, and C, respectively), at position 286 of the GH7 A and B, and  
326 at position 287 of the GH7 C (**Fig. 4A**). A sequence alignment showed that these positions are  
327 conserved between these GH7 homologs (**Fig. 5A**). These Met were oxidized with similar levels of  
328 Met<sup>16</sup>O (between 28% and 38%) and Met<sup>16</sup>O<sub>2</sub> (between 9% and 13%). We also detected seven other  
329 oxidized Met on these GH7s, four were uniquely on the GH7 A, one on the GH7 B, and two were in a  
330 position conserved in GH7 A and B. Since we had assessed the overall level of Pro oxidation, we  
331 quantified the percentage of oxidation of individual Pro, considering only those labeled with <sup>18</sup>O  
332 (**Dataset 4**). GH7 A had four oxidized Pro, two of which were in positions conserved into the GH7 B  
333 (**Fig. 5A**). Interestingly, we found that oxidation of Met and/or Pro occurred on homologs of GH16\_2,  
334 PL35, proteases and GH152 (**Fig. 4; Fig. S2**). This represented 11 proteins among the 33 proteins  
335 identified having oxidized residues (28 with Met with or without Pro and 5 with Pro only) (**Fig. 4; Fig.**  
336 **S2; Datasets 3; 4**). Moreover, 19 out of the 33 identified proteins were oxidized on multiple residues,  
337 from two to 10. A GH152 was oxidized on 8 Pro, while the GH7 A was the protein with the highest  
338 number of oxidation sites (10), including six Met and four Pro (**Fig. 4; Fig. 5; Fig. S2; Datasets 3; 4**).  
339 Finally, several oxidation sites were located close to each other in the protein sequences, forming  
340 “hotspots” like the Pro343 and Met345, and three other Pro of the GH7 A (**Fig. 5A**). In total, we counted  
341 24 occurrences of sites containing two oxidized residues in the protein sequences (**Fig. 5B**). Analysis of  
342 a tridimensional model of GH7 A revealed that oxidized residues distant on the sequence may be close  
343 on the structure, as the Met130 and Met160, the Met153 in the vicinity of three Pro, and the Met286  
344 clustered with the Pro343 and Met345 (**Fig. 5C**). Altogether, these results indicate that most of the  
345 oxidation affects a relatively small set of CAZymes that can be oxidized at multiple positions, with some  
346 hotspots. Moreover, the fact that oxidized residues were found at similar positions within the  
347 homologs of *P. cinnabarinus* secreted proteins suggests that these residues have been conserved to  
348 fulfill antioxidant or regulatory roles.

349

350



351

352 **Figure 5. Partial sequence alignment of GH7, numbers of oxidation sites and hotspots in identified**  
 353 **secreted proteins and tridimensional model of GH7. A)** Oxidized Met and Pro residues are on *blue*  
 354 and *orange* background, respectively. *Dark* and *light grey* backgrounds represent strictly conserved  
 355 and similar amino acids, respectively. Position and percentage of oxidation of the residues are  
 356 indicated above the sequence. For Pro, the percentage presented correspond to the sum of all oxidized  
 357 forms, either  $^{16}\text{O}$  or  $^{18}\text{O}$  at day 3, excepted the one indicated with 'D5', corresponding to Pro oxidation  
 358 at day 5. Underlined sequences represent peptides detected by LC-MS/MS. Accession numbers are on  
 359 the left of the sequences. **B)** Values correspond to the total number of oxidizable Met and Pro sites  
 360 found per protein. Hotspots correspond to the number of times that two oxidizable sites were located  
 361 within a window of ten amino acids on the protein sequence. All data are presented in **Datasets 3 and**  
 362 **4.** **C)** 3D model of the GH7 A0A060SFM6 downloaded from Alphafold database (62). The position of  
 363 the cellonanoase substrate (in *blue*) was modeled using the experimentally determined structure of  
 364 *Trichoderma reesei* Cel7A (PDB #4C4C). Met and Pro found oxidized are represented as *cyan* and  
 365 *orange* sticks, respectively. Catalytic residues are represented as *red* sticks. Percentage of oxidation at  
 366 day 3 are in brackets. Some parts of the protein were rendered transparent for clarity.

367

368 **Conservation suggests a list of residues with antioxidant or regulatory roles.**

369 To determine whether oxidized residues found in *P. cinnabarinus* proteins were widely  
370 conserved, we searched for homologs of the 33 proteins having oxidized residues in homologs from  
371 other fungi and quantified the conservation of the 89 oxidized residues. Our hypothesis was that  
372 oxidizable residues playing antioxidant or regulatory roles are more likely to be conserved among  
373 distantly related organisms, as shown for Met of actin or of the ffh/SRP proteins (63–65). To avoid  
374 overrepresentation of sequences coming from closely related organisms, the search was made in 120  
375 representative fungal species, covering the nine fungal phyla (**Dataset 5**). Overall, the conservation of  
376 Met or Pro ranged from 1% to 100%, with a mean of 49%. Considering only residues with conservation  
377 values above 75%, and found in more than 80 sequences, we obtained a list of nine Met and nine Pro  
378 at conserved positions in 14 secreted proteins (**Table 1**). The GH7 A has four conserved Met, among  
379 which Met 169 conserved in 99% of the homologs, and a conserved Pro. Other Met are conserved in  
380 the GH7 B and C, and in two GH16\_2, one GH18, one AA3\_2 and a peptidase M36, present in all fungi  
381 (**Table 1; Dataset 5**). Regarding Pro, three are conserved in all homologs, at positions 614, 143 and 441  
382 of a GH16\_2, a GH28 and a laccase, respectively. Conserved Pro were also found in a AA3\_2, an  
383 unknown protein and a GH152 (**Table 1; Dataset 5**). These results allowed identification of proteins  
384 with oxidizable Met and Pro residues strongly conserved across the fungal phyla.

385

386 **Table 1. *P. cinnabarinus* secreted proteins with oxidized residues conserved across fungal proteins.**  
 387 Only proteins for which the number of identified homolog sequences identified was higher than 80  
 388 were considered. The complete list of identified secreted proteins is presented in **Dataset 5**.

<i>Accession Uniprot (JGI)</i>	<i>Description</i>	<i>Repartition (Nb. of sequences)</i>	<i>Residue</i>	<i>Conservation (%)</i>
AOA060SFM6 (8640)	GH7	Dikarya (98)	Met 130	80
			Met 169	99
			Met 286 <sup>a</sup>	76
			Met 378	83
			Pro 430 <sup>b</sup>	99
AOA060ST00 (6809)	GH7	Dikarya (98)	Met 287 <sup>a</sup>	76
AOA060SMU8 (5845)	GH7	Dikarya (98)	Pro 431 <sup>b</sup>	99
AOA060SIS0 (301)	GH16_2	Basidiomycota (100)	Met 286 <sup>a</sup>	76
AOA060S933 (3097)	GH16_2	Basidiomycota (91)	Met 615	92
			Pro 614	100
AOA060S933 (3097)	GH16_2	Basidiomycota (91)	Met 583	83
AOA060SC99 (6004)	GH18	Dikarya, Mucor., Zoo. (99)	Met 342	86
AOA060S4J1 (1984)	AA3_2	Agaricomycetes (100)	Met 124	83
AOA060SF78 (8374)	Pep. M36	All fungi (100)	Met 387	81
AOA060S2S5 (8001)	GH28	Dikarya (99)	Pro 143	100
Q9UVQ2 (8672)	Laccase	Basidiomycota (100)	Pro 284	96
			Pro 441	100
AOA060SC37 (3147)	AA3_2	Dikarya (98)	Pro 594	97
AOA060S4R6 (3419)	Unknown	Dikarya, Mucor., Chytridio., Neo. (100)	Pro 170	83
AOA060SF67 (3180)	GH152	Dikarya, Mucoromycota (99)	Pro 184 <sup>c</sup>	94
AOA060SY89 (5455)	GH152	Dikarya, Mucor., Chytridio. (99)	Pro 200 <sup>c</sup>	96

389 <sup>a, b, c</sup> Conserved positions between the *P. cinnabarinus* homologs. *Mucor.*, *Mucoromycota*; *Zoo.*, *Zoopagomycota*,  
 390 *Chytridio.*, *Chytridiomycota*, *Neo.*, *Neocallimastigomycota*.

391

## 392 Discussion

393 Taking a white-rot wood decayer as model, we showed that fungal secreted proteins have a  
394 strongly reduced Met content (~1.4%) compared to the non-secreted (~2.1%) and that this feature is  
395 not conserved in metazoans and plants. This decreased Met content suggests that protection of  
396 proteins secreted by fungi is achieved by avoiding oxidation, in line with the previously proposed  
397 mechanism (16). Interestingly, these data contrast with those obtained from animal studies which  
398 showed that proteins encoded by the mitochondrial genome may contain up to 6-10% Met. This high  
399 content could act as an endogenous antioxidant with the exposed Met scavenging ROS which are then  
400 removed by methionine sulfoxide reductases in a cycle of oxidation and reduction (66). A similar  
401 mechanism was proposed in bacteria (67, 68). The absence of external system of MetO reduction  
402 preclude the use of such mechanism with fungal secreted proteins (24). This protection is most likely  
403 accompanied by the secreted catalase we found (**Fig. 3**), in line with previous assumptions (16, 69–71).  
404 The secretion of a catalase by *P. cinnabarinus* was unexpected, as it has not been previously detected  
405 (41, 42), nor has extracellular activity been measured for the white-rot *Phanerochaete chrysosporium*  
406 (72). However, searching for secreted catalase homologs, we found that it is widespread in  
407 basidiomycota, and particularly in white-rot fungi (**Table S5**), contrary to previous phylogenetic  
408 analysis (73). Lack of detection of the protein or its activity may be due to specific expression  
409 conditions. In white-rot, it is surprising that a catalase was expressed along with H<sub>2</sub>O<sub>2</sub> producing and  
410 consuming enzymes. However, catalases have Km values in the mM range (70), and their action could  
411 result in lowering the H<sub>2</sub>O<sub>2</sub> concentration below a levels that is damaging to proteins, but allows the  
412 activity of H<sub>2</sub>O<sub>2</sub>-driven oxidoreductases.

413 Using H<sub>2</sub><sup>18</sup>O<sub>2</sub>-based proteomics, we identified oxidizable Met and highlighted that CAZymes  
414 were the most oxidized secreted proteins during wood-decay, and that Met oxidation could occur on  
415 several residues, sometimes clustered in hotspots. Clustering was also observed in bacterial proteins  
416 (74), suggesting a universal phenomenon. The blocking strategy we used is based on a saturation of



417 reduced Met with  $^{18}\text{O}$ . However, saturation was difficult to achieve, with only 4 Met saturated with  $^{18}\text{O}$   
418 in the day 3 secretome (**Fig. 4**). An initial denaturation step would have probably allowed  $\text{H}_2^{18}\text{O}_2$  to  
419 access the Met buried in the protein core (28). However, as our goal was to identify secreted proteins  
420 with potential oxidizable Met we chose not to denature them. Our idea was that an oxidizable Met  
421 should be accessible to an oxidant, either natural or  $\text{H}_2^{18}\text{O}_2$ . The results at day 3 argue for the  
422 accessibility of the Met to  $\text{H}_2^{18}\text{O}_2$ , as the percentage of  $^{18}\text{O}$  was higher than that of  $^{16}\text{O}$  for 43 Met out  
423 of 46. As artifactual  $^{16}\text{O}$ -oxidation cannot be ruled out, the percentage of  $^{16}\text{O}$  was necessarily lower or  
424 equal to those measured. In our study, the mean percentage of  $^{16}\text{O}$  was 18%, while in studies on  
425 animals using similar approaches, it was ~4% (27, 28), arguing for potential artifactual oxidation.  
426 However, while intracellular proteins are protected by methionine sulfoxide reductases and ROS  
427 scavengers, the MetO in secreted proteins cannot be reduced, and no  $\text{H}_2\text{O}_2$ -consuming was detected  
428 in the secretome at day 3. Thus, to find high levels of MetO in fungal secreted proteins is expected.  
429 Interestingly, nine Met were oxidized as  $^{16}\text{O}$  sulfone among which five were also oxidized as  $\text{Met}^{18}\text{O}_2$   
430 (**Fig. 4**), confirming their propensity to form sulfone during wood decomposition.

431         Some of the Met we found oxidized in *P. cinnabarinus* are conserved throughout the fungal  
432 kingdom, suggesting potentially important roles (**Table 5**). They could act as antioxidants, as for the  
433 GH7 whose Met and Pro are located at the surface of the proteins and in the substrate cleft (**Fig. 5C**),  
434 potentially preventing critical residues from being oxidized, as proposed for a glutamine synthetase  
435 (23). Oxidizable Met could also have a functional role altering enzyme activity, like for a GH18 in which  
436 a conserved oxidized Met is located near the catalytic residue (**Fig. S3**), as shown for a GH27  
437 galactosidase of *T. reesei* (25). In conclusion, our findings highlight a common mechanism in fungi  
438 preventing oxidation of secreted proteins and, pinpoint redox-regulated CAZymes to be further  
439 characterized.

440

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449

450 **CRedit author statement**

451 **Lise Molinelli:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data  
452 Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, **Elodie Drula:** Software,  
453 Methodology, Formal analysis, Investigation, Data Curation, **Jean-Charles Gaillard:** Formal analysis,  
454 Investigation, **David Navarro:** Investigation, Resources, **Jean Armengaud:** Formal analysis, Resources,  
455 Data Curation, Writing - Review & Editing, **Jean-Guy Berrin:** Resources, Writing - Review & Editing,  
456 Funding acquisition, **Thierry Tron:** Conceptualization, Resources, Writing - Review & Editing,  
457 Supervision, Funding acquisition, **Lionel Tarrago:** Conceptualization, Methodology, Software,  
458 Validation, Formal analysis, Investigation, Resources, Data Curation, Writing - Original Draft, Writing -  
459 Review & Editing, Visualization, Supervision, Funding acquisition.

460

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