



Antioxidant activity and ACE-inhibitory of Class II hydrophobin from wild strain *Trichoderma reesei*

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Abstract: There are several possible uses of the Class II hydrophobin HFBII in clinical applications. To fully understand and exploit this potential however, the antioxidant activity and ACE-inhibitory potential of this protein need to be better understood and have not been previously reported. In this study, the Class II hydrophobin HFBII was produced by the cultivation of wild type *Trichoderma reesei*. The crude hydrophobin extract obtained from the fermentation process was purified using reversed-phase liquid chromatography and the identity of the purified HFBII verified by MALDI-TOF (molecular weight: 7.2 kDa). Subsequently the antioxidant activity of different concentrations of HFBII (0.01-0.40 mg/mL) were determined. The results show that for HFBII concentrations of 0.04 mg/mL and upwards the protein significantly reduced the presence of ABTS+ radicals in the medium, the IC50 value found to be 0.13 mg/mL. Computational modeling highlighted the role of the amino acid residues located in the conserved and exposed hydrophobic patch on the surface of the HFBII molecule and the interactions with the aromatic rings of ABTS. The ACE-inhibitory effect of HFBII was found to occur from 0.5 mg/mL and upwards, making the combination of HFBII with strong ACE-inhibitors attractive for use in the healthcare industry.

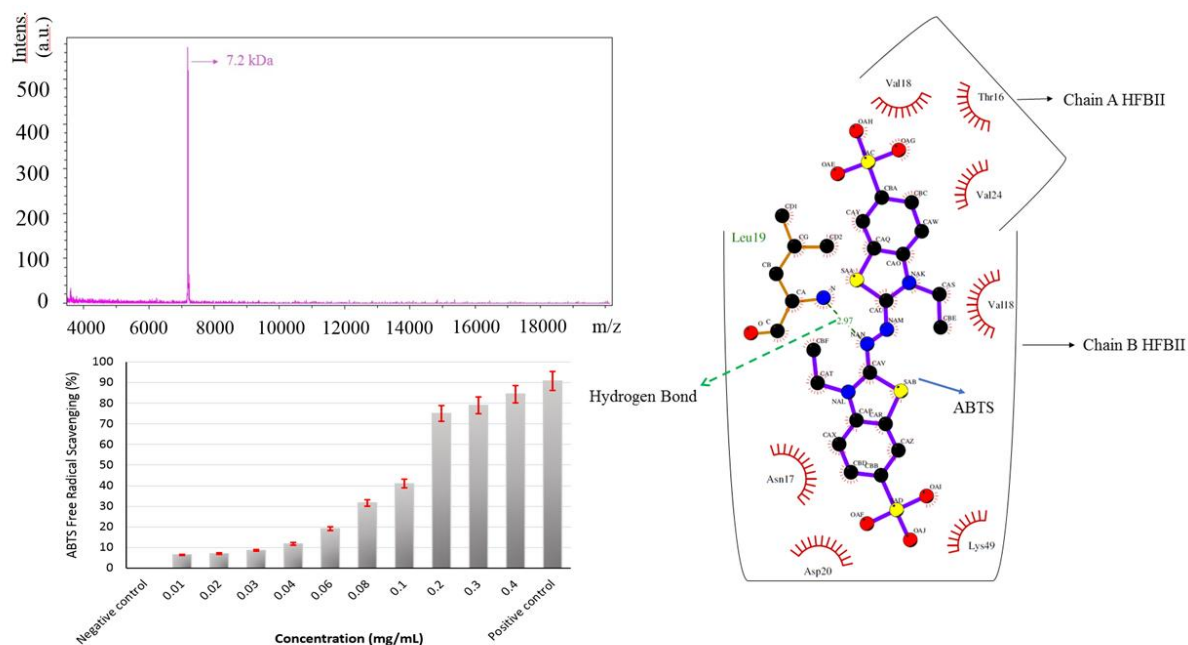
Highlights

- ✓ Production of Class II hydrophobin by wild strain fungi for positive applications.
- ✓ Using of hydrophobin HFBII as a novel protein based antioxidant from fungi.
- ✓ Remarking HFBII as a **low** ACE inhibitor.
- ✓ Computational modeling for understanding the interactions between HFBII and ABTS.

Abstract

There are several possible uses of the Class II hydrophobin HFBII in clinical applications. To fully understand and exploit this potential however, the antioxidant activity and ACE-inhibitory potential of this protein need to be better understood and have not been previously reported. In this study, the Class II hydrophobin HFBII was produced by the cultivation of wild type *Trichoderma reesei*. The crude hydrophobin extract obtained from the fermentation process was purified using reversed-phase liquid chromatography and the identity of the purified HFBII verified by MALDI-TOF (molecular weight: 7.2 kDa). Subsequently the antioxidant activity of different concentrations of HFBII (0.01-0.40 mg/mL) were determined. The results show that for HFBII concentrations of 0.04 mg/mL and upwards the protein significantly reduced the presence of ABTS⁺ radicals in the medium, the IC₅₀ value found to be 0.13 mg/mL. Computational modeling highlighted the role of the amino acid residues located in the conserved and exposed hydrophobic patch on the surface of the HFBII molecule and the interactions with the aromatic rings of ABTS. The ACE-inhibitory effect of HFBII was found to occur from 0.5 mg/mL and upwards, making the combination of HFBII with strong ACE-inhibitors attractive for use in the healthcare industry.

Graphical abstract



1 **Antioxidant activity and ACE-inhibitory of Class II hydrophobin from** 2 **wild strain *Trichoderma reesei***

3
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19 20 21 **Abstract**

22 There are several possible uses of the Class II hydrophobin HFBII in clinical applications. To
23 fully understand and exploit this potential however, the antioxidant activity and ACE-
24 inhibitory potential of this protein need to be better understood and have not been previously
25 reported. In this study, the Class II hydrophobin HFBII was produced by the cultivation of
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36 with strong ACE-inhibitors attractive for use in the healthcare industry.

37
38 **Keywords:** Hydrophobin; Antioxidant; ACE-inhibitory.

41 **1. Introduction**

42 Oxidation is an important issue in food science, causing significant chemical and textural
43 deterioration of food products [1]. As a consequence of this phenomenon, free radicals are
44 generated which increase risk factors associated with many hazardous diseases, *e.g.* different
45 types of cancers [2], cardiovascular disease [3] and Alzheimer's disease [4]. Antioxidants
46 have been reported to lower the risk of such diseases developing [5] with these protective
47 agents being found naturally in many foods consumed by humans and are, sometimes, taken
48 as supplements in the human diet.

49
50 Antioxidant activity and ACE (angiotensin I-converting enzyme) inhibitory effects have been
51 noticed together in several studies [6,7,8]. ACE is a dipeptidyl carboxypeptidase which
52 inactivates bradykinin (a potent vasodilator) by removal of its carboxyl terminal
53 Phenylalanyl-Arginine dipeptide [9,10]. On the other hand, ACE increases the rate of
54 formation of angiotensin II by removing the Histidyl-Leucine dipeptide from angiotensin I.
55 These effects serve to reduce blood pressure, thus prescription of ACE-inhibitors with good
56 oral bioavailability is required [11,12,13]. Additionally, it has been observed that ACE-
57 inhibitors alleviate the symptoms of type 1 diabetes, hypertension, atherosclerosis, and
58 myocardial infarction [14].

59
60 Cultivation of microorganisms for biosynthesis of protein-based compounds as a novel class
61 of antioxidants and/or ACE-inhibitors as alternatives to plant sourced extracts has become a
62 matter of significant interest for biotechnologists and research activity has progressively
63 increased in recent years [15]. There are several reports concerning the use of microbial
64 antioxidants [16,17,18,19], including different microbial extracts and secondary metabolites
65 such as citrinin, protocatechuic, and curvulic acid [20].

66
67 Filamentous fungi produce different types of hydrophobins, a family of low molecular weight
68 (MW) proteins consisting four disulfide bridges and a large conserved and exposed
69 hydrophobic patch [21], this structure and the resulting properties conferred to hydrophobins
70 gives these low MW proteins a number of applications in bio-industry [22]. Hydrophobins
71 are split into Class I and Class II, on the basis of their solubility and elasticity of their films
72 [23,24]. Class I hydrophobins are rarely soluble in water whereas the proteins from Class II
73 are easily dissolved in the aqueous phase and their films are highly elastic, such that can

74 cover and protect lipophilic micelles in a hydrophilic environment [25,26]. The large
75 potential of Class II hydrophobins in clinical applications has been described in the literature
76 [27]. Furthermore, Class II hydrophobins have been successfully used for generating the
77 stabilized foams in rich-foam products where control of the air phase is especially important
78 [28,29,3]. In contrast, the formation of stabilized CO₂ nano-bubbles by Class II hydrophobins
79 have been reported to provoke the gushing phenomenon, and considered to be a negative
80 function of this protein in the carbonated beverages industry [31,32,33].

81

82 In drug delivery system adsorption of amphiphilic Class II hydrophobins around the
83 lipophilic micelle-liquid interface results in three special effects: (i) retarding droplet growth,
84 (ii) enhancing the droplet stability, and (iii) increasing the functionality of particles inside the
85 droplet by providing an elastic skin [33]. Velo et al. (2010) [34] report that the Class II
86 hydrophobin HFBII can enhance the stability of itraconazole as a model of a hydrophobic
87 drug. In the same way, researchers used the hydrophobin HFBII from wild strain
88 *Trichoderma reesei* (*T. reesei*) to enhance the retention time of ocimene, a non-polar aroma
89 compound derived from hops, in an aqueous phase [35]. Khalesi et al. (2015) concluded that
90 this postponing effect may correspond to the formation of the new stable micelles with
91 certain diameters (mostly in the range of 600-2000 nm), surrounded by HFBII mono- or
92 multi-layer films with a high surface elasticity (0.5 N/m²) [23]. However, in those studies (*i.e.*
93 references 34-35) the antioxidant potential of hydrophobins, which may be also the cause of
94 the retention of the volatile compounds, has not been considered.

95

96 Therefore, experimental determination of the antioxidant activity of HFBII, supported by the
97 data obtained from computational modeling of the system, as well as measurement of the
98 ACE-inhibitory effect of Class II hydrophobins, as a model of microbial proteins produced by
99 a natural fungus, are presented in this study.

100

101 **2. Materials and methods**

102

103 *2.1. Microbial culture*

104 A fermenter (KGW-Isotherm, Schieder GmbH, Germany) with a 1 L capacity was used for
105 fermentation of *T. reesei* MUCL 44908 (BCCM/MUCL Agro-Industrial Fungi & Yeast

106 Collection, Belgium). The system was equipped with a RW20 digital agitator (IKA GmbH,
107 Germany) and a type R 1389 agitator (PTFE-coated) fitted with a 3-bladed propeller stirrer
108 (Stirrer: 45 mm, Shaft: 8 mm, Shaft length: 350 mm). The stirrer was operated in the range
109 1000-1400 rpm, to maintain dissolved oxygen levels. Fermentation was performed at 29 °C
110 for 7 days with an air flow rate of 1 L/min. The aqueous medium consisted of peptone 4 g/L,
111 yeast extract 1 g/L, KH₂PO₄ 4 g/L, (NH₄)₂SO₄ 2.8 g/L, MgSO₄·7H₂O 0.6 g/L, CaCl₂ 0.6 g/L,
112 CoCl₂·6H₂O 4.0 mg/L, MnSO₄·H₂O 3.2 mg/L, ZnSO₄·7H₂O 6.9 mg/L and FeSO₄·7H₂O 10.0
113 mg/L [36,37]. For the production of hydrophobin HFBII lactose (40 g/L) was added as the
114 carbon source, this is correlated to the biochemical routes of producing HFBII by *T. reesei*.
115 For further information, see Khalesi et al. 2014 [38] and 2015 [39]. Phosphoric acid was
116 added to maintain a pH of 4.5-5.0. Inoculation of 1 L fresh sterilized medium in the fermenter
117 was performed with an overnight culture of *T. reesei*. At the end of fermentation (7 days), the
118 medium culture was separated from the mycelium by centrifugation (8000 g for 25 min at 6
119 °C, Beckman model J2-21, USA) and the supernatant stored at 2 °C prior to further
120 purification.

121

122 2.2. Protein purification

123 HFBII present in the supernatant was purified by reverse phase liquid chromatography, using
124 a Bio-Rad system (Bio-Rad laboratories n.v., Eke, Belgium) and programmed with a Biologic
125 duoflow software. A SOURCE 30RPC column (25 × 450 mm, Pall Life Science, Zaventem,
126 Belgium) was installed for the elimination of the impurities. The sample obtained from the
127 fermentation process (100 mL) was injected to the column. Elution of the proteins was
128 carried out with a linear gradient from 0% to 60% acetonitrile (ACN) containing 0.1%
129 trifluoroacetic acid (TFA). The flow rate was adjusted to 10 mL/min. Peak elution was
130 monitored by UV detection at 214 nm. Table 1 details the characteristics of the column as
131 well as the experimental conditions used in the purification of hydrophobin HFBII.

132

133 2.3. Identification of hydrophobin HFBII

134 Positive identification of the produced hydrophobins was carried out using MALDI-TOF
135 (Brüker Daltonics, GmbH, Germany) in the range of 0-40 kDa MW. The eluted fractions
136 from the chromatography step were collected and dehydrated by vacuum centrifuge (Univapo
137 150 Ech and Multitrap, Canada). The dried samples were then re-dissolved in 100 µL MilliQ

138 water with 5% ACN containing 0.5% formic acid (FA). To prevent protein aggregation, the
139 samples were vortexed using a bench stirrer (30 s, 1000 rpm) and sonicated for 5 min using
140 an ultrasonic bath (Branson 2510, Wareham, USA). A matrix was prepared by the addition of
141 10 mg α -cyano-4-hydroxy cinnamic acid to 200 μ L of a solution containing 50% MilliQ
142 water and 50% ACN with 0.5% FA. A 1 μ L sample was spotted onto a target plate (MTP 384
143 ground steel, Brüker Daltonics, GmbH, Germany) and mixed with 1 μ L matrix solution. The
144 fractions which contained only the proteins with the MW of 7.0-7.4 kDa were taken as pure
145 HFBII (κ -grade).

146

147 *2.4. Measurement of antioxidant activity*

148 The ABTS⁺ [2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonicacid)] radical scavenging
149 activity assay is the standard method for measuring the antioxidant activity of different
150 compounds. To perform this analysis, 7 mM ABTS (Sigma-Aldrich, Munich, Germany)
151 solution was oxidized in water by treatment with 2.45 mM potassium persulfate (Sigma-
152 Aldrich, Munich, Germany), left for 12-16 h in a dark room. This solution exhibited an
153 absorbance of 0.70 ± 0.05 at 734 nm measured by a microplate reader (Biotek powerwave
154 XS2, BioTek Instruments Inc., Vermont, USA) [40,41]. To evaluate the antioxidant activity
155 of Class II hydrophobins, previously produced and purified κ -HFBII at concentrations of
156 0.01, 0.02, 0.03, 0.04, 0.06, 0.08, 0.1, 0.2, 0.3 and 0.4 mg/mL were mixed with 200 μ L of the
157 prepared ABTS⁺⁺ solutions. These mixtures were incubated at 25°C for 6 min and the
158 absorbance measured at 734 nm, relative to a blank of ABTS⁺⁺ solution alone. The positive
159 control used was a mixture of bioactive peptides extracted from Persian walnut with a
160 concentration of 0.05 mg/mL. In the current study, we used the bioactive walnut peptides as a
161 plant-source model of antioxidants, and in the discussion we compare this to a protein from a
162 fungus as a potential microbial antioxidant, *i.e.* HFBII. These two experiments were run in
163 parallel following the same methodology.

164

165 *2.5. Computer-aided molecular modelling*

166 To identify the possible bindings between the Class II hydrophobin HFBII and ABTS,
167 Molecular Docking was performed using AutoDock 4.2 and AutoDockTools *version 1.5.4*
168 using standard parameters. The crystal structure of HFBII in a dimer state was obtained from
169 the protein data bank (PDB code 2B97). The ligand structure was constructed and the energy

170 minimized using a PRODRG online server. Images were generated with the PMV and the
171 LigPlot *version 4.5.3*.

172

173 *2.6. Measurement of ACE-inhibitory activity*

174 The ACE-inhibitory activity of different concentrations of κ -HFBII from *T. reesei* was
175 determined based on the method described previously by Vermeirssen et al. (2002) [42].
176 Rabbit lung extract (Sigma-Aldrich, Munich, Germany) as a source of ACE was prepared by
177 dissolving 1 g of rabbit lung acetone powder in 10 mL of potassium phosphate buffer (50
178 mM, pH 8.3) containing 5% (v/v) glycerol. After overnight stirring at 4 °C, the mixture was
179 centrifuged (40000 g, 40 min) using an ultracentrifuge (Refrigerated Centrifuge Sigma 3-30k,
180 ATRBiotech Inc. Laurel, MD, USA). The upper phase was collected and stored at 4 °C prior
181 to analysis. An aliquot (150 μ L) of furanacryloyl tripeptide (1 mM) (Sigma-Aldrich,
182 Munich, Germany) was dissolved in 50 mM Tris-HCl buffer (pH 8.3) containing 0.3 mM
183 NaCl. Different concentrations of κ -HFBII (0.1, 0.2, 0.4, 0.5 and 0.6 mg/mL) were then
184 added to the plates. ACE extract was added to each mixture and pre-incubated at 37 °C for 2
185 min. A microplate reader (Biotek powerwave XS2, BioTek Instruments Inc., Vermont, USA)
186 was used to record the absorbance at a wavelength 340 nm before and after a period of 30
187 min. The negative control was taken to be the sole enzyme and its substrate (N-[3-(2-
188 Furyl)acryloyl]-L-phenylalanyl-glycyl-glycine, FAPGG). A mixture of peptide fractions from
189 Persian walnut (purchased from Hamedan, Iran) with MWs lower than 30 kDa was taken as a
190 positive control. This mixture had been previously purified in our laboratory and the ACE-
191 inhibitory activity verified. The ACE activity of each sample was calculated based on the
192 following equation [43]:

$$193 \quad \text{ACE-inhibition (\%)} = \left(1 - \frac{\delta A \text{ inhibitor}}{\delta A \text{ negative control}} \right) \times 100$$

194 where δA inhibitor and δA negative control represent the slopes of the declining in
195 absorbance at 340 nm for the test and for the negative control samples, respectively.

196

197

198

199

200 2.7. *Statistical analysis*

201 All experiments were carried out in triplicate. All the data are presented as a mean value \pm
202 standard deviation (SD). The significance of the differences between measured values was
203 determined with a t-test (P value < 0.05) using SPSS *version 15.0*.

204

205 **3. Results and Discussion**

206

207 3.1. *Verification of Class II hydrophobin HFBII*

208 The fermentation process using *T. reesei* was stopped after 7 days of production, achieving a
209 final biomass concentration of 53.2 ± 2.7 g/L. The biomass was discarded after centrifugation
210 and the supernatant used for further analysis. After injection of 100 mL supernatant phase to
211 the SOURCE 30RPC column, all the fractions of interest, which eluted at ACN 35-50% (v/v),
212 were collected. An example of the chromatograms generated during the purification step is
213 represented in Fig. 1.

214

215 The collected fractions eluted at 35-50 % ACN were submitted to the MALDI-TOF. A MW
216 of 7.2 kDa was obtained in some of the fractions (Fig. 2). These were considered as κ -grade
217 HFBII (the purest fractions). The obtained HFBII concentration was determined to be $0.20 \pm$
218 0.01 mg/mL.

219

220 3.2. *κ -HFBII and its antioxidant activity*

221 The antioxidant activity of different concentrations of κ -HFBII in the range 0.01-0.4 mg/mL
222 was measured. Although HFBII concentrations below 0.04 mg/mL were found to have a very
223 small scavenging effect on ABTS radicals, higher concentrations lowered the amount of
224 radicals (Fig. 3). Interestingly, the concentration of 0.04 mg/mL is equal to the critical
225 micelle concentration (CMC) of HFBII. Thus, our results demonstrate that significant
226 antioxidant activity of HFBII starts at the CMC. Based on the data obtained for different
227 concentrations of κ -HFBII regarding the antioxidant activity, the IC₅₀ value was determined
228 to be 0.13 ± 0.02 mg/mL. This value compares favorably with the results for other sources of
229 antioxidants, *e.g.* crude extract, Albumins, Globulins, Prolamins and Glutelins extracted from

230 plant source Adzuki bean seeds with IC50 values equal to 0.65 ± 0.04 , 0.89 ± 0.07 , $0.54 \pm$
231 0.02 , 0.06 ± 0.00 , 1.12 ± 0.03 mg/mL, respectively [44].

232

233 Given the result of the current experiment and the previous report regarding the enhanced
234 solubility of non-polar compounds in a water phase by using Class II hydrophobin HFBII as
235 an encapsulant [34,35], it is proposed that the film created by HFBII around the volatile and
236 lipophilic compounds in food products and medicines, not only reduces the escaping ratio of
237 those compounds, but also prevents their oxidation.

238

239 Many studies used a mixture of peptides as a natural antioxidant, this is due to the fact that in
240 most of cases, a sole peptide has a very small effect on oxidants. Thus, by use of several
241 peptides with different structures or sequences, synergistic effects may occur. By mixing the
242 peptides, however, the fundamental mechanism of antioxidant activity is masked and will
243 remain unclear. It is i) easier, ii) safer, and iii) perhaps more cost effective to produce and
244 purify a microbial antioxidant than to extract a mixture with potential of antioxidant activity
245 from a seed or a plant, iv) also the legal approval of the application of a pure protein is more
246 likely, and finally v) some proteins, in particular hydrophobins, possess several extracellular
247 characteristics:

248

249 i) It is easier to produce antioxidants from a microbial source since the cultivation of
250 microorganisms, *i.e.* producers of secondary metabolites, is a well-regulated and understood
251 biotechnological production route.

252

253 ii) It is safer to produce proteins from a microbial source than extract from plants as,
254 unfortunately, it is not always possible to identify all the fractions of the protein mixtures
255 obtained from plants. There are sometimes allergens or toxic products as for instance,
256 chitinase Ib and lipid transfer protein from Chestnut [45], profiling, lipid transfer protein, and
257 11S globulin-like protein from Hazelnut [46], ochratoxin A (OTA) in liquorice products
258 [47,48,49], and aflatoxin B₁ in pistachio [50]. Thus complex analysis and a very sensitive
259 method are required to ensure that the obtained product is safe. In the case of bioactive
260 peptides from walnuts, tested in this research as a positive control for antioxidant activity,
261 there is a risk of the presence of 2S albumin which is an allergen [51]. This nut is also always
262 exposed to the mycotoxins produced by different storage molds, for instance *Aspergillus* sp.

263 which produce OTA [52]. Thus, it is possible to overcome the allergy effect of plant extracts
264 through the development of new natural sources of antioxidants.

265

266 iii) Production of microbial antioxidants is potentially cheaper than getting antioxidants
267 directly from plants. In the case of the production cost of antioxidant obtained from seeds or
268 plants the following stages have to be considered: implanting, harvesting and post-harvesting
269 processing of the plant, extraction, purification, as well as analysis of the safety of the
270 extracted product. Normally after extraction of the antioxidant from the plants or the seeds
271 they are no longer fit for consumption because the extraction process transforms the texture
272 and the nutritional properties of the products. In the case of production of natural antioxidants
273 by microorganisms, it is only required to describe a protocol for the growth of the
274 microorganisms and releasing the product as well as a purification step. It is also possible to
275 increase the yield of produced microbial metabolites by using recombinant microorganisms,
276 improving the design of bioreactors and optimization of the fermentation conditions, through
277 the application of biochemical engineering.

278

279 iv) It should be mentioned that for both cases, *i.e.* getting the antioxidant from plants or
280 production by microorganisms, the use of final product needs to be authorized before
281 industrial application. Of course, meeting these criteria is more difficult with a mixture of
282 compounds, than a sole compound.

283

284 v) The additional advantage of using hydrophobin HFBII, as an alternative of other sources
285 of antioxidants, is its extracellular structures and the special characteristics [53]. This protein
286 possesses several functionalities in real products; it can lower the surface tension of the water
287 due to its amphipathic nature [54], it creates highly stable foams in foods and beverages [55],
288 it can be applied as a carrier of pharmaceuticals [56] and food ingredients [35], and has also
289 recently been reported to possess anti-tumor activity [57].

290

291 *3.3. Computational modeling of HFBII and ABTS: Possible interactions*

292 Computational modeling using AutoDock was carried out to understand the possible
293 mechanism of antioxidant activity of hydrophobin HFBII in the presence of an ABTS
294 molecule. In the model HFBII was considered to be a dimer. This state was previously
295 reported for the current working concentration [58,59]. The results showed that more than

296 150 situations may occur when both HFBII dimer and ABTS are combined in an isolated box
297 of the experiment. Nevertheless, the most probable situation with the lowest activation energy
298 revealed that only few amino acids of HFBII are involved in the possible interactions. These
299 include Threonine₁₆, Valine₁₈, Valine₂₄ from Chain A HFBII, and Asparagine₁₇, Valine₁₈,
300 Leucine₁₉, Aspartic acid₂₀, Lysine₄₉ from Chain B HFBII. More importantly, the result
301 showed that Leucine₁₉ may also create a hydrogen bond (2.97 Å) to interact with ABTS (Fig.
302 4). Another interesting observation was the intention of the molecule ABTS to approach into
303 the hydrophobic patch of HFBII, re-emphasizing this patch as an active site of HFBII.

304

305

306 3.4. ACE-inhibitory of κ -HFBII

307 Fig. 5. shows the ACE-inhibitory effects of different concentrations of hydrophobin HFBII in
308 the range of 0.1-0.6 mg/mL.

309

310 The obtained pure Class II hydrophobin HFBII showed a very small ACE-inhibitory effect in
311 comparison with positive control, *i.e.* a mixture of bioactive peptides extracted from the
312 Persian walnut. A concentration of 0.05 mg/mL of extract from the latter reduced ACE
313 activity by 47±1%. When using high concentrations of hydrophobin HFBII, the ACE activity
314 started dropping. The decline is significant from HFBII concentrations of 0.5 mg/mL and
315 upwards. This point is approximately 12 times higher than the CMC reported for HFBII (*i.e.*
316 0.041 mg/mL). In comparison with the Persian walnut, HFBII is a weak ACE-inhibitor.
317 Nevertheless, since hydrophobin is a natural non-toxic product with a straightforward
318 procedure of production, showing also antioxidant activity and other functionalities, it is
319 proposed to combine HFBII with drugs, like angiotensin receptor blockers and applying it to
320 treat a broad variety of disorders.

321

322 As the structure of κ -HFBII has been widely studied, it is easy to postulate the exact
323 mechanism of the action of this protein on ACE rather than any peptide mixtures extracted
324 from the plants. The ACE-inhibitory effects of HFBII may due to the presence of a
325 significant hydrophobic patch, which enhances binding of HFBII to the active site of ACE.
326 The low ACE-inhibitory of Class II hydrophobins may correspond to the poor content of
327 Prolin amino acid at carboxyl terminal and its large size in comparison with the peptides.

328

329 **4. Conclusion**

330 The use of the Class II hydrophobin HFBII as a coating agent of different molecules has been
331 previously reported. In our previous work, we examined the retention time of ocimene, a non-
332 polar volatile compound from the hops, in a water phase containing hydrophobin HFBII. Our
333 results clearly showed that HFBII postponed the escape of ocimene molecules from the water
334 phase. In that research we concluded that due to the hydrophobicity of ocimene, HFBII may
335 cover the ocimene via the hydrophobic patches, and prevents its micellization and volatility
336 of the molecules. Our new findings in the current study claims that the conclusion from the
337 previous work was incomplete due to the fact that the antioxidant activity of hydrophobin
338 may also prevent the possible oxidation phenomenon, and this is probably another reason for
339 maintaining the ocimene in the system for a longer period. Here, we showed that HFBII can
340 play a role as an antioxidant and reduce the presence of free radicals of ABTS in the
341 environment. We used ABTS[•] to study this effect, but it is important to note that HFBII does
342 not coat ABTS[•]. Actually, the HFBII dimer interacts with ABTS through specific amino acids
343 residues (*i.e.* Threonine₁₆, Valine₁₈, Valine₂₄ from Chain A HFBII, and Asparagine₁₇,
344 Valine₁₈, Leucine₁₉, Aspartic acid₂₀, Lysine₄₉ from Chain B HFBII), modeled by AutoDock.
345 In this study, we also demonstrated that this protein possesses ACE-inhibitory effects at high
346 HFBII concentrations. Thus, by using Class II hydrophobin HFBII as an encapsulant, first of
347 all, the elastic skin formed retards the escape of lipophilic volatile compounds. Secondly, it
348 prevents harmful deterioration processes *e.g.* oxidation. The fact that the antioxidants
349 extracted from natural plants or seeds may be allergens encourages biotechnologists to
350 characterize novel microbial antioxidants. We showed that Class II hydrophobin HFBII is a
351 good candidate in this case and that this functionality corresponds significantly to the
352 hydrophobic patch of HFBII. It seems that in the near future, bioproduction of antioxidants
353 and ACE-inhibitors by native microorganisms will gain wider attention from the healthcare
354 industry.

355

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360

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Table and Table Caption.**Table 1**

Characteristics of the SOURCE 30RPC polymer based matrix and the purification condition.

Matrix	Poly(styrene/divinyl benzene)
Bead form	Rigid, spherical, porous, monodisperse
Particle size	30 μm
Dynamic capacity	14 mg BSA/mL
Operating pH	4.7
Operating temperature	25 $^{\circ}\text{C}$

Figures and Figure captions.

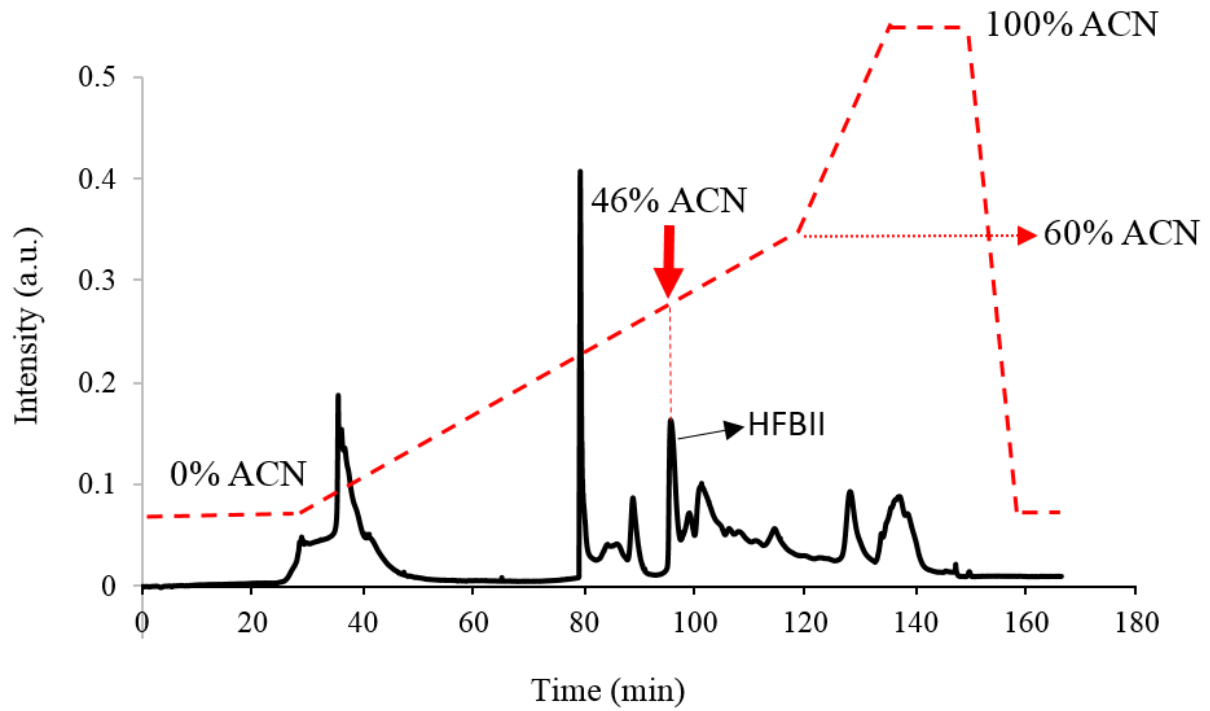


Fig. 1. An example of chromatograms for the culture medium containing hydrophobin HFBII resulting from fermentation of *T. reesei* after purification by SOURCE 30RPC column, recorded at a wavelength of 214 nm. The dashed line shows the ACN% gradient. HFBII was eluted with solvent containing 46% ACN and 0.1% TFA.

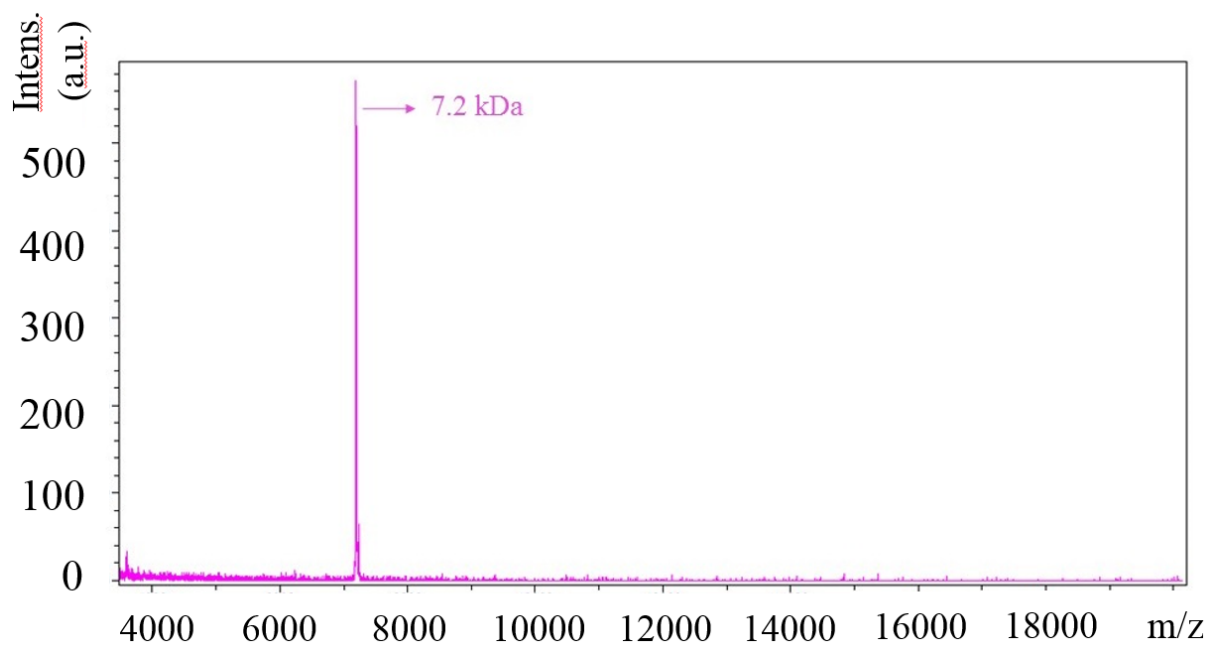


Fig. 2. MALDI-TOF spectrum of κ -HFBII obtained from *T. reesei* after the purification step using chromatography.

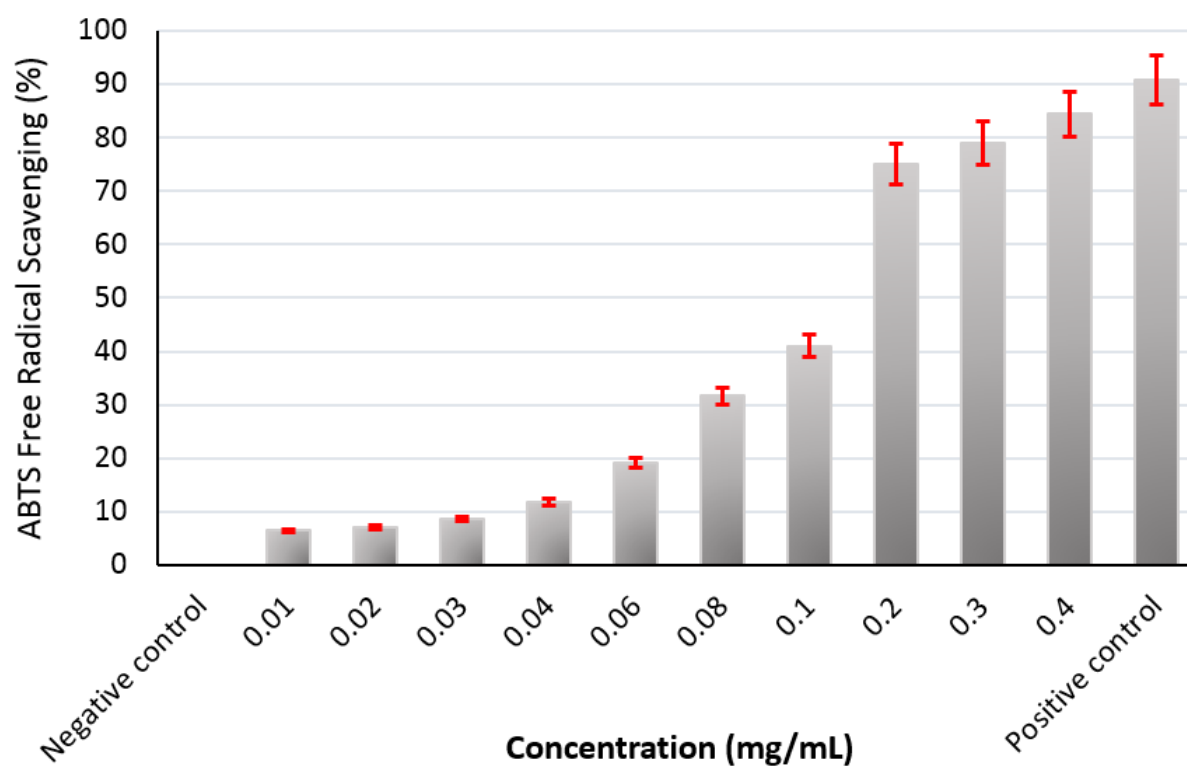


Fig. 3. Radical scavenging properties of Class II hydrophobin κ -HFBII from wild strain *T. reesei*. The negative control was considered to be the ABTS^{•+} solution without addition of κ -HFBII. The positive control was considered to be a mixture of bioactive peptides extracted from the Persian walnut with a concentration of 0.05 mg/mL.

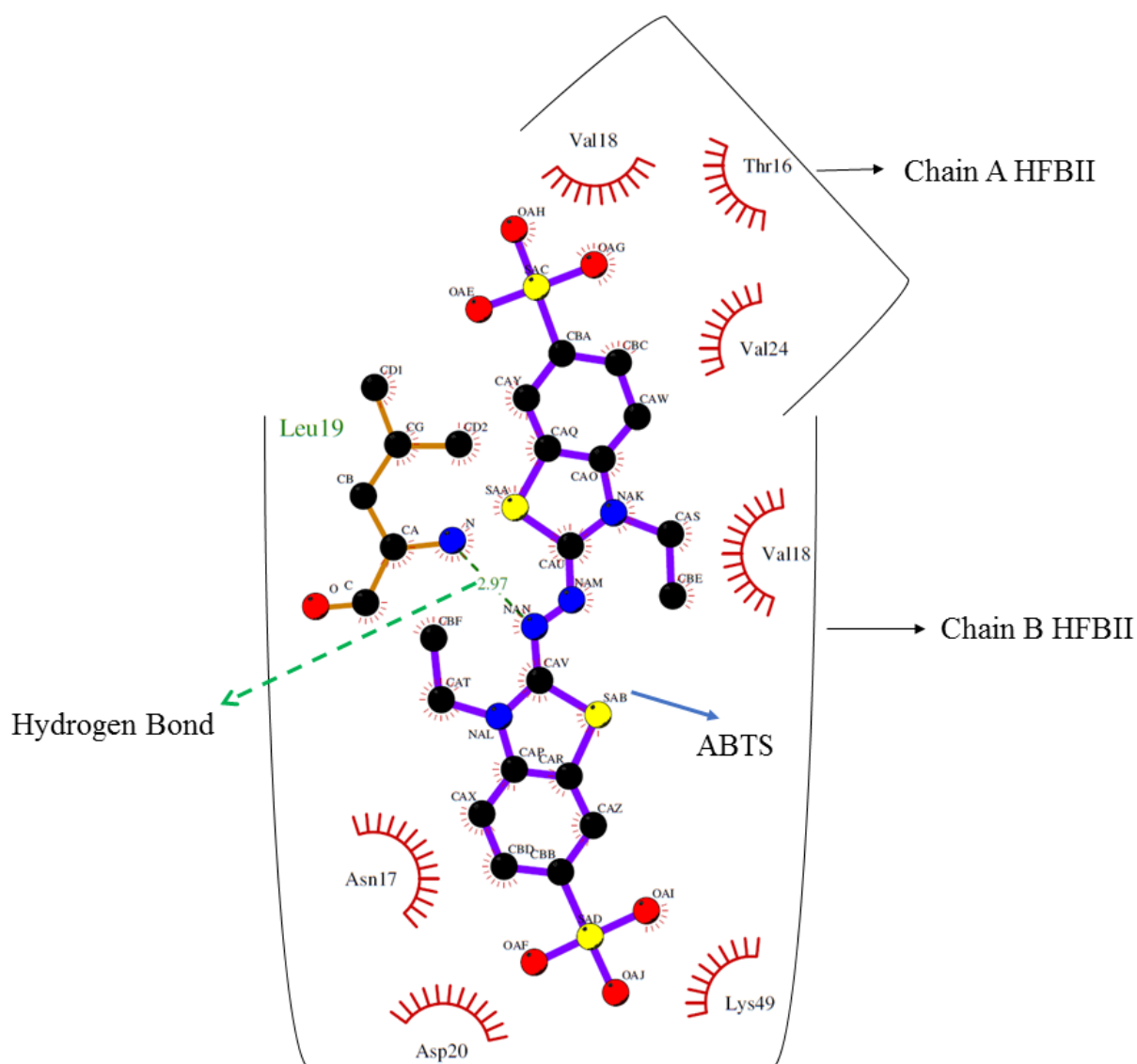


Fig. 4. Two dimensional image presentation of peripheral site residues of HFBII (dimer) and the molecule of ABTS. The most probable interacting residues of HFBII include Threonine₁₆, Valine₁₈, Valine₂₄ from Chain A HFBII, and Asparagine₁₇, Valine₁₈, Leucine₁₉, Aspartic acid₂₀, Lysine₄₉ from Chain B HFBII. A hydrogen bond between Leucine₁₉ from chain B and ABTS is occurred.

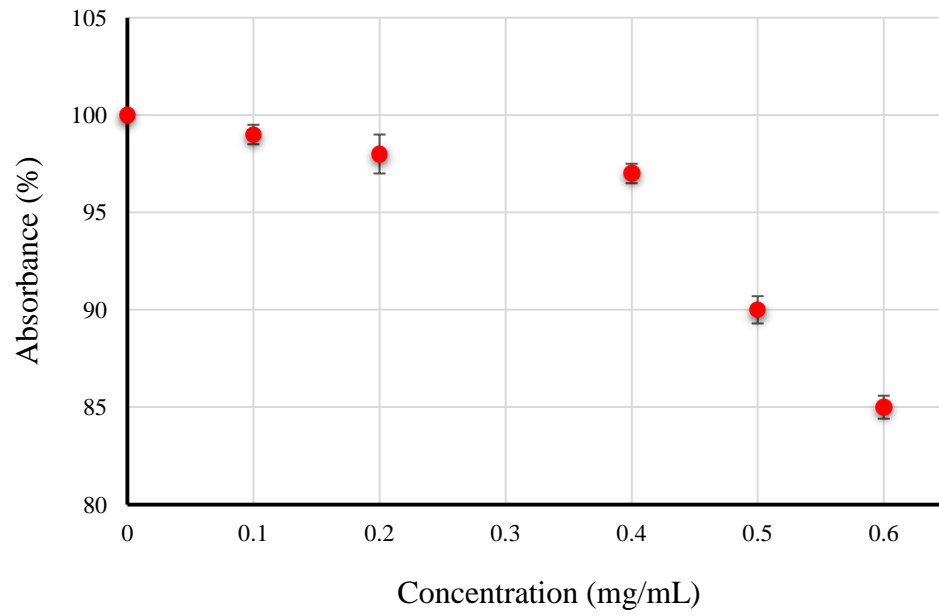


Fig. 5. ACE inhibitory of pure Class II hydrophobin HFBII produced by wild strain *T. reesei*.