Identification of Angiotensin-Converting Enzyme Inhibitory Proteins from Mycelium of *Pleurotus pulmonarius* (Oyster Mushroom)

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Introduction

Hypertension, also referred to as high blood pressure, is a medical condition in which the blood pressure level in the arteries is consistently above the normal range (> 140/90 mmHg). It has been listed as the 13th leading cause of death in the USA [1] and represents a major risk for cardiovascular maladies, which remains the number one leading cause of death worldwide [2]. Due to hypertension’s prevalence and its risks to human life, it is important to maintain a healthy lifestyle to reduce the risk of hypertension. However, anti-hypertensive drugs are concomitantly required to treat this disorder [3].

The regulation of blood pressure involves several mechanisms. RAAS is one of the key regulators of blood pressure, which involves a series of enzymatic reactions. ACE in RAAS is one of the enzymes that controls blood pressure by converting inactive angiotensin I to the potent vasoconstrictor angiotensin II [4]. Exploiting the inhibitor of ACE as one curative means for hypertension is therefore paramount. At present, ACE inhibitors have become a pharmacological target for the treatment of hypertension in addition to other beneficial effects on glucose and lipid metabolism, such as decreasing insulin requirements in diabetes and increasing exercise tolerance [5].

To date, ACE inhibitors such as captopril, enalapril, and lisinopril are the first-line synthetic drugs used as clinical antihypertensive medications [6]. Despite their remarkable efficacy as antihypertensive drugs, they may also cause deleterious effects such as dizziness, cough, allergies, and more serious complications such as proteinuria and disturbance of kidney function [7]. Hence, research and development to find safer hypertension remedies is greatly needed.

Abstract

Pleurotus pulmonarius (grey oyster mushroom) has been acknowledged as a recuperative agent for many diseases in addition to its recognition as a nutritious provision. We performed a study on P. pulmonarius mycelium for an antihypertensive effect via the angiotensin-converting enzyme inhibitory activity. The preliminary assay on the mycelial water extract demonstrated that the angiotensin-converting enzyme inhibitory activity had an IC50 value of 720 µg/mL. Further protein purifications via ammonium sulphate precipitation and RP-HPLC resulted in 60× stronger angiotensin-converting enzyme inhibitory activity than that of the mycelial water extract (IC50 = 12 µg/mL). Protein identification and characterisation by MALDI-TOF/TOF, later corroborated by LC-MS/MS, indicated three proteins that are responsible for the blood pressure lowering effects via different mechanisms: serine proteinase inhibitor-like protein, nitrite reductase-like protein, and DEAD/DEAH box RNA helicase-like protein.

Abbreviations

ACE: angiotensin-converting enzyme
BCA: bicinchoninic acid
BSA: bovine serum albumin
CHAMP: cardiac helicase activated by MEF2 protein
HIV: human immunodeficiency virus
ITS: internal transcribed spacer
MEA: malt extract agar
NOS: nitric oxide synthase
RAAS: renin angiotensin aldosterone system
XOR: xanthine oxidoreductase

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Key words

ACE inhibitor
bioactive protein
hypertension
fungi
Pleurotaceae
Pleurotus pulmonarius
oyster mushroom

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Recent interests have considered the isolation and identification of ACE inhibitors from natural products and foods as an alternative for synthetic drugs. These sources are acknowledged to carry a wide range of functional and biological properties with more safety and economical advantages. Several natural products from plant extracts [8,9] and fungi [10–12], as well as food products such as fermented foods [13] and milk proteins [14] have demonstrated ACE inhibitory potency and are proven to be amongst the most powerful known vasodilators. *Pleurotus pulmonarius* (Fr.) Quél (oyster mushroom) is one of the commonly recognised and therapeutically valuable Basidiomycetes mushrooms. Often mistakenly described as *Pleurotus sajor-caju* by mycologists and cultivators [15], this mushroom contains many mineral elements, fibre, and carbohydrates, which makes it suitable for dietary supplements [16]. Owing to its opulence in bioactive compounds and crude proteins, it has demonstrated many medicinal properties including an antiproliferative effect on hepatoma and leukaemia cells [17], antioxidant activity [18], and antiviral properties against HIV-1 reverse transcriptase [19]. Of late, the health promoting effects of mushroom mycelia have been cited in quite a few research reports [20–23]. However, no study has been conducted to determine the ACE inhibitory activity from the *P. pulmonarius* mycelium. Hence, this study was designed to investigate the ACE inhibitory property from the proteins of *P. pulmonarius* mycelium.

**Results and Discussion**

In our preliminary study on ACE inhibitory activity performed on *P. pulmonarius* mycelial water and broth extracts, a mycelial water extract was shown to have inhibited ACE activity with an IC50 value of 720 µg/mL when tested between the concentration of 0 to 3 mg/mL, an approximately two times higher activity than that of the broth extract tested at the same concentrations (data not shown). Our mycelial water extract indicated stronger ACE inhibitory activity than the water extracts of mycelia from six different mushrooms, namely *Cathathelus ventricosum*, *Lactarius camphoratus*, *Pholiota adipose*, *Phellinus linteus*, *Thelephora ganbajan*, and *Tricholoma giganteum*, with their IC50 values ranging from 1.28 to 5.25 mg/mL [24]. The mycelial water extract was thus selected for a further anti-ACE activity study. To probe for the active ACE inhibitors from the mycelial water extract, bioassay-guided protein purifications were successively incorporated to solely examine proteins with ACE inhibitory properties, thus eliminating unrelated proteins and other compounds. First, proteins from the mycelial water extract were fractionated via the salting-out method, with salt concentrations ranging from 10% to 100%, and the fraction yields were labelled as A to J. All fractions were tested for the ACE inhibitory assay at the concentration of 15 µg/mL, this being D6 with 57.7%. In Fig. 1, the most active fraction is indicated with an arrow. The inhibitory activity exhibited by D6 was significantly different from the other protein fractions (p < 0.05) and was thus selected for subsequent analysis. The IC50 value of D6 was evaluated. From the comparison of the IC50 values, it was confirmed that further protein purifications showed a greater efficacy of the ACE inhibitory activity. The IC50 value of fraction D6 indicated an enhanced ACE inhibitory effect by 60× compared to the mycelial water extract (Table 2). Hence, we have postulated the presence of ACE inhibitors that function individually and/or specific peptide inhibitors of ACE, which denote a good ACE inhibitor(s).

Likewise, the inhibitory effect shown by D6 was stronger than some other naturally occurring ACE inhibitors discovered in other medicinal mushrooms. For instance, the ACE inhibitory activity of D6 was approximately 10× stronger than an active peptide from *Grifola frondosa* (IC50 = 0.13 mg/mL) [26]. Other reported ACE inhibitors were from an active tripeptide of *T. giganteum* (IC50 = 0.04 mg/mL) [27], an active pentapeptide of *Pholiota adiposa* (IC50 = 0.044 mg/mL) [28], and two oligopeptides from *Pleurotus cornucopiae* (IC50 = 0.46 and 1.14 mg/mL, respectively) [29]. SDS-PAGE was then performed on fractions D and D6. The results indicated four distinct protein bands visualised from D6, and all of the bands were analogous with the bands from fraction D (Fig. 2). These four bands were labelled D6-a, D6-b, D6-c, and D6-d, and underwent protein identification by MALDI-TOF/TOF mass spectrometry. Based on MALDI-TOF/TOF MS data, a Mascot search showed a number of proteins for each protein band. Three antihypertensive-related proteins were then identified, i.e., a nitrite reductase-like protein from D6-c, which was identified from SwissProt-Fungi, and serine proteinase inhibitor-like protein and DEAD/DEAH box helicase-like protein from D6-d, which were identified from NCBI-Pleurotus ostreatus and NCBI-Fungi, respectively (Table 3). Studies by other researchers have found the contribution of these proteins to the regulation of blood pressure through several mechanisms. The role of each protein candidate will be discussed afterwards.

The meagre protein scores by a Mascot search (Table 3) were due to inadequate mushroom and/or fungal databases available for proteomic and genomic studies, which has also resulted in adopting more than one species database to expand the search

<table>
<thead>
<tr>
<th>Protein fraction</th>
<th>ACE inhibitory activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>39.5 ± 3.6*</td>
</tr>
<tr>
<td>B</td>
<td>42.9 ± 0.7**</td>
</tr>
<tr>
<td>C</td>
<td>37.5 ± 3.2**</td>
</tr>
<tr>
<td>D</td>
<td>61.2 ± 2.4**</td>
</tr>
<tr>
<td>E</td>
<td>38.6 ± 2.7**</td>
</tr>
<tr>
<td>F</td>
<td>48.1 ± 1.6**</td>
</tr>
<tr>
<td>G</td>
<td>42.3 ± 1.6**</td>
</tr>
<tr>
<td>H</td>
<td>32.5 ± 3.1**</td>
</tr>
<tr>
<td>I</td>
<td>31.2 ± 2.1**</td>
</tr>
<tr>
<td>J</td>
<td>33.1 ± 2.7**</td>
</tr>
</tbody>
</table>

The results are expressed as mean ± standard deviation (n = 3); Values are expressed as means ± SEM of triplicate values; Means with different alphabetical letters (a–c) denote a significant difference at p < 0.05
capacity. This discrepancy of protein sequences beyond the homology or conserved regions may also contribute to the drawback, especially when comparing different species [30]. However, an excess of 40% sequence similarity shown by DEAD/DEAH box RNA helicase and nitrite reductase-like protein has warranted a hypothetically correct match [31].

Fraction D6 was also subjected to LC-MS/MS to validate the proteins identified from MALDI-TOF/TOF MS. Intriguingly, only one antihypertensive protein that had been identified by MALDI recurred in LC-MS/MS, which was the DEAD/DEAH box RNA helicase-like protein. The presence of this protein as one of the ACE inhibitors in *P. pulmonarius* mycelium was substantiated by its detection in both proteomic tools. Based on the overall study performed here, DEAD/DEAH box RNA helicase-like protein has been shown to dwarf the other two proteins in regard to its more concrete data obtained from the proteomic platforms. However, all proteins are acceptable as potential ACE inhibitors in view of the active in vitro ACE inhibitory assays that conclusively demonstrated a strong IC$_{50}$ value of peak D$_6$.

The IC$_{50}$ values were determined within tested concentrations of 0 to 3 mg/mL (n = 3) for the mycelial water extract and 0 to 150 µg/mL for fractions D and D$_6$ (n = 3). An extremely strong IC$_{50}$ value of captopril was well inferred, as it is a first-line clinical ACE inhibitor.

### Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC$_{50}$ value (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycelial water extract</td>
<td>720</td>
</tr>
<tr>
<td>Fraction D (salting-out method)</td>
<td>22</td>
</tr>
<tr>
<td>Fraction D$_6$ (RP-HPLC)</td>
<td>12</td>
</tr>
<tr>
<td>Captopril (positive control)</td>
<td>$1.0 \times 10^{-11}$</td>
</tr>
</tbody>
</table>

The IC$_{50}$ values were determined within tested concentrations of 0 to 3 mg/mL (n = 3) for the mycelial water extract and 0 to 150 µg/mL for fractions D and D$_6$ (n = 3). An extremely strong IC$_{50}$ value of captopril was well inferred, as it is a first-line clinical ACE inhibitor.

The DEAp/DEAH box RNA helicase protein has long been known to be involved in RNA metabolism and cellular functions. However, an earlier study by Sahni et al. [32] postulated an antihypertensive property of DEAD/DEAH box RNA helicase-like protein through the regulation of cardiac cellular mechanisms, via upregulation of CHAMP which is an RNA helicase (belonging to the DEAD box proteins) that acts through general cell cycle machinery by inhibiting cell cycle proliferation. Antiproliferative activity by CHAMP is mediated by the cell cycle inhibitor p21$^{CDI}$ and the ATPase domain [33]. In cardiac hypertrophy cases, the inhibitory activity weakens the wall stress and prevents or reverses the hypertrophic phenotype [34]. This may allow restoration of blood flow and its pressure effect. A study performed by Mohamad An- sor et al. reported anti-ACE activity from the DEAD/DEAH box RNA helicase-like protein in *Ganoderma lucidum* mycelium and

![Fig. 1](image1.png)

**Fig. 1** RP-HPLC profile of fraction D at wavelength 220 nm. The collected peaks are labelled as D$_1$–D$_8$ sequentially. The strongest ACE inhibitory activity (D$_6$) is indicated by an arrow. (Color figure available online only.)

![Fig. 2](image2.png)

**Fig. 2** SDS-PAGE of fraction D and RP-HPLC peak, D$_6$. PM refers to the protein marker (broad range), while D$_6$-a, D$_6$-b, D$_6$-c, and D$_6$-d represent protein bands that emerged from fraction D$_6$. (Color figure available online only.)
postulated the presence of a synergistic inhibitory effect of ACE and a cardiac cellular hypertensive effect [35]. Hence, we have hypothesised the same for our DEAD/DEAH box RNA helicase-like protein.

Noticeably, there was a discrepancy between the estimated molecular masses of DEAD/DEAH box RNA helicase demonstrated in MALDI, LCMS/MS, and band D6-d of SDS-PAGE. This was predictable as the size of the helicases is different among species, often caused by N- and/or C-terminal extensions [36]. Degradation that occurs intracellularly, such as during apoptosis [37] or during the experimental procedures, might also contribute to protein fragmentations.

The second antihypertensive protein is a nitrite reductase-like protein. It is involved in the hypertensive mechanism via the NOS independent pathway by converting NO2⁻ to NO. In the human microvascular system, NO mediates endothelium-dependent vasodilation and inhibits smooth muscle cell proliferation [38]. A study by Gladwin et al. [39] reported maximum reductase activity by the nitrite reductase enzyme under hypoxic conditions to support vasodilation, thus restoring blood flow and oxygen demand to the cells. Another clinical study showed an increase of erythrocyte XOR-nitrite reductase activity with enhanced vasodilator activity and a blood pressure lowering effect in spontaneously hypertensive rats [40].

Nitrite reductase-like protein may contribute to ACE inhibitory activity through the interaction between the NO pathway and RAAS via vascular (endothelial) dysfunction mechanisms [41]. However, there is as yet no supporting data for our hypothesis asserting the synergistic effect of nitrite reductase as an ACE inhibitor. Yet, there is room for further investigation due to complex mechanisms in the cardiovascular system.

Concern due to divergence on the estimated molecular mass of nitrite reductase between MALDI (Table 3) and the SDS-PAGE band (D6-c, Fig. 2) is understandable. Apparently, in several studies, a reduced molecular mass of nitrite reductase has been observed for various reasons, mainly protein denaturation [42, 43]. The denaturation of large proteins or proteins with subunits leads to the detection of truncated protein by the gel [44]. Protein degradation occurring due to technical reasons also causes molecular mass reduction [45].

The final antihypertensive protein is a serine proteinase inhibitor-like protein. Proteinase (protease) inhibitors have long been investigated for the treatment of HIV, hypertension, and other cardiovascular diseases such as atherosclerosis [46]. Serine proteinase inhibitors are directly involved in RAAS as suppressors of serine proteinase, an enzyme that is responsible for the formation of cardiac angiotensin II via the ACE-independent enzymatic pathway. Chymase, for example, is a type of efficient serine proteinase that cleaves angiotensin I to form angiotensin II at the same site as ACE [47].

Although information about proteinases from mushrooms and their inhibitors is still limited, several proteinase inhibitors have been found in mushrooms, and many have shown unique characteristics, which may be exclusive to Basidiomycetes [48]. Two serine proteinase inhibitors with respective molecular masses of 8307 and 8244 were discovered from P. ostreatus [49]. Our serine proteinase inhibitor-like protein corresponds with fragments 11 to 19 of a total 76 amino acids of serine proteinase inhibitors from P. ostreatus, with two amino acid differences. Another serine proteinase inhibitor was found from Lentinus edodes. This inhibitor did not show similar characteristics to any major group of serine proteinase inhibitors available in the databases, and was tested to show a positive result for blood coagulation and fibrinolysis [50].

Studies reported a high resistance of serine proteinase to ACE inhibitors but no report confirmed a resistance of ACE towards serine proteinase inhibitors [51]. According to Petrov et al. [52], a neutral serine proteinase inhibitor called leupeptin was reported to inhibit ACE activity in human T-lymphocytes. Based on the overall function of the serine proteinase inhibitor in RAAS, we have hypothesised synergistic effects towards serine proteinase and ACE indicated by our serine proteinase inhibitor-like protein. An in vitro study has proven that P. pulmonarius mycelium contains highly potential antihypertension-related proteins. Hence, this study forms a basis for in vivo validation. In addition, a profound research is required especially on the isolation of pure ACE inhibitory protein(s). A thorough mechanistic approach is also necessary to elucidate how the identified proteins are involved in the blood pressure machinery, concomitantly to in vivo studies of the proteins, prior to the claim of P. pulmonarius mycelium becoming an alternative to synthetic antihypertensive remedies.

Materials and Methods

Cultivation of Pleurotus pulmonarius mycelium

P. pulmonarius mycelium culture was kindly obtained from the Mushroom Research Centre, University of Malaya. Based on morphological characters and DNA sequence data of the ITS regions, the species studied was identified as P. pulmonarius and deposited in the Mycological Laboratory of the Mushroom Research Centre, University of Malaya, with the registration number KUM61119. A voucher specimen was deposited in the University of Malaya Herbarium under the registration number KLU-M1234. The culture was grown and maintained on MEA (Thermo Fisher Scientific) at 25 °C. For submerged fermentation purposes, 7-day-old plate cultures were used by piercing ten mycelial plugs (10 mm diameter) from the periphery of the culture using a sterile cork borer and inoculating them into sterilised 500 mL Erlenmeyer flasks, each containing 100 mL of brown sugar–spent yeast liquid media in the percentage ratio of 1:2 (w/v). The flasks were incubated for seven days at room temperature under agitation at 150 rpm using an SK 300 rotary shaker (Jeio Tech Co. Ltd.). The culture was then harvested by separating the mycelial biomass from the whole broth. The mycelial biomass was washed with running distilled water, and both the mycelial biomass and whole broth were freeze-dried at −52 °C (Labconco). The freeze-dried samples were ground into powdered form and stored at 4 °C.

<table>
<thead>
<tr>
<th>Putative protein</th>
<th>Accession number</th>
<th>Protein score (%)</th>
<th>MW (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrite reductase (D6-c)</td>
<td>P22944 (SwissProt-Fungi)</td>
<td>48</td>
<td>123994</td>
</tr>
<tr>
<td>Serine proteinase inhibitor (D6-d)</td>
<td>sp/Q7M4 T5 (NCBI-P. ostreatus)</td>
<td>6</td>
<td>8190</td>
</tr>
<tr>
<td>DEAD/DEAH box RNA helicase (D6-d)</td>
<td>gi</td>
<td>242767802 (NCBI-Fungi)</td>
<td>67</td>
</tr>
</tbody>
</table>
Preparation of mycelial water extract
Mycelial water extract was prepared by direct infusion of the freeze-dried mycelial biomass (10 mg) in 100 mL distilled water (ratio of 1:10). The extract was blended and then stirred vigorously on an ice bath for one hour to achieve homogeneity. The supernatant was collected by centrifugation at 5000 rpm for 10 min at 4°C and employed freshly for further analysis. Freshly prepared mycelial water extract was diluted to an appropriate concentration for the preliminary ACE inhibitory test.

Protein fractionation of the mycelial water extract by the salting-out method
Proteins from the mycelial water extract were fractionated by the salting-out method using ammonium sulphate, which was increased gradually from 10% to 100% of the salt saturation. The precipitated protein from each salt saturation was obtained by constant stirring for 30 min on an ice bath, followed by centrifugation at 10000 rpm for 15 min at 4°C. The pellet was then dissolved in 4 mL distilled water and then dialysed four times against 1000 mL distilled water using Snakeskin Dialysis Tubing™ (Thermo Fisher Scientific), with a 3500 molecular weight cutoff for 48 h. Protein fractions were freeze-dried and stored at −20°C for further analysis.

Protein estimation
The protein concentration was estimated using the Pierce® BCA protein assay kit (Thermo Fisher Scientific), per the manufacturer’s instructions. The absorbance value was read at 562 nm using a Sunrise™ ELISA microplate reader (Tecan Group Ltd.). The protein concentration was determined by referring to the standard curve graph of BSA.

Angiotensin-converting enzyme inhibitory assay
The ACE inhibitory activity of the mycelial water extract and the protein fractions was analysed using ACE Kit-WST (Dojindo Laboratories) as instructed in the manufacturer’s protocol [53]. The assay used 3-hydroxybutryrylglycyl-glycyl-glycine (3HB-GGG) as a substrate for the screening of the ACE inhibitor. The absorbance value of the assay reaction was read at 450 nm using a Sunrise™ ELISA microplate reader. Captopril ≥ 98% (C4202-25 g) (Sigma-Aldrich Co.) was used as a positive control in this study.

Identification and reconfirmation of ACE inhibitor proteins by MALDI-TOF/TOF MS
Potent ACE inhibitors from the RP-HPLC peak were identified by MALDI-TOF/TOF MS. The resolved bands of the RP-HPLC peak from SDS-PAGE gel were excised and in-gel digested with 6 ng/µL trypsin (37°C overnight) and then desalted using Zip Tip (C18) (EMD Milipore Co.). The samples were analysed using a 4800 Plus MALDI TOF/TOF MS Proteomics Analyser (Applied Biosystems) combined with Mascot database software (Matrix Science Ltd., London, UK). Protein identification was carried out using the following acquisition settings: Databases – Fungi (SwissProt), Fungi (NCBI), and P. ostreatus (NCBI); protein mass range – 0 kDa to 100 kDa; mass tolerance between 0.5 Da to 1.15 Da – one missed cleavage.

SDS-PAGE analysis of mycelial protein fractions
Protein fractions from ammonium sulphate precipitation and RP-HPLC that demonstrated the strongest ACE inhibitory activity were further separated using SDS-PAGE by Laemmli [54]. The sample buffer and protein fractions were mixed at a ratio of 1:3 (v/v) and preheated at 95°C for 5 min prior to electrophoresis. The samples and prestained SDS-PAGE standards broad range molecular weight (Bio-Rad Laboratories Inc.) were then loaded into the gel, and electrophoresis was performed at a constant voltage of 60 V for the stacking gel (4% polyacrylamide) followed by 100 V for the separating gel (16% polyacrylamide). The gel was silver stained to visualise the protein bands.

Statistical analysis
Mean values for all the parameters tested were obtained and subjected to one-way ANOVA using Minitab statistical software (Minitab, Inc.). The mean values were tested for significance using the multiple range tests at 95% and least significant difference, p < 0.05.

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Conflict of Interest

The authors declare that they have no conflicts of interests.

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