Characterisation of novel angiotensin-I-converting enzyme inhibitory tripeptide, Gly-Val-Arg derived from mycelium of Pleurotus pulmonarius

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A R T I C L E  I N F O

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ACE inhibitor
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A B S T R A C T

It has been shown that fraction D6 from Pleurotus pulmonarius has the potential to inhibit ACE. After this discovery, additional studies were conducted to obtain peptides from that fraction, as ACE inhibitors. By size exclusion chromatography, single peak was resolved and termed as Psec. The IC50 of Psec was assessed to be 4.50 μg/mL, which was 2.5 times lower than that of D6. When Psec was resolved by SDS-PAGE, three bands with estimated molecular weight of 63 kDa, 55 kDa and 11 kDa were observed. The protein bands were subjected to MALDI-ToF MS/MS for protein identification. By using the BIOPEP database for predicting in silico digestion of gastrointestinal (GI) enzymes, four stable tripeptides with ACE inhibitor potential resulting from GI enzyme digestion were identified, namely GVR, VVR, NPR, and VVL. The IC50 was estimated to be 55 μg/mL, 93 μg/mL, 110 μg/mL and > 250 μg/mL individually. Based on a Lineweaver-Burk plot, tripeptide GVR was determined to be a competitive inhibitor and this was confirmed by molecular docking analysis. At 100 mg/kg of body weight (bw), the tripeptide GVR reduced SBP 33.5 mm Hg in SHRs. The results suggested that this tripeptide is potentially beneficial as an antihypertensive agent.

1. Introduction

Hypertension affects up to 30% of adults in most countries, with more than 50% of hypertensive persons unaware of their illness [1]. High blood pressure also affects the aging Malaysian population. Its occurrence in the elderly in city and rural regions is 62% and 26%, respectively. Around 58.3% of the aging population in northern Malaysia have high blood pressure. The occurrence of high blood pressure in central Malaysia is 25.6% and 51.1% in the community and in nursing homes, respectively. Around 58.3% of the aging population in northern Malaysia have high blood pressure. The occurrence of high blood pressure in central Malaysia is 25.6% and 51.1% in the community and in nursing homes, respectively. The rennin-angiotensin system (RAS) is a highly documented humoral system for blood pressure control. Its' malfunctions are linked to the pathophysiology of hypertension [3]. Angiotensin-I converting enzyme (ACE) plays a significant role in blood pressure regulation and high blood pressure. The inactive angiotensin-I is converted into a potent vasoconstrictor, angiotensin-II, by ACE. ACE also deactivates the vasodilator bradykinin [4]. Inhibition of ACE activity is thus a beneficial therapeutic method in the treatment of high blood pressure. Several synthetic ACE inhibitors like captopril and enalapril, have been used widely as antihypertensive medicines. The synthetic ACE inhibitors however cause many adverse effects such as dry cough, angioedema and many other dysfunctions of human organs. There is still the need to discover new antihypertensive drugs which are inexpensive and have minimal side effects [5,6].

Food origin antihypertensive peptides have attracted much attention from both investigators and users. The most well recognised mechanism underlying the blood-pressure-lowering effect of food-origin peptides appears to be inhibition of ACE activity [7]. Recently, many investigators have revealed the ACE inhibitory activity of peptides from food protein, such as grass carp protein [8], Spanish dry-cured ham [9], Acaudina molpadioidea [10], mycelium of the oyster mushroom [11] and salmon [12]. Through enzymatic digestion in the animal or human model or in vitro simulation of enzymatic digestion, protein can be digested into peptides and can be used to exert the physiological action. These peptides thus signify possible health-improving nutraceuticals for diet and therapeutic uses. Since peptides originating from food proteins may possess an ACE inhibiting action, they potentially can be used as natural substitutes for ACE inhibitor medications [13].

Pleurotus pulmonarius (Pp) is one of the most famous palatable
mushrooms in the South East Asia countries, including Malaysia. It is well-known for the delicious and succulent taste and regularly incorporated into many dishes. Apart from being nutritious, this species was shown to have several nutraceutical properties. The methanol extract of Pp’s fruiting bodies was shown to have antioxidant, anti-cholesterolesterase and anti-inflammatory activities, which may due to the phenolic compounds in the sample [45]. Pp aqueous extract that is high with antioxidant activity was also able to inhibit oxidation of low-density lipoprotein, which is the early event for atherosclerosis development [46]. In vivo study also demonstrated the anti-cancer property of Pp water extract where it inhibits the liver cancer cell line Huh7 on nude mice [47]. Apart from its antioxidant potential, the protein extract of Pp has an anti-diabetic property where it can inhibit enzymes involved in carbohydrate digestion [48]. As for anti-hypertension, both fruiting body and mycelial extract of the species was shown to inhibit ACE activity [49,11].

In a previous study, we have shown the ACE inhibitory activity of fraction D₆ using reverse phase chromatography of Pleurotus pulmonarius mycelium extract [11]. Continuing from this study, further analyses were carried out on the fraction in order to discover a pure peptide with potential ACE inhibiting activity. This study was hence designed to investigate the ACE inhibitory activity of stable tripeptides resulting from digestion by gastrointestinal enzymes from P. pulmonarius mycelium, and their effect on spontaneously hypertensive rats (SHRs).

2. Materials and methods

2.1. Preparation of fraction D₆ from Pleurotus pulmonarius mycelium

The method used was as described previously by [11]. Briefly, Pleurotus pulmonarius (KUM61119) mycelium was grown in brown sugar-spend yeast liquid media for 7 days at room temperature, with agitation. The mycelial biomass was then harvested, freeze-dried and ground into powder. A crude water extract was prepared by dissolving the powder in distilled water at 10% concentration. The mixture was blended and stirred vigorously for an hour to achieve homogeneity and this was followed by collection of the supernatant after centrifugation.

To prepare the F40 fraction, ammonium sulphate was added to the supernatant up to 30% saturation and stirred for 30 min in an ice bath. The supernatant was collected by centrifugation and more ammonium sulphate was added until 40% salt saturation was reached. The protein precipitate from this fraction was collected by centrifugation at 10,000 rpm for 15 min at 4 °C. After dialysis four times against distilled water using 3500 MWcutoff Snakeskin Dialysis Tubing™ (Thermo Fisher Scientific, Illinois, USA), the fraction was freeze-dried.

To prepare fraction D₆, F40 was subjected to RP-HPLC using a C18 SemiPrep column (C18, 100 × 10 mm, 2 μm, 130 Å; Merck KGaA, Darmstadt, Germany), as previously described. Fraction D₆ was collected, dried by evaporation and kept at −20 °C.

2.2. Size exclusion chromatography (SEC) of fraction D₆

Fraction D₆ was further fractionated by SEC with a Biosep SEC-S2000 column (300 × 7.8 mm, Phenomenex, Torrance, CA, USA) on a HPLC system fitted with a SCL-10AVP system controller, LC-10ATVP solvent delivery unit, SPD-M10AVP UV–vis diode array detector and equipped with a DGU-12A degasser (Shimadzu, Kyoto, Japan).

The isocratic mode was employed using 45% acetonitrile with 0.1% TFA as the mobile phase while the flow rate was maintained at 1.0 ml/min. The effluent was monitored at the wavelength of 214 nm and the observed peak was then collected, freeze-dried and kept at −20 °C [14].

2.3. Protein content estimation

Using a Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Illinois, USA), the estimation of protein concentration was performed as outlined by the manufacturer. With a Sunrise™ ELISA microplate reader (Tecan Group Ltd, Männedorf, Switzerland), the absorbance of the sample and a bovine serum albumin (BSA) standard were measured at 562 nm and the protein concentration of the sample was estimated using the BSA standard curve graph.

2.4. ACE inhibitory activity

The inhibition of the ACE activity was determined using the ACE inhibitory assay kit (ACE kit-WST, Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer’s protocol and the absorbance of the sample at 450 nm was read on a Sunrise™ ELISA microplate reader (Tecan Group Ltd). The concentration of the stock sample used in this analysis was 14 μg/mL. The percentage of ACE inhibitory activity of the sample was calculated using the formula provided by the manufacturer. The IC₅₀ value of the sample was determined from the plotted graph.

2.5. SDS-PAGE analysis of protein fraction from SEC

The protein fraction from SEC was further separated using SDS-PAGE, as described by [15]. The sample was preheated at 95 °C for 5 min prior to the electrophoresis. The electrophoresis was performed at a constant voltage of 60 V for the stacking gel (4% polyacrylamide) followed by 100 V for the separating gel (15% polyacrylamide). At the end of the electrophoresis, the bands were developed using a silver staining method [16]. A prestained SDS-PAGE broad range molecular weight standard (BIO RAD, California, US) was used to estimate the molecular weight of the produced bands.

2.6. Protein identification by MALDI-TOF/TOF MS

The preparation of samples for protein identification using MALDI-TOF/TOF MS was carried out according to [17]. The samples were analysed with an ABI 4800 Plus (Applied Biosystems, Foster City, CA) coupled with Mascot database software (Matrix Science Ltd., London, UK). Protein identification was performed based on the SwissProt (Fungi) and NCBI (Fungi) databases.

2.7. In silico gastrointestinal enzyme digestion analysis

The parent peptides identified by MALDI-TOF/TOF MS were subjected to in silico analysis using the BIOPEP database to produce gastrointestinal enzyme-digestion stable triptides [18]. Using that database, each parent peptide from MALDI-TOF/TOF MS data was tested against trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1) and pepsin (pH > 2) (EC 3.4.23.1). Apart from resistance to gastrointestinal enzyme digestion, another criterion the peptides were chosen on was based on the potent amino acids composition in the sequence as described by [19].

2.8. Peptide synthesis and IC₅₀ determination of the synthesised peptides

Four triptides with ACE inhibitory potential (identified by the BIOPEP database) and have not been reported previously for their ACE inhibitory activity were chosen and chemically synthesised by Mimotopes, Australia. The purity of the synthesised peptides was > 95%, as measured by RP-HPLC. The peptides were then tested for ACE inhibitory activity as described above.

2.9. Determination of the ACE activity inhibition pattern

Determination of the type of enzyme inhibition of ACE by GVR was
adopted from [20] with some modifications. HHL (Sigma, Missouri, USA) concentrations used were 0.375, 0.75, 1.50 and 3.00 mM. HHL was incubated with ACE in the presence of GVR, at a concentration of 0.031 and 0.25 mg/mL or without GVR (control). From the Lineweaver-Burk plots, the ACE inhibition pattern was determined.

2.10. Molecular docking of tripeptide GVR and ACE

Molecular docking studies were performed using AutoDock 4.2 [21] and AutoDockTools 1.5.6 software [22]. The 3D structure of ACE protein was downloaded from the Protein Data Bank [23] (PDB ID: 1UZE with 1.82 Å resolution) and was used as the receptor in this study. All hetero atoms including water molecules were removed. The atomic coordinates of 1UZE were stored in a separate file and used as input to AutoDockTools, where polar hydrogens, Kollman charges and solvation parameters were then added. The ligand GVR was constructed using ACD/ChemSketch Freeware (Advanced Chemistry Development Inc. Ontario, Canada) and geometry-optimised with MMFF94 force field [24] using Avogadro Software [25]. The ligand was then prepared for docking analysis by merging the non-polar hydrogen atoms and defining its rotatable bonds. Grid maps of 126 × 126 × 126 with 0.375 Å grid spacing were used to define the binding site. The grid box was centred at coordinates (40.54, 37.55, 43.48). The Larmarckian genetic algorithm with local search was used as the search engine, with a total of 100 runs. In each run, a population of 150 individuals with 27,000 generations and 250,000 energy evaluations were employed. Operator weights for crossover, mutations and elitism were set at 0.8, 0.02, and 1.0, respectively. The ligand-protein complex was visualised and analysed using AutoDockTools.

2.11. In vivo activity of tripeptide GVR in spontaneously hypertensive rats

Twenty-five SHRs aged 11 weeks (weight 280–296 g) were purchased from the Animal Experimental Unit, University of Malay. The principles and guidelines for animal care, research conduct and sacrifice procedure approved by the institution were followed (2015–161103/IBS/R/SM). The rats were separated into five groups: negative control, positive control (captopril, 5 mg/kg of body weight) and three experimental peptide dosages (10, 50, 100 mg/kg). They were kept in a 12 h light-dark cycle, with temperature and humidity at 23 ± 1 °C and 50 ± 5%. A diet of Altromin No. 1324, (Altromin GmbH, Lage, Germany) and tap water were supplied if the identification of fraction D6 was confirmed that fraction D6 contained 0.98 μg/mL of protein Psec. When ACE inhibitory activity was ascertained, fraction Psec yielded an IC50 value of 4.5 μg/mL, which was 2.5 times better than fraction D6 (IC50 12 μg/mL).

2.12. Statistical analysis

One way ANOVA was performed using SPSS 16, for short term administration of peptide.

3. Results

3.1. Purification of fraction D6 using size exclusion chromatography

Peak D6 was successfully collected from the reverse phase chromatography and dried. The lyophilised peak D6 was then reconstituted in Milli Q water and injected into HPLC for size exclusion chromatography. Only one prominent peak was observed at minute 10, which was collected and labelled as Psec (Fig. 1).

3.2. Protein content estimation and ACE inhibitory assay of fraction from SEC

Based on the bovine serum albumin (BSA) standard, it was estimated that fraction D6 contained 0.98 μg/mL of protein Psec. When ACE inhibitory activity was ascertained, fraction Psec yielded an IC50 value of 4.5 μg/mL, which was 2.5 times better than fraction D6 (IC50 12 μg/mL).

3.3. SDS-PAGE of Psec and peptide identification by MALDI-T of MS

Psec was resolved into three bands as compared to four bands for D6, when resolved with SDS-PAGE. As previously published, the four bands were labeled as D6-a, D6-b, D6-c and D6-d (Fig. 2) but the D6-c band was observed only in fraction D6 and was not present in Psec. From the SDS-PAGE of Psec, it could be suggested that the peak Psec from size exclusion chromatography was still a mixture of proteins.

The resolved bands of D6-a, D6-b, and D6-d were carefully excised and processed for MALDI-TOF/TOF MS analysis to identify the possible protein(s) of the bands and, more importantly, to screen the peptide sequences having potential ACE inhibitory characteristics (Table 1). The results obtained further supported the proposal that fraction Psec was a mixture of proteins, which also explained the low score obtained for protein identity. The peptide sequences acquired from the analysis were then screened for ACE inhibitory characteristics.

3.4. In silico gastrointestinal enzyme digestion analysis

The BIOPEP database was used to predict the stability of the peptides derived from MALDI-TOF/TOF MS. Some peptide sequences were not chosen in spite of their stability based on the in silico digestion; if the digestion products did not possess amino acids or a significant arrangement of amino acids in the N and C terminals of the sequences that are responsible for ACE inhibition. Based on the in silico analysis, four tripeptide sequences with the characteristic of ACE inhibitory activity were selected. Table 2 represents the enzymes’ action on the peptides, as predicted by the BIOPEP database. In Table 2, some peptide sequences are highlighted to show the selected peptide sequences for further studies.

3.5. ACE inhibitory activity of the synthesised tripeptides

To confirm if the identified tripeptide sequence would be able to inhibit ACE, an inhibition assay was performed on the synthesised tripeptides and the IC50 value was determined. Among the four tripeptides, GVR was the best in inhibiting ACE with an IC50 value of 55 μg/mL (Table 3). This indicated the possible relationship between the structure of the peptides and its’ ACE inhibitory activity.

3.6. Determination of ACE inhibition by GVR

To further characterise the tripeptide GVR, its enzyme inhibition mechanism was determined based on the Lineweaver-Burk plot. As indicated in Fig. 3, all three lines representing enzyme reaction with GVR (with the concentration of 0, 0.031 and 0.25 mg/mL), intercept the same point on the y-axis. This confirms the tripeptide GVR competitively inhibited ACE, which means the peptide can bind to ACE’s active sites.

3.7. Molecular docking of ligand and protein

To further confirm the Lineweaver-Burk plot, molecular docking analysis was performed. Table 4 shows that the lowest binding energy forms 100 runs of molecular docking simulation. The conformation with the lowest binding energy was then used for the binding mode analysis of the GVR-ACE complex.
Out of the 100 docking simulations, the results were ranked according to the docking binding energy. The lowest energy of the 10 binding energies, $-8.16$ Kcal/mol with five hydrogen (H) bonds, was chosen. The bulkiness of the ACE protein is shown in Fig. 4, with the binding pocket having the ligand (GVR) inside.

From Fig. 5, Panel A represents the protein structure as a helix, in order to see the binding site. The binding site is enlarged to show all the interactions between the ligand and the H bonds. There are 3 hydrogen-interacting residues and 5 hydrogen bonds within them (Panel B) that stabilise the structure. The predicted hydrogen bonding and the

Table 1
Selected peptide sequences from MALDI-TOF analysis.

<table>
<thead>
<tr>
<th>Peptide sequence</th>
<th>Sample (Band)</th>
<th>Putative protein</th>
<th>Accession</th>
<th>Score (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VTVGLVVR</td>
<td>D6-a</td>
<td>Protein STU1</td>
<td>gi</td>
<td>50551313</td>
</tr>
<tr>
<td>ALGVR</td>
<td>D6-b</td>
<td>Hypothetical protein NCU08737</td>
<td>gi</td>
<td>85110719</td>
</tr>
<tr>
<td>VVLRNNK</td>
<td>D6-b</td>
<td>Hypothetical protein AN0945.2</td>
<td>gi</td>
<td>67517345</td>
</tr>
<tr>
<td>ATGNLNPVR</td>
<td>D6-a</td>
<td>Galactose-1-phosphate uridylyltransferase GAL7_YEAST</td>
<td>gi</td>
<td>50551313</td>
</tr>
</tbody>
</table>

Table 2
Gastrointestinal enzymes-digestion stable peptides production through in silico approach using BIOPEP database.

<table>
<thead>
<tr>
<th>Peptide sequence</th>
<th>Enzymes’ action</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trypsin</td>
</tr>
<tr>
<td>ALGVR</td>
<td>ALGVR</td>
</tr>
<tr>
<td>VTVGLVVR</td>
<td>VTVGLVVR</td>
</tr>
<tr>
<td>VVLRNNK</td>
<td>VVLR</td>
</tr>
<tr>
<td>ATGNLNPVR</td>
<td>ATGNLNPVR</td>
</tr>
</tbody>
</table>

Table 3
IC$_{50}$ of synthetic gastrointestinal enzymes-digestion stable peptides.

<table>
<thead>
<tr>
<th>Tripeptide</th>
<th>Molecular weight (Da)</th>
<th>IC$_{50}$ (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GVR</td>
<td>330.38</td>
<td>55</td>
</tr>
<tr>
<td>VVR</td>
<td>372.46</td>
<td>93</td>
</tr>
<tr>
<td>NPR</td>
<td>385.42</td>
<td>110</td>
</tr>
<tr>
<td>VVL</td>
<td>329.44</td>
<td>&gt; 250</td>
</tr>
</tbody>
</table>
3.8. Single oral dose administration of peptide in SHRs

The tripeptide GVR was chosen for an in vivo study of single oral dose administration (Fig. 6). Administration of 10 mg/kg lead to a systolic blood pressure reduction of 16.2 mmHg of within 4 h. At doses of 50 mg/kg and 100 mg/kg, systolic blood pressure reductions of 31.5 mmHg and 33.5 mmHg respectively were recorded within 6 h. Statistically, there was no significant difference between 50 mg/kg and 100 mg/kg in comparison with the positive control. For the positive control, a maximum reduction of 36.2 mmHg of systolic blood pressure was recorded within 6 h of peptide administration. There was not much change in systolic blood pressure for the negative control group.

4. Discussion

It has been previously shown that fractions of *P. pulmonarius* mycelium extract were able to inhibit ACE. To investigate in more detail the potential of this extract in lowering blood pressure, further purification and characterisation was carried out. Fraction D6 of *P. pulmonarius* was subjected to size exclusion chromatography to narrow down the possible potent ACE inhibitory protein(s). Fraction D6 was resolved into a single prominent peak of Psec and a few other insignificant peaks which were not well resolved, and thus only Psec was collected.

Compared to D6, the Psec fraction produced better ACE inhibition. Higher inhibition of ACE probably indicated the fraction was purer after size exclusion chromatography. The Psec fraction significantly inhibited ACE compared to some other edible mushrooms, such as *Grifola frondosa* [26], *Tricholoma giganteum* [27], *Pholiota adiposa* [28] and *Pleurotus cornucopiae* [29].

To our knowledge, the only other published data on ACE inhibition by mycelium extract other than *P. pulmonarius* was on *Ganoderma lucidum*, described by [30]. Fraction C3, C4, C5 of RP-HPLC of mycelium of *G. lucidum* produced 10, 18 and 12.5 μg/mL of IC50 respectively. The Psec fraction thus produced better IC50 compared to *G. lucidum* mycelial extracts.

When subjected to SDS-PAGE, the proteins in fraction Psec were resolved into three bands, as compared to four bands for the fraction D6. Band D6-c was no longer observed in the sample after size exclusion chromatography, indicating that protein(s) in this band might not be responsible for the observed ACE inhibition. According to [11] the band may belong to nitrite reductase proteinlike protein, which has anti-hypertensive activity. Until now it’s direct effect on ACE has never been reported. As observed on SDS-PAGE, the protein(s) responsible for ACE inhibition were high molecular weight protein(s). If these proteins were administered orally, they would not be absorbed into the blood circulation in an intact form; they would be digested. No matter how good the inhibition is, the effect may not be observed once the protein is subjected to the digestive system. To predict stability of the proteins in the digestive system, the peptide sequences of the proteins in each band were determined using MALDI-TOF/TOF MS.

The peptide sequences data were then screened for sequences with potential ACE inhibitory characteristics. The characteristics were based on what was described by [19]. First, the peptide’s length must be short, within 2–12 amino acids. Second, the C-terminal of the peptide is preferably a hydrophobic amino acid, since it is responsible for the binding inside the active site. Third, it has to have at least one proline (a hydrophobic amino acid). Fourth, the peptide should have leucine, preferably a hydrophobic amino acid, since it may contribute to the inhibition of ACE. Fifth, there should be positively charged arginine at the C terminal since it is proven to reduce ACE activity [19]. Four peptide sequences from MALDI-TOF/TOF MS data which fit these criteria and which have never been reported before were selected, viz. ALGVR, VTVGLVVR, VVLRNNK and ATGNLNPR.

Stability of the selected peptide sequences was predicted using the BIOPEP database where trypsin, chymotrypsin (A) and pepsin were selected for this analysis. Trypsin was chosen because it is commonly used to produce biologically active peptides using an in vitro method [31]. Although trypsin was used to digest the sample during sample preparation for MALDI-TOF/TOF MS, trypsin was chosen again because it could be possible that total digestion by trypsin may not occur completely during the in-gel digestion of the sample. Pepsin and a-chymotrypsin were chosen to simulate digestion in the stomach and small intestine. With the aid of pepsin in the acidic environment, the
Pepsin has di-trypsin. Both enzymes cleaved the peptide sequences at leucine, but trypsin which does not cleave at alanine at the C-terminal as compared to pepsin. With BIOPEP simulation, these enzymes were seen to digest in-gel proteins. As for chymotrypsin (A) and pepsin (B), there are several enzymatic cleavage sites for these enzymes even at pH > 2.

The pH of 2 was chosen because generally the patient will consume food before taking the oral anti-hypertensive drugs.

In the digestive tract, proteases and peptidases would degrade or lessen the activity of peptide, therefore it is of extreme importance that the peptide is unaffected by the enzymes, as an intact peptide is required to inhibit ACE. During digestion, the acidic nature of the stomach optimises enzymatic activity of the pepsin to digest proteins and peptides. The action of pancreatic proteases, trypsin, α-chymotrypsin, elastase, and carboxypeptidases A and B then further cuts polypeptides at a more alkaline pH. With the help of an in vitro model, the bioavailability of bioactive peptides is usually established by successive hydrolysis with pepsin and pancreatic enzymes, in an attempt to mimic the gastrointestinal environment. It has been shown that in the course of simulated gastrointestinal digestion, the peptide inhibitory activity drops [19,34].

A forecast of how stable a peptide is against gastrointestinal digestion may be of help predicting the availability of the peptide sequence once orally administrated. From Table 2, tripeptides GVR, VVL, VVR, NPR were chosen after being tested against the three gastrointestinal enzymes. This was due to the short peptide sequences which were stable against the three enzymes and which probably could preserve their activity and be easily absorbed into the blood circulation.

Although there are several novel ACE inhibitory peptides reported previously originated from different starting materials [1,8,10,14,20,27,29,42], to our best knowledge, the four tripeptides used in this study have never been reported before of their ACE inhibitory activity. Furthermore, ACE inhibitory peptides/proteins purified from mycelium of mushrooms are not well studied. Focus were given more to the fruiting bodies [14,20,27,29] which are easily obtained.

Four peptides were synthetically synthesised and subjected to an ACE inhibitory assay. The synthesised peptide GVR produced strong inhibitory activity. Furthermore, ACE inhibitory peptides/proteins purified from mycelium of mushrooms are not well studied. Focus were given more to the fruiting bodies [14,20,27,29] which are easily obtained.

For BIOPEP analysis, pH 2 was chosen because the optimal pH of about 2 permits the pepsin to function in a natural acidic environment [33]. After food intake, the pH of the stomach will increase to more than 2 compared to before the intake of food, which is around pH 1.3. The pH of 2 was chosen because generally the patient will consume food before taking the oral anti-hypertensive drugs.

In the digestive tract, proteases and peptidases would degrade or lessen the activity of peptide, therefore it is of extreme importance that the peptide is unaffected by the enzymes, as an intact peptide is required to inhibit ACE. During digestion, the acidic nature of the stomach optimises enzymatic activity of the pepsin to digest proteins and peptides. The action of pancreatic proteases, trypsin, α-chymotrypsin, elastase, and carboxypeptidases A and B then further cuts polypeptides at a more alkaline pH. With the help of an in vitro model, the bioavailability of bioactive peptides is usually established by successive hydrolysis with pepsin and pancreatic enzymes, in an attempt to mimic the gastrointestinal environment. It has been shown that in the course of simulated gastrointestinal digestion, the peptide inhibitory activity drops [19,34].

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Four peptides were synthetically synthesised and subjected to an ACE inhibitory assay. The synthesised peptide GVR produced strong IC50 compared to the other three peptides. Based on the IC50 values listed in Table 3, there was some relationship between the structure of amino acids and their ACE inhibitory activity.

In peptides GVR and VVR, the difference in amino acid present at the N-terminal of these sequences made a huge difference in terms of IC50 value. The presence of glycine at the N-terminal produced a better IC50 value compared to that of valine. Similarly, for peptides VVR and VVL, the presence of arginine at the C-terminal produced a better result compared to that of leucine. It was difficult to make a comparison among tripeptides with glycine at the C-terminal, because not many tripeptides are reported with glycine at the C-terminal. It was reported that tripeptide GPL and GLP produced IC50 values of 2.65 and 1.62 μM respectively, which proved the effect of proline in inhibiting ACE at the penultimate position [35].

According to [19], for a long or oligopeptide structure, the organisation of the C terminal tripeptide is vital, so that the peptide can bind strongly to the ACE active site. Based on our result, this was also applicable to short tripeptides. The tripeptide NPR produced an IC50 value...
of 110 μg/mL and this is in agreement with [36] and [35]; where the existence of the proline at the penultimate position will lead the peptide to attach weakly to the ACE active site.

Based on the inhibitory results of the peptide used in this experiment, the mode of inhibition of ACE activity was investigated via a kinetic study. From the Lineweaver-Burk plots in Fig. 3, the inhibition pattern exhibited was competitive, which means the peptide could bind to the ACE active sites. According to [37], ACE appears to attract substrates or competitive inhibitors containing hydrophobic amino acid residues at antepenultimate, penultimate and ultimate positions (last three locations of a sequence) of the C-terminal, which will bind with S1, S2 and S3 subsites within the ACE active site. According to [19], the positive charge on the side chains of arginine and lysine residues at the C-terminus have been well-known to favour the possible ACE inhibitory activity of a peptide. For example, the tripeptide MKR was reported to competitively inhibit ACE [38]. The tripeptide MKR contains arginine at the ultimate position. Methionine is a hydrophobic amino acid whereas lysine is a hydrophilic but positively charged amino acid.

The GVR ligand was determined to bind to ACE at the active site of the protein. The binding pocket is defined as the amino acid residues within 5 Å from the ligand [39]. We found that the binding pocket was walled by 23 amino acid residues which are Tyr146, Glu162, Thr166, Asn277, Trp279, Gln281, Asn285, Lys352, His353, Ala354, Gln369, Lys370, Thr371, Thr372, Asn374, Glu376, Asp377, Val380, His383, Glu384, Tyr385, Lys511 and His513. GVR was buried inside the cavity (FIG) and stabilised through five hydrogen bonds as shown in Fig. 5B. Glu384, which is one of the amino acid residues responsible for the biocatalytic property of ACE [40], was also identified within the proximity of the binding pocket. Since there are hydrophobic amino acid residues within the binding pocket of GVR-ACE, we would assume that hydrophobic interaction would also involve stabilising the complex conformation.

Based on the IC₅₀ value, the tripeptide GVR was chosen for our single oral dose administration study on SHR rats. For 10 mg/kg of GVR, the maximum reduction of SBP was at the fourth hour, compared to the sixth hour for other groups. This was possibly due to the lower concentration of peptide administered to the rats, where the amount of peptide was unable to produce a longer reduction in SBP. In this study 5 mg/kg of captopril was administered to the rats and the maximum reduction of SBP was at the sixth hour. According to [41], after the administration of 100 mg/kg of captopril, the maximum reduction in SBP was recorded at the second hour whereas [42] administered 10 mg/kg of captopril but the maximum reduction in SBP was recorded at the fourth hour. These three findings show that the amount of drug administered to the rats also plays an important role in the hypotensive effect, apart from other factors like gender, rate of metabolism and health of the subject (severity of the SBP); which also influence the effectiveness of the drug on each individual. Based on this study, there was no significant difference between captopril and 100 mg/kg of GVR, although in vitro analysis revealed a significant difference in their IC₅₀ value.

Very few studies have carried out ACE inhibitory peptides identification through in silico platform [43,44]. From the literature survey, it was very rare to find peptide produced through in silico platform being tested in vivo. Peptides produced from in silico platform must be studied for its in vivo effect before the antihypertensive effect can be claimed. By using in silico platform, the selection of sequence of interest would be easier. According to [43], a quantitative structure activity relationship (QSAR) model of ACE inhibitory tripeptides from milk was produced by good predictive ability. In the study, [43] had chosen four tripeptides based on the constructed model and tested their activity in vitro. Out of the four tripeptides, IVP and VIP were additionally tested for their possible blood pressure lowering effect in the animal model and proven to reduce the elevated blood pressure.

In another study, two penta-peptides, KTGGY and KRIHF were produced from computer-aided (in silico) simulation of pepsin hydrolysis of yam Dioscoria. Through in vitro assay, these two peptides produced more than 50% ACE inhibitory activity. When tested in SHR rats, these two peptides significantly reduced elevated systolic blood pressure [44]. These two in vivo studies have proved the ability of the in silico platform to produce ACE inhibitory peptides. Similarly, in current study, tripeptide GVR which was selected through in silico platform had shown to have antihypertensive activity when tested in vitro and in vivo.

5. Conclusion

The mycelium of the P. pulmonarius comprises naturally occurring bioactive ingredients that are potentially valuable for the uses as a health supplements. Tripeptide GVR produced significant antihypertensive effect through inhibition of the ACE in vitro and in vivo. This tripeptide also produced competitive inhibition and confirmed through the molecular docking analysis thus, it competes with the angiotensin I for the ACE’s active sites. These findings showed that this tripeptide potentially able to be developed as a nutraceutical product to attenuate hypertension.

Conflict of interest

Authors would like to declare there is no competing interest in this research.

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S. Manoharan et al. Process Biochemistry xxx (xxxx) xxx–xxx