JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Proteomic Analysis of Antihypertensive Proteins in Edible Mushrooms

Ching-Ching Lau, Noorlidah Abdullah,* Adawiyah Suriza Shuib, and Norhaniza Aminudin

Mushroom Research Centre, Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

ABSTRACT: Mushrooms are high in protein content, which makes them potentially a good source of antihypertensive peptides. Among the mushrooms tested, protein extracts from *Pleurotus cystidiosus* (E1Pc and E5Pc) and *Agaricus bisporus* (E1Ab and E3Ab) had high levels of antihypertensive activity. The protein extracts were fractionated by reverse-phase high-performance liquid chromatography (RPHPLC) into six fractions. Fraction 3 from E5Pc (E5PcF3) and fraction 6 from E3Ab (E3AbF6) had the highest antihypertensive activities. SDS-PAGE analysis showed E5PcF3 consisted mainly of low molecular weight proteins, whereas E3AbF6 contained a variety of high to low molecular weight proteins. There were 22 protein clusters detected by SELDI-TOF-MS analysis with five common peaks found in E5PcF3 and E3AbF6, which had m/z values in the range of 3940−11413. This study suggests that the antihypertensive activity in the two mushroom species could be due to proteins with molecular masses ranging from 3 to 10 kDa.

KEYWORDS: *Agaricus bisporus, Pleurotus cystidiosus, ACE inhibitory peptides, SELDI-TOF-MS*

INTRODUCTION

Hypertension or high blood pressure has been on the rise worldwide. According to the World Health Organization (WHO), nearly 1 billion people globally have high blood pressure, and it is predicted to increase to a total of 1.56 billion in the year 2025.[1] Hypertension is one of the major risk factors for cardiovascular diseases such as stroke, coronary heart disease, and peripheral artery disease.[2]

Angiotensin converting enzyme (ACE) is a dipeptidyl carboxypeptidase of the renin−angiotensin system that plays a key role in blood pressure homeostasis and the salt balance of mammals. ACE is involved in the conversion of angiotensin I to angiotensin II. This reaction ultimately causes the contraction of blood vessels and thereby leads to hypertension. Today, specific synthetic inhibitors of ACE are used to treat hypertension, congestive heart failure, and myocardial infarction.[3] However, these inhibitors can induce powerful side effects, such as angioedema.[4]

Nutritional factors play an important role in the prevention and treatment of hypertension. In addition to pharmacological treatment, a moderate lowering of blood pressure can be obtained by a nutritional approach. Many ACE inhibitory peptides have been detected in food proteins such as milk,[5] buckwheat,[6] potato,[7] tuna,[8] oyster,[9] broccoli,[10] cheese,[11] and chickpea.[12] Furthermore, naturally occurring ACE inhibitory proteins have been proven to be effective as in vivo antihypertensive agents.[13] Proteins with a high content of hydrophobic amino acids are good sources of ACE inhibitory peptides because high hydrophilicity will make the proteins inaccessible to the active site of ACE.[14,15] Structural analysis of tripeptides with high ACE inhibitory activity revealed they usually contain amino acid residues with aromatic and hydrophobic side chains at the carboxyl and amino termini, respectively, whereas positively charged amino acids are preferred for the middle position.[16] These peptides have been found to be less active than the synthetic ACE inhibitors; however, they might play an important role as antihypertensive agents as they can be part of the daily diet in the form of functional foods and are perceived as natural and safe by consumers.[17]

Mushrooms are favorite dishes in Oriental and Western countries. Besides their nutritional value, the unique color, taste, and aroma of mushrooms are the reasons that attract their consumption by humans.[18] Mushrooms are high in protein content; the amount of protein in mushrooms is below that in most animal meats but above that in most other foods, such as wheat and milk.[19] Previous studies have shown successful purification of ACE inhibitory peptides from the fruiting bodies of edible mushrooms, such as *Tricholoma giganteum*,[20] *Grifola frondosa*,[21] and *Pleurotus cornucopiae*.[22] Hence, mushrooms can be a good source of bioactive peptides. Currently, there are no studies available profiling antihypertensive peptides in edible mushrooms, and proteomics is an excellent tool to do this. Therefore, the objective of the current study was to compare the antihypertensive activity of proteins in edible mushrooms and carry out a proteomic analysis of selected potent antihypertensive proteins.

MATERIALS AND METHODS

Materials. Sporocarps (or fruiting bodies) of edible mushrooms *Agaricus bisporus* (button mushroom), *Flammulina velutipes* (golden needle), and *Lentinus edodes* (shiitake) were purchased from a local supermarket, whereas *Hericium erinaceus* (monkey's head mushroom), *Pleurotus citrinopileatus* (yellow oyster mushroom), *Pleurotus cystidiosus* (abalone mushroom), *Pleurotus flabellatus* (pink oyster mushroom), *Pleurotus florida* (white oyster mushroom), and *Pleurotus sajor-caju* (gray oyster mushroom) were purchased from a local mushroom farm, Ganofarm Sdn Bhd, Malaysia. α-Cyano-4-hydroxycinnamic acid (CHCA) (used for SELDI-TOF-MS analysis) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals, such as ammonium sulfate,

Received: October 4, 2012
Revised: November 28, 2012
Accepted: November 28, 2012
Published: November 28, 2012

trifluoroacetic acid (TFA), methanol, and reagents for silver staining, were purchased from Merck (Darmstadt, Germany).

Preparation of Protein Extracts from Fruiting Bodies of Edible Mushrooms. Fresh fruiting bodies of the nine edible mushroom species were cleaned and sliced. Each mushroom fruiting body was blended with distilled water at a ratio of 1:2. The mixture was filtered and centrifuged to remove unwanted debris. Proteins were precipitated out from the water extracts using the ammonium sulfate precipitation method. The concentration of ammonium sulfate was increased stepwise from 10 to 100% salt saturation, and the precipitated protein was recovered at each step by centrifugation at 10000 rpm for 15 min at 4 °C. Then the protein extracts were dialyzed using snakeskin dialysis tubing with a cutoff of 3500 Da (Thermo Scientific, Rockford, IL, USA) to remove the salt from the sample. The protein extracts were dialyzed in a cold room (4 °C) against distilled water under continuous stirring with a magnetic stirrer for 48 h. Water was changed four times throughout the process. Dialyzed protein extracts were freeze-dried and stored at −20 °C for further analysis.

Estimation of Protein Content in the Protein Extracts of Edible Mushrooms. Protein content was estimated using the Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific) according to the protocols provided by the manufacturer. The absorbance values were measured with a Sunrise ELISA microplate reader (Tecan, Grödig, Austria) at 562 nm. Protein content was determined by comparing the absorbance value of the samples with a standard curve of bovine serum albumin (BSA).

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis of Protein Extracts from Edible Mushrooms. SDS-PAGE was used as a parameter to pool the protein extracts precipitated from different percentages of salt saturation. It was also used to visualize the molecular weights of the proteins in reverse-phase high-performance liquid chromatography (RPHPLC) fractions exhibiting the highest ACE inhibitory activities. The method was carried out in a vertical slab gel apparatus according to the modified method of Laemmli.[23] Sample buffer and crude protein were mixed at a ratio of 1:3 (v/v) and boiled for 5 min. The mixture and broad range SDS-PAGE standard markers (Bio-Rad Laboratories, Hercules, CA, USA) were then loaded into the wells. Electrophoresis was conducted at a constant current of 60 V to move the proteins through the stacking gel (4% polyacrylamide), followed by 100 V to resolve the proteins in the separating gel (16% polyacrylamide). Following electrophoresis, the gel was fixed with a solution consisting of 40% (v/v) methanol and 10% (v/v) acetic acid for 30 min. The protein bands were then stained by a silver staining method.

ACE Inhibitory Activity of Water Extracts and Protein Extracts from Edible Mushrooms. The protein extracts were tested using an ACE inhibitory assay kit (ACE kit-WST, Dojindo Laboratories, Kumamoto, Japan). The water and protein extracts were tested at concentrations of 10 mg/mL and 10 μg/mL protein, respectively. RPHPLC fractions were tested at 1 μg/mL protein. The assay was carried out according to the protocol provided by the manufacturer. The absorbances of the samples were measured with a Sunrise ELISA microplate reader (Tecan) at 450 nm. The ACE inhibitory activity of the samples tested was calculated using the formula given in the protocol.

Purification of Protein Extracts from *Pleurotus cystidiosus* and *Agaricus bisporus* with the Highest Percentage of ACE Inhibitory Activity. Purification of four selected protein extracts (E1Pc, E5Pc, E1Ab, and E3Ab) with active ACE inhibitory activities was carried out using an HPLC system equipped with an SCL-10AVP system controller, an LC-10ATVP solvent delivery unit, an SPD-M10AVP UV−vis diode array detector, and a DGU-12A degasser (Shimadzu, Kyoto, Japan). The column used in the current study was a Chromolith SemiPrep RP-18 column (100−10 mm, Merck, Darmstadt, Germany). First, the crude proteins were filtered through a syringe filter before being injected into the column. The crude proteins were eluted with an increasing gradient of acetonitrile containing 0.05% TFA, from 0 to 50% in 10 min at a flow rate of 3.5 mL/min. The UV absorbance of the eluent was monitored at 220 nm. All of the protein extracts were fractionated according to the peaks obtained. The fractions were then freeze-dried and stored at −20 °C for further analysis.

Proteomic Analysis of Selected RPHPLC Fractions with Active ACE Inhibitory Activity. RPHPLC fractions from *P. cystidiosus* (E5PcF3) and *A. bisporus* (E3AbF6) were analyzed using SDS-PAGE and surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF-MS). SDS-PAGE was performed according to the method described above, whereas SELDI-TOF-MS analysis was carried out using hydrophobic H50 ProteinChip arrays (Bio-Rad Laboratories). The arrays were first equilibrated with binding buffer, which consisted of 0.5% TFA in 50% acetonitrile (v/v). Then, 5 μL samples containing 0.5 μg of protein were spotted on the arrays. After the arrays were air-dried, 2 μL of CHCA in 0.5% TFA in 50% acetonitrile (v/v) was added onto the arrays, and they were left to air-dry. The analyses were carried out on the Bio-Rad ProteinChip SELDI system PCS4000. Data collection was carried out in positive ion mode using the following acquisition settings: mass range, 0−20 kDa; focus mass, 10 kDa. Laser energies used for the shot sequences were based on the following settings: warming shots, 1000 nJ; data shots, 900 nJ. Spectra were calibrated using an external calibration against a mixture of

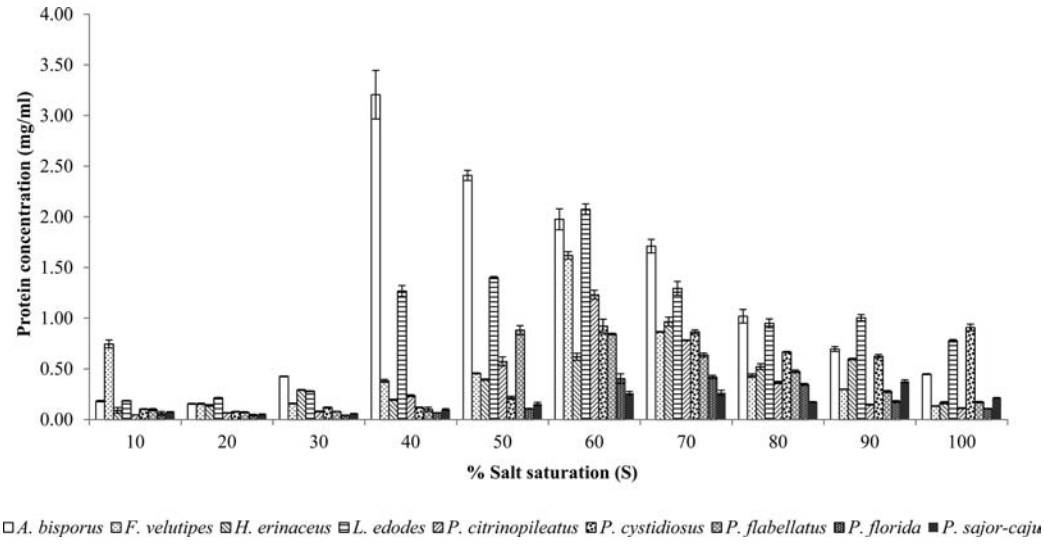

Figure 1. Comparison of protein concentrations (mg/mL protein in 100 g fresh weight of fruiting bodies) between protein extracts of edible mushrooms precipitated at 10 different salt saturations. Values are expressed as the mean ± standard deviation (n = 3).

standards consisting of somatostatin (1637.9 Da), arg-insulin (5969.0 Da), and cytochrome *c* (12263.3 Da).

Statistical Analysis. The analyses of protein content and ACE inhibitory activity were carried out in triplicate, and results were reported as the mean ± standard deviation (SD). Mean differences of protein content and ACE inhibitory activity in mushrooms were analyzed using one-way ANOVA in Statgraphics Plus 3.0 at $p < 0.05$. Expression difference map (EDM) analysis on the SELDI-TOF-MS spectra was performed using ProteinChip Data Manager 3.5 (Bio-Rad Laboratories). Peaks with mass to charge ratio (m/z) between 2000 and 30000 were analyzed. Mean peak intensities from four replicate samples were used to determine the p value. Peak clusters were done using second-pass peak selection with S/N > 2, within 0.1% of the mass window and allowing estimated peaks to be included; $p < 0.01$ was considered to be statistically significant.

Table 1. Protein Content and Percentages of ACE Inhibitory Activity in Water Extracts from Fruiting Bodies of Edible Mushrooms[a]

mushroom species	protein content (g/100 g fresh weight)	ACE inhibitory activity[b] (%)
A. bisporus	1.70 ± 0.01 a	87.2 ± 0.8 b
F. velutipes	1.53 ± 0.01 b	95.6 ± 0.3 g
H. erinaceus	0.96 ± 0.01 c	90.1 ± 0.6 f
L. edodes	2.70 ± 0.02 d	95.2 ± 0.3 g
P. citrinopileatus	2.67 ± 0.07 d	71.9 ± 1.0 d
P. cystidiosus	2.07 ± 0.10 e	81.3 ± 0.8 a
P. flabellatus	2.47 ± 0.09 f	75.4 ± 1.3 c
P. florida	0.73 ± 0.02 g	87.6 ± 1.4 b
P. sajor-caju	0.75 ± 0.01 g	85.2 ± 0.6 e

[a]All values are expressed as the mean ± standard deviation ($n = 3$). Mean values with different letters within a column are significantly different ($p < 0.05$). [b]ACE inhibitory activity was tested at 10 mg/mL.

Table 2. Protein Extracts (S10−S100) Pooled According to the Similarity of Protein Bands Observed by SDS-PAGE Profile

mushroom species	pooled protein extracts (E)					
	1	2	3	4	5	6
A. bisporus	S10−20	S30	S40	S50−60	S70−100	
F. velutipes	S10−30	S40	S50−60	S70	S80	S90−100
H. erinaceus	S10−30	S40−50	S60−70	S80−100		
L. edodes	S10−20	S30−40	S50	S60	S70−80	S90−100
P. citrinopileatus	S10−40	S50	S60−70	S80−100		
P. cystidiosus	S10−20	S30−50	S60	S70−80	S90	S100
P. flabellatus	S10−40	S50	S60	S70−80	S90−100	
P. florida	S10−40	S50	S60−70	S80	S90−100	
P. sajor-caju	S10−20	S30−50	S60	S70	S80−100	

RESULTS AND DISCUSSION

Comparisons of Protein Content and ACE Inhibitory Activity in Water Extracts from Fruiting Bodies of Edible Mushrooms. The concentrations of proteins in water extracts of edible mushrooms were estimated using the BCA protein assay kit. As shown in Table 1, the protein contents of the nine edible mushroom species were in the range of 0.73−2.70 g/100 g fresh weight; *L. edodes* and *P. citrinopileatus* had the highest protein content. These were followed by *P. flabellatus* and *P. cystidiosus*. The most commonly cultivated *Pleurotus* species in Malaysia, *P. florida* and *P. sajor-caju*, had the lowest protein contents compared to the other mushroom species tested in the current study. *A. bisporus* had a similar protein content compared to samples of *A. bisporus* from Italy (1.63 g/100 g fresh weight) and northeastern Portugal (1.23 g/100 g fresh weight).[24,25] *L. edodes* also showed a similar protein content compared to samples of *L. edodes* from Turkey, which had protein concentrations in the range of 2.07−2.94 g/100 g fresh weight.[26] However, lower protein contents for *L. edodes* was reported in samples from Italy (1.53 g/100 g fresh weight) and northeastern Portugal (0.89 g/100 g fresh weight).[24,25] *P. sajor-caju* in the current study had a lower protein content compared to *P. sajor-caju* as determined by Cağlarirmak[26] and Bonatti et al.,[27] who reported 1.76 and 1.54 g/100 g fresh weight, respectively. Substrates used for mushroom cultivation, temperature, and humidity during growth as well as the age of the harvested mushroom have been reported to influence the chemical composition and nutritional value of mushrooms.[19,28] Hence, different pre- and post-harvest conditions may cause variations in the protein content of mushrooms.

The ACE inhibitory activity of water extracts from the nine mushroom species were tested at a concentration of 10 mg/mL. Referring to Table 1, the percentages of ACE inhibitory activity were in the range of 71.9−95.6%. The highest ACE inhibitions were exhibited by *F. velutipes* and *L. edodes*, followed by *A. bisporus* and *P. florida*. The lowest ACE inhibitory activity was shown by *P. citrinopileatus*. The antihypertensive activity reported in the current study was higher compared to previous reports. Water extracts of *A. bisporus* and *P. sajor-caju* were reported to inhibit 27.3 and 38.7% of ACE, respectively, at a concentration of 25 mg/mL, whereas different strains of *F. velutipes* inhibited ACE activity in the range of 13.7−32.9%.[20]

Table 3. Percentages of ACE Inhibitory Activity in Pooled Protein Extracts from Edible Mushrooms[a]

mushroom species	pooled protein extracts (E)					
	1	2	3	4	5	6
A. bisporus	**78.4 ± 4.4 s**	8.3 ± 3.2 abcd	**71.4 ± 0.9 r**	39.3 ± 1.3 p	8.1 ± 2.8 abcd	
F. velutipes	18.3 ± 3.5 fghi	15.8 ± 0.8 efg	12.6 ± 2.2 cdef	13.7 ± 3.2 def	16.4 ± 3.7 efgh	19.8 ± 0.5 ghij
H. erinaceus	5.4 ± 3.4 a	5.9 ± 2.9 a	6.3 ± 1.8 ab	6.9 ± 4.6 abc		
L. edodes	11.7 ± 2.1 bcde	16.6 ± 6.3 efgh	24.9 ± 2.8 jkl	5.7 ± 2.9 a	3.3 ± 1.9 a	4.6 ± 2.6 a
P. citrinopileatus	41.1 ± 4.4 p	20.9 ± 1.6 ghij	15.2 ± 4.9 efg	20.2 ± 1.5 ghij		
P. cystidiosus	**96.2 ± 0.6 u**	47.5 ± 2.7 q	31.6 ± 6.1 mno	31.5 ± 2.3 mno	**86.7 ± 2.8 t**	48.3 ± 2.6 q
P. flabellatus	28.4 ± 3.1 klm	36.5 ± 4.1 op	21.8 ± 3.9 hij	19.9 ± 3.0 ghij	22.0 ± 3.4 hij	
P. florida	25.5 ± 2.3 jkl	24.2 ± 8.5 jk	17.0 ± 3.1 efgh	22.9 ± 4.7 ijk	4.5 ± 4.2 a	
P. sajor-caju	41.2 ± 7.3 p	30.6 ± 0.8 lmn	35.9 ± 2.2 nop	32.2 ± 4.2 mno	5.5 ± 1.7 a	

[a]All values are expressed as the mean ± standard deviation ($n = 3$). Different letters indicate significant differences ($p < 0.05$) analyzed with one-way ANOVA. ACE inhibitory activity was tested at 10 μg/mL protein. Protein extracts highlighted in bold were selected for further purification.

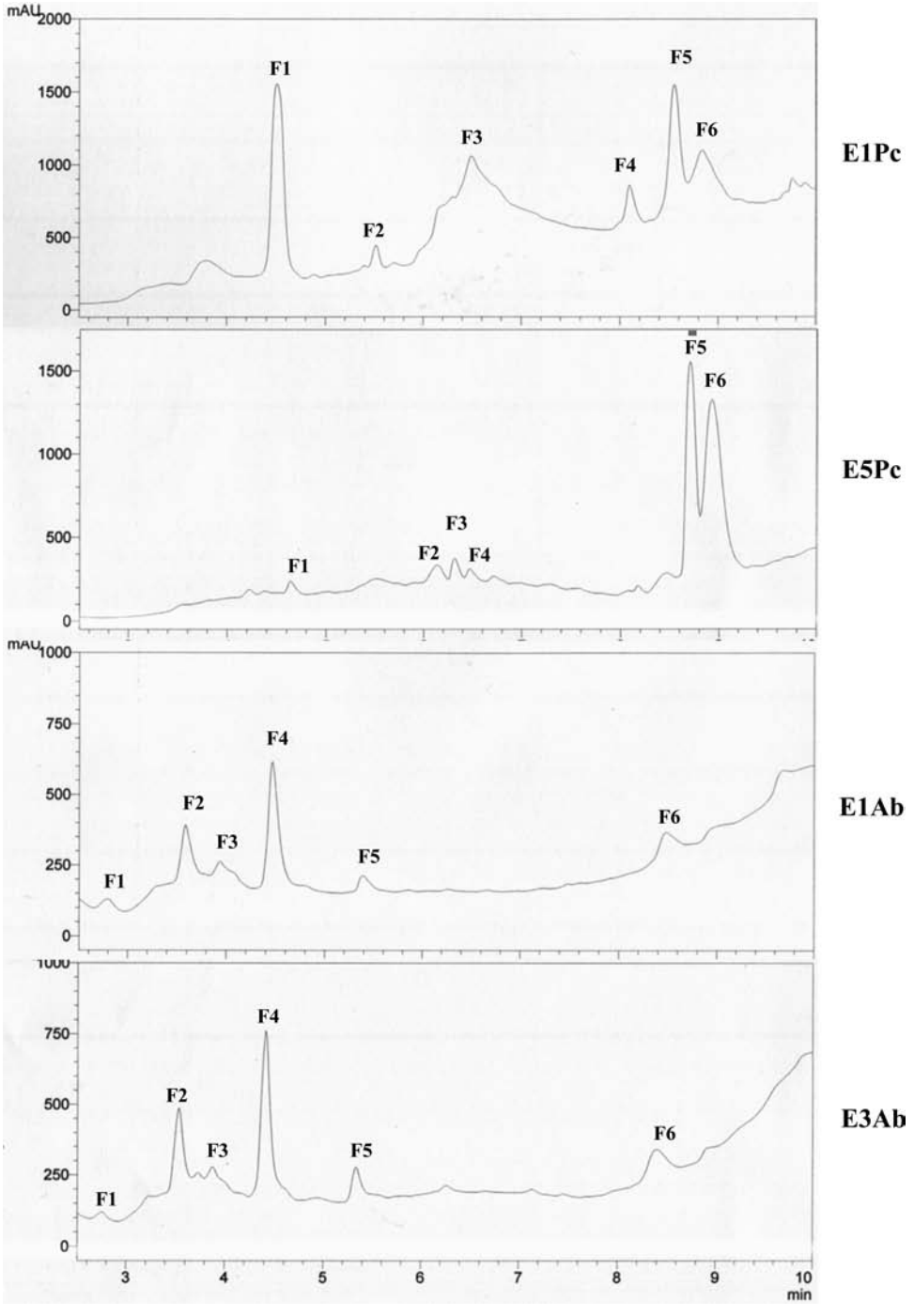

Figure 2. RPHPLC chromatograms of protein extracts from *P. cystidiosus* (E1Pc and E5Pc) and *A. bisporus* (E1Ab and E3Ab). Fractions collected were labeled as F1−F6.

Kim and co-workers[29] also reported lower ACE inhibitory activities for *L. edodes* and *F. velutipes*, where 50% of ACE activity was inhibited at concentrations of 18.4−39.3 and 7.4−22.6 mg/mL, respectively. The conditions of extraction of ACE inhibitor from mushrooms have been reported to influence the level of ACE inhibitory activity. The optimum extraction conditions for *T. giganteum* and *Pholiota* sp. were at 30 °C for 3 and 1 h, respectively.[20] *G. frondosa* required a longer extraction time, that is, 12 h.[21]

In the current study, there was no correlation between the protein content and the ACE inhibitory activity (R^2 = 0.1442). This is supported by a report by Apostolidis et al.,[30] which showed no correlation between the two parameters. This may indicate that other compounds in the crude water extract could have interfered or competed with proteins to bind to the active site of ACE. Therefore, protein purification is important to eliminate other compounds that might hinder the true effect of these proteins.

Protein Extraction from Edible Mushrooms. Proteins were precipitated from the water extracts of the nine edible mushrooms by different percentages of salt saturation ranging from 10 to 100% (S10−S100). Figure 1 shows a comparison of the protein concentrations in the protein extracts of each mushroom species tested. Most mushroom species in the current

dx.doi.org/10.1021/jf3042159 | J. Agric. Food Chem. 2012, 60, 12341−12348

Table 4. Percentages of Recovery Yield and Percentages of ACE Inhibitory Activity of the RPHPLC Fractions[a]

	protein extracts							
	E1Pc		E5Pc		E1Ab		E3Ab	
RPHPLC fraction	% recov	% inh	% recov	% inh	% recov	% inh	% recov	% inh
F1	15.75	3.3 ± 0.6 a	5.53	19.2 ± 4.5 de	9.62	9.2 ± 3.8 bc	6.06	7.7 ± 2.2 abc
F2	6.16	7.7 ± 5.0 ab	4.95	28.4 ± 5.9 fg	12.00	7.4 ± 2.9 abc	10.16	12.9 ± 2.3 cd
F3	21.78	10.6 ± 3.2 abc	5.36	**30.8 ± 5.6 g**	7.22	1.2 ± 2.7 a	6.90	14.4 ± 7.6 cd
F4	9.74	25.9 ± 2.9 efg	8.33	21.4 ± 4.5 def	19.14	4.4 ± 2.1 ab	17.18	12.2 ± 5.7 cd
F5	15.06	21.9 ± 5.4 def	14.46	19.1 ± 4.7 de	6.69	3.8 ± 4.7 ab	5.35	16.8 ± 2.7 d
F6	13.24	14.7 ± 6.8 bcd	17.48	17.4 ± 4.1 cd	7.84	19.3 ± 6.8 de	6.15	**25.7 ± 4.2 e**
total	81.73		56.11		62.51		51.80	

[a]All values were tested in triplicate and expressed as the mean ± standard deviation (n = 3). Different letters within the same mushroom species indicate significant differences ($p < 0.05$) in percentage of ACE inhibitory activity analyzed with one-way ANOVA. ACE inhibitory activity of RPHPLC fractions was tested at 1 μg/mL protein. RPHPLC fractions highlighted in bold were selected for further analysis.

study had the highest concentration of protein precipitated at S50−S70, except for *A. bisporus* (S40) and *P. sajor-caju* (S90). Theoretically, proteins with large numbers of hydrophobic residues should precipitate out first. Hence, mushrooms tested in the current study contain high amounts of amphipathic proteins. This is in line with a report that mushrooms contain several unique proteins intermediate in hydrophobicity, including lectins, lignocellulotic enzymes, and hydrophobins.[31]

The 10 protein extracts from the 9 mushroom species were subjected to SDS-PAGE analysis. Protein extracts with similar SDS-PAGE protein band profiles were pooled together in the same group as shown in Table 2. Protein extracts from *H. erinaceus* and *P. citrinopileatus* were pooled into four groups, whereas *A. bisporus*, *P. flabellatus*, *P. florida*, and *P. sajor-caju* were pooled into five groups. *F. velutipes*, *L. edodes*, and *P. cystidiosus* were divided into six groups.

Comparisons of ACE Inhibitory Activity in Pooled Protein Extracts from Edible Mushrooms. The ACE inhibitory activity of the pooled protein extracts from the mushrooms was tested at a concentration of 10 μg/mL protein. Previously in Table 1, water extracts of the different species showed 71.9−95.2% of ACE inhibitory activity at 10 mg/mL, that is, a maximum difference of 23.7% among the species. However, protein extracts separated on the basis of variations in hydrophobicity by ammonium sulfate precipitation (Table 3) showed significant differences in their percentages of antihypertensive activity. According to the study by Barbosa-Filho et al.,[32] natural compounds showing active ACE inhibition include proteins, flavonoids, alkaloids, sesquiterpenoids, and terpenes. Among these, protein was the most reported compound showing active ACE inhibitory activity. In addition, proteins with a high content of hydrophobic amino acids are good sources of ACE inhibitory peptides.[14,15]

Among the protein extracts, E1 from *P. cystidiosus* (E1Pc) showed the highest ACE inhibitory activity, blocking 96.2% of ACE activity in the inhibition assay. This was followed by E5 of *P. cystidiosus* (E5Pc) with 86.7% ACE inhibition. E1 and E3 of *A. bisporus* (E1Ab and E3Ab) also showed significantly higher ACE inhibitory activities compared to the other mushroom species, 78.4 and 71.4%, respectively. Thus, to further characterize the proteins, the four protein extracts with the most potent ACE inhibitory activity were selected for further purification by RPHPLC.

Purification of ACE Inhibitor from *Pleurotus cystidiosus* and *Agaricus bisporus* by RPHPLC. RPHPLC chromatograms of E1Pc, E5Pc, E1Ab, and E3Ab are shown in Figure 2. The two protein extracts of *P. cystidiosus* had different peak patterns, but the two protein extracts of *A. bisporus* showed very similar peak patterns. This could be because the percentages of salt saturation for E1Ab (S10−S20) and E3Ab (S40) were quite close. Thus, some proteins with similar characteristics could be precipitated within these salt saturation solutions.

Six RPHPLC fractions (F1−F6) were collected from each protein extract. They were freeze-dried and evaluated for ACE-inhibitory activity. Referring to Table 4, RPHPLC fractions collected from E1Pc and E1Ab had higher recovery rate percentages compared to E5Pc and E3Ab, respectively. The total percentage of proteins collected showed that not all of the proteins were recovered from the four protein extracts. Previous work has shown that proteins denature and lose their bioactivity in 50% aqueous−acetonitrile solution.[33] In view of this complication, we eluted proteins from the RPHPLC column only to a maximum of 50% acetonitrile. Proteins eluted by >50% acetonitrile were therefore eliminated from analysis.

ACE inhibitory activities of the protein fractions collected from RPHPLC are shown in Table 4. Approximately 3.3−25.9% of ACE was inhibited by the RPHPLC fractions from E1Pc, whereas the antihypertensive activity of the RPHPLC fractions from E5Pc was in the range of 17.4−30.8%. RPHPLC fractions from E1Ab and E3Ab inhibited ACE activity in the ranges of 1.2−19.3 and 7.7−25.7%, respectively. Apparently, in the current study, proteins with intermediate hydrophobicity (based on the percentage of ammonium sulfate used) precipitated in E5Pc and E3Ab had higher ACE inhibitory activity compared to RPHPLC fractions collected from E1Pc and E1Ab, respectively. This is supported by a report that proteins with higher hydrophobicity are good ACE inhibitors.[14] In addition, studies by Wang et al.[34] revealed that proteins isolated from apricot almond meal, which contain 50.08% hydrophobic amino acids, had good ACE inhibitory activity, inhibiting 50% of ACE activity at a concentration of 0.138 mg/mL.

In previous studies, different ACE inhibitory peptides had been purified from different species of edible mushrooms.[20−22] This shows that different proteins in the mushroom species were responsible for the ACE inhibitory activity. Hence, the RPHPLC fractions with the highest ACE inhibitory activities from *P. cystidiosus* (E5PcF3) and *A. bisporus* (E3AbF6) were selected for further analysis by SDS-PAGE and SELDI-TOF-MS to compare their protein characteristics.

Proteomic Analysis of Selected RPHPLC Fractions with Active ACE Inhibitory Activity. The protein characteristics of E5PcF3 and E3AbF6 analyzed by SDS-PAGE and SELDI-TOF-MS

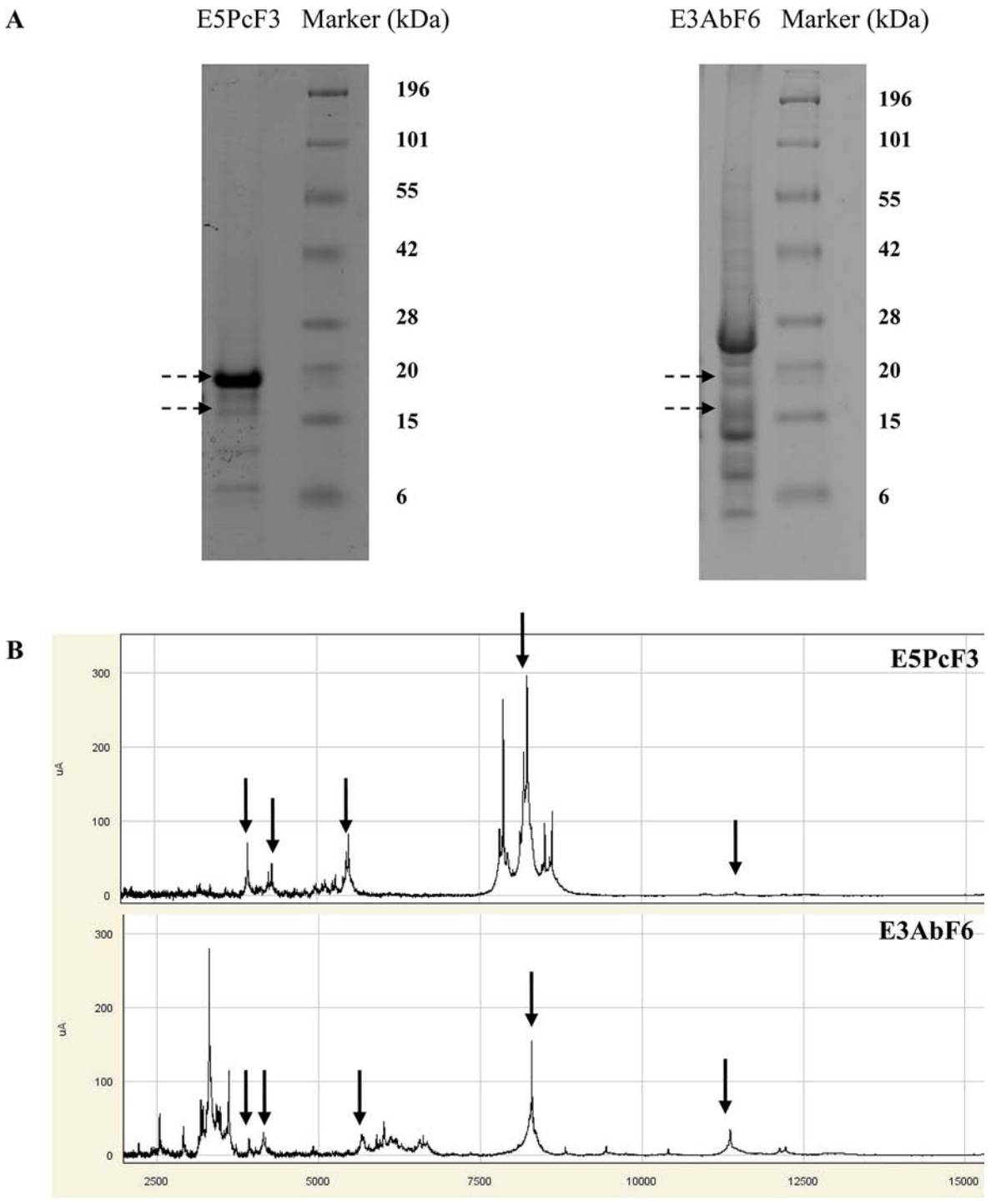

Figure 3. Proteomic analysis of E5PcF3 and E3AbF6 by (A) SDS-PAGE and (B) SELDI-TOF-MS. Dashed line arrow, similar proteins present in SDS PAGE of E5PcF3 and E3AbF6; solid line arrow, similar proteins present in SELDI-TOF-MS spectra of E5PcF3 and E3AbF6; SELDI-TOF-MS spectra, the *x*-axis of the spectra indicates the mass-to-charge ratio (m/z) of protonated proteins, and the *y*-axis indicates the relative intensities of mass spectral signals.

are shown in Figure 3. Referring to Figure 3A, SDS-PAGE of the two RPHPLC fractions revealed mostly proteins of low molecular mass (<30 kDa). Most previous studies on food-derived peptides with ACE inhibitory abilities also feature relatively low molecular weight proteins.[5−12] Proteins with molecular masses of 3−10 kDa have been reported to exhibit moderate ACE inhibitory activity.[35,36] In the current study, E5PcF3 and E3AbF6 have protein bands with molecular masses of 7 and 12 kDa as well as 5, 8, and 13 kDa, respectively. Therefore, it is possible to suggest that these low molecular weight proteins could be responsible for triggering the ACE inhibitory activity in E5PcF3 and E3AbF6.

There were two protein bands common to E5PcF3 and E3AbF6 of molecular masses of 16 and 18 kDa. Besides the two common bands, E5PcF3 contained a distinct 17 kDa band, which was not visualized in E3AbF6. On the other hand, E3AbF6

dx.doi.org/10.1021/jf3042159 | J. Agric. Food Chem. 2012, 60, 12341−12348

contained eight protein bands not detected in E5PcF3. They consisted mostly of high molecular weight proteins. According to Natesh et al.,[37] large peptide molecules are restricted for fitting into the active site of ACE. Therefore, the ACE inhibitory activity in E3AbF6 might not be attributable to these high molecular weight proteins.

SELDI-TOF-MS has been reported to be the most effective method for profiling low molecular mass proteins and peptides (<30 kDa),[38] providing a complementary visualization technique to SDS-PAGE data. SELDI-TOF-MS spectra of E5PcF3 and E3AbP6 are shown in Figure 3B. EDM analysis of the two RPHPLC fractions had identified 22 protein clusters (Table 5).

Table 5. EDM Analysis of Peak Intensities Observed in SELDI-TOF-MS Spectra of E5PcF3 and E3AbF6

		peak intensities[c] (μA)	
protein cluster[a] m/z	p value[b]	E5PcF3	E3AbF6
2552.0 ± 0.9	0.021	ND	59.9 ± 5.7
2919.2 ± 0.9	0.021	ND	39.4 ± 4.3
3189.0 ± 1.3	0.021	ND	70.9 ± 11.4
3223.2 ± 1.1	0.021	ND	60.1 ± 11.9
3317.7 ± 0.9	0.021	ND	295.7 ± 7.7
3621.2 ± 1.0	0.021	ND	102.0 ± 19.2
3940.6 ± 2.8	0.021	66.9 ± 2.8	22.4 ± 2.3
4254.8 ± 1.0	0.021	42.1 ± 2.6	30.6 ± 3.0
5291.2 ± 0.5	0.021	25.9 ± 0.5	ND
5496.6 ± 0.4	0.021	85.0 ± 5.4	35.2 ± 4.0
5906.9 ± 2.3	0.021	ND	30.1 ± 2.0
6022.5 ± 0.8	0.021	ND	42.5 ± 7.4
7876.5 ± 0.6	0.021	226.2 ± 39.4	ND
8193.5 ± 0.6	0.021	189.2 ± 5.4	ND
8223.6 ± 1.1	0.021	141.5 ± 4.9	ND
8244.2 ± 0.6	0.021	284.9 ± 13.5	ND
8291.8 ± 2.3	0.040	ND	76.5 ± 3.9
8305.8 ± 1.4	0.021	88.5 ± 2.5	155.3 ± 14.7
8328.2 ± 22.1	0.021	80.3 ± 2.6	50.1 ± 4.5
8516.6 ± 0.6	0.021	80.3 ± 13.9	ND
8629.5 ± 0.7	0.021	95.2 ± 14.9	ND
11413.5 ± 2.7	0.021	5.0 ± 0.3	39.6 ± 3.4

[a]Proteins with similar molecular masses were clustered during EDM analysis. The m/z value for each protein cluster is presented as the mean ± standard deviation (n = 4). Protein clusters highlighted in bold are common proteins detected in E5PcF3 and E3AbF6. [b]Values with $p < 0.05$ indicate the protein peaks were statistically significant in the two samples. [c]Peak intensities are presented as the mean ± standard deviation (n = 4). ND, peaks were not detected. Intensities of ND peaks are only estimated values for clustering purposes during EDM analysis. Thus, their intensities are not shown.

The SELDI-TOF-MS spectra of E3AbF6 contain SELDI peaks of high intensity lower than m/z 3500. These peaks were not detected in E5PcF3. In the current study, proteins lower than 3500 Da should have been removed during dialysis of the protein extracts by snakeskin dialysis tubing (3500 Da cutoff). As reported previously, intact protein structures may become unstable in 50% aqueous−acetonitrile mixtures.[39] On the basis of the location of the peak in the RPHPLC chromatogram (Figure 2), the fraction was collected from the gradient in close to a 50% water−acetonitrile mixture. The fact that proteins smaller than 3500 Da could represent broken down or fragmented proteins might explain their detection in the SELDI spectra of E3AbF6.

There were five common peaks with m/z values 3940, 4254, 5496, 8300, and 11413 detected in E5PcF3 and E3AbF6. The high intensity of these common peaks shows they could play a role in the high levels of ACE inhibitory activity exhibited by the two RPHPLC fractions. Besides the five common peaks, there were seven additional peaks with m/z values of 5291, 7876−8244, and 8516−8629 detected only in E5PcF3, whereas E3AbF6 contained three additional peaks with m/z values of 5906, 6022, and 8291 that were not detected in E5PcF3. Interestingly, the m/z values of these proteins were in the range of 3000−10000. Therefore, this further supported the SDS-PAGE results, which showed the ACE inhibitory activity in E5PcF3 and E3AbF6 could be attributed to proteins ranging from 3 to 10 kDa. In addition, the greater quantity of peptides with molecular mass ranging from 3000 to 10000 Da detected in E5PcF3 might explain the higher ACE inhibitory activity in E5PcF3 compared to E3AbF6.

In conclusion, mushrooms are a good source of antihypertensive proteins. This study demonstrated that proteins from *P. cystidiosus* and *A. bisporus* exhibited the highest ACE inhibitory activity. Proteomic analysis of ACE inhibitory proteins from *P. cystidiosus* showed they consist mainly of low molecular weight proteins with molecular mass ranging from 3 to 10 kDa. In contrast, the ACE inhibitory proteins from *A. bisporus* not only consist of proteins ranging from 3 to 10 kDa but also high molecular weight proteins. The detection of different ACE inhibitory proteins from *P. cystidiosus* and *A. bisporus* shows that these potentially active proteins possessed different characteristics. This suggests that the protein structures and the type of amino acid residues that make up the protein sequence may make contributions to the inhibition of ACE enzyme. Therefore, it is important to validate the structure and amino acid characteristics to relate the protein to antihypertensive activity.

AUTHOR INFORMATION

Corresponding Author

*Phone: +603-7967 4371. Fax: +603-7967 4178. E-mail: noorlidah@um.edu.my.

Funding

We thank the University of Malaya (Grant PPP: PS238/2008C, PS478/2010B, PV073-2011B) and the Ministry of Higher Education Malaysia (HIR-MOHE: F000002-21001) for financial support of this project.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The SELDI-TOF-MS analysis was facilitated by access to the Medical Biotechnology Laboratory, Faculty of Medicine, University of Malaya and University of Malaya Centre for Proteomics Research (UMCPR), University of Malaya.

REFERENCES

(1) Kearney, P. M.; Whelton, M.; Reynolds, K.; Muntner, P.; Whelton, P. K.; He, J. Global burden of hypertension: analysis of worldwide data. *Lancet* **2005**, *365*, 217−223.

(2) Hernández-Ledesma, B.; Contreras, M. d. M.; Recio, I. Antihpertensive peptides: production, bioavailability and incorporation into foods. *Adv. Colloid Interface Sci.* **2011**, *165*, 23−25.

(3) Turner, A. J.; Hooper, N. M. The angiotensin-converting enzyme gene family: genomics and pharmacology. *Trends Pharmacol. Sci.* **2002**, *23*, 177−183.

(4) Antonios, T. F.; MacGregor, G. A. Angiotensin converting enzyme inhibitors in hypertension: potential problems. *J. Hypertens.* **1995**, *13*, S11−16.
(5) Nakamura, Y.; Yamamoto, N.; Sakai, K.; Okubo, A.; Yamazaki, S.; Takano, T. Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk. *J. Dairy Sci.* **1995**, *78*, 777−783.
(6) Ma, M.-S.; Bae, I. Y.; Lee, H. G.; Yang, C.-B. Purification and identification of angiotensin I-converting enzyme inhibitory peptide from buckwheat (*Fagopyrum esculentum* Moench). *Food Chem.* **2006**, *96*, 36−42.
(7) Pihlanto, A.; Akkanen, S.; Korhonen, H. J. ACE-inhibitory and antioxidant properties of potato (*Solanum tuberosum*). *Food Chem.* **2008**, *109*, 104−112.
(8) Lee, S.-H.; Qian, Z.-J.; Kim, S.-K. A novel angiotensin I converting enzyme inhibitory peptide from tuna frame protein hydrolysate and its antihypertensive effect in spontaneously hypertensive rats. *Food Chem.* **2010**, *118*, 96−102.
(9) Wang, J.; Hu, J.; Cui, J.; Bai, X.; Du, Y.; Miyaguchi, Y.; Lin, B. Purification and identification of a ACE inhibitory peptide from oyster proteins hydrolysate and the antihypertensive effect of hydrolysate in spontaneously hypertensive rats. *Food Chem.* **2008**, *111*, 302−308.
(10) Lee, J.-E.; Bae, I. Y.; Lee, H. G.; Yang, C.-B. Tyr-Pro-Lys, an angiotensin I-converting enzyme inhibitory peptide derived from broccoli (*Brassica oleracea Italica*). *Food Chem.* **2006**, *99*, 143−148.
(11) Saito, T.; Nakamura, T.; Kitazawa, H.; Kawai, Y.; Itoh, T. Isolation and structural analysis of antihypertensive peptides that exist naturally in Gouda cheese. *J. Dairy Sci.* **2000**, *83*, 1434−1440.
(12) Barbana, C.; Boye, J. I. Angiotensin I-converting enzyme inhibitory activity of chickpea and pea protein hydrolysates. *Food Res. Int.* **2010**, *43*, 1642−1649.
(13) Hata, Y.; Yamamoto, M.; Ohni, M.; Nakajima, K.; Nakamura, Y.; Takano, T. A placebo-controlled study of the effect of sour milk on blood pressure in hypertensive subjects. *Am. J. Clin. Nutr.* **1996**, *64*, 767−771.
(14) He, R.; Ma, H.; Zhao, W.; Qu, W.; Zhao, J.; Luo, L.; Zhu, W. Modeling the QSAR of ACE-inhibitory peptides with ANN and its applied illustration. *Int. J. Pept.* **2012**, DOI: 10.1155/2012/620609.
(15) Li, G.-H.; Le, G.-W.; Shi, Y.-H.; Shrestha, S. Angiotensin I-converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacological effects. *Nutr. Res. (N.Y.)* **2004**, *24*, 469−486.
(16) Wu, J.; Aluko, R. E.; Nakai, S. Structural requirements of angiotensin I-converting enzyme inhibitory peptides: quantitative structure-activity relationship modeling of peptides containing 4−10 amino acid residues. *QSAR Comb. Sci.* **2006**, *25*, 873−880.
(17) Wu, J.; Ding, X. Hypotensive and physiological effect of angiotensin converting enzyme inhibitory peptides derived from soy protein on spontaneously hypertensive rats. *J. Agric. Food Chem.* **2001**, *49*, 501−506.
(18) Guillamón, E.; García-Lafuente, A.; Lozano, M.; D'Arrigo, M.; Rostagno, M. A.; Villares, A.; Martínez, J. A. Edible mushrooms: role in the prevention of cardiovascular diseases. *Fitoterapia* **2010**, *81*, 715−723.
(19) Chang, S.-T.; Miles, P. G. *Mushrooms: Cultivation, Nutritional Value, Medicinal Effect and Environmental Impact*; CRC Press: Boca Raton, FL, 2004; Vol. *109*, pp 255−256.
(20) Lee, D. H.; Kim, J. H.; Park, J. S.; Choi, Y. J.; Lee, J. S. Isolation and characterization of a novel angiotensin I-converting enzyme inhibitory peptide derived from the edible mushroom *Tricholoma giganteum*. *Peptides* **2004**, *25*, 621−627.
(21) Choi, H. S.; Cho, H. Y.; Yang, H. C.; Ra, K. S.; Suh, H. J. Angiotensin I-converting enzyme inhibitor from *Grifola frondosa*. *Food Res. Int.* **2001**, *34*, 177−182.
(22) Jang, J.-H.; Jeong, S.-C.; Kim, J.-H.; Lee, Y.-H.; Ju, Y.-C.; Lee, J.-S. Characterisation of a new antihypertensive angiotensin I-converting enzyme inhibitory peptide from *Pleurotus cornucopiae*. *Food Chem.* **2011**, *127*, 412−418.
(23) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.
(24) Manzi, P.; Aguzzi, A.; Pizzoferrato, L. Nutritional value of mushrooms widely consumed in Italy. *Food Chem.* **2001**, *73*, 321−325.
(25) Reis, F. S.; Barros, L.; Martins, A.; Ferreira, I. C. F. R. Chemical composition and nutritional value of the most widely appreciated cultivated mushrooms: an inter-species comparative study. *Food Chem. Toxicol.* **2012**, *50*, 191−197.
(26) Cağlarirmak, N. The nutrients of exotic mushrooms (*Lentinula edodes* and *Pleurotus* species) and an estimated approach to the volatile compounds. *Food Chem.* **2007**, *105*, 1188−1194.
(27) Bonatti, M.; Karnopp, P.; Soares, H. M.; Furlan, S. A. Evaluation of *Pleurotus ostreatus* and *Pleurotus sajor-caju* nutritional characteristics when cultivated in different lignocellulosic wastes. *Food Chem.* **2004**, *88*, 425−428.
(28) Dundar, A.; Acay, H.; Yildiz, A. Yield performances and nutritional contents of three oyster mushroom species cultivated on wheat stalk. *Afr. J. Biotechnol.* **2008**, *7*, 3497−3501.
(29) Kim, J.; Ra, K.; Noh, D.; Suh, H. Optimization of submerged culture conditions for the production of angiotensin converting enzyme inhibitor from *Flammulina velutipes*. *J. Ind. Microbiol. Biotechnol.* **2002**, *29*, 292−295.
(30) Apostolidis, E.; Kwon, Y.-I.; Shetty, K. Potential of select yoghurts for diabetes and hypertension management. *J. Food Biochem.* **2006**, *30*, 699−717.
(31) Erjavec, J.; Kos, J.; Ravnikar, M.; Dreo, T.; Sabotič, J. Proteins of higher fungi-from forest to application. *Trends Biotechnol.* **2012**, *30*, 259−273.
(32) Barbosa-Filho, J. M.; Martins, V. K. M.; Rabelo, L. A.; Moura, M. D.; Silva, M. S.; Cunhua, E. V. L; Souza, M. F. V.; Almeida, R. N.; Medeiros, I. A. Natural products inhibitors of the angiotensin converting enzyme (ACE). A review between 1980−2000. *Rev. Bras. Farmacogn.* **2006**, *16*, 421−446.
(33) Scopes, R. K. *Protein Purification: Principles and Practice*,3rd ed.; Springer-Verlag: New York, 1994.
(34) Wang, C.; Tian, J.; Wang, Q. ACE inhibitory and antihypertensive properties of apricot almond meal hydrolysate. *Eur. Food Res. Technol.* **2011**, *232*, 549−556.
(35) Chiang, W.-D.; Lee, M.-J.; Guo, W.-S.; Tsai, T.-C. Protein hydrolysate batch production with angiotensin-I-converting enzyme inhibitory activity from egg whites. *J. Food Drug Anal.* **2006**, *14*, 385−390.
(36) Jiang, Z.; Tian, B.; Brodkorb, A.; Huo, G. Production, analysis and *in vivo* evaluation of novel angiotensin-I-converting enzyme inhibitory peptides from bovine casein. *Food Chem.* **2010**, *123*, 779−786.
(37) Natesh, R.; Schwager, S. L. U.; Sturrock, E. D.; Acharya, K. R. Crystal structure of the human angiotensin-converting enzyme-lisinopril complex. *Nature* **2003**, *421*, 551−554.
(38) Issaq, H. J.; Conrads, T. P.; Prieto, D. A.; Tirumalai, R.; Veenstra, T. D. Peer reviewed: SELDI-TOF MS for diagnostic proteomics. *Anal. Chem.* **2003**, *75*, 148A−155A.
(39) Gekko, K.; Ohmae, E.; Kameyama, K.; Takagi, T. Acetonitrile-protein interactions: amino acid solubility and preferential solvation. *Biochim. Biophys. Acta* **1998**, *1387*, 195−205.