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Angiotensin-I converting enzyme inhibitory activity of mushroom *Lentinus polychrous* Lév. and its development of healthy drink recipes

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ABSTRACT

Hypertension is a major health problem affecting all populations around the world. Angiotensin I-converting enzyme (ACE-I), an important enzyme in renin angiotensin system (RAS) catalysis, yields a product that increases blood pressure in our bodies. Many mushrooms have been reported to have blood pressure lowering properties. However, such activities have not been investigated in *Lentinus polychrous* Lév., one of the most consumed mushrooms in Thailand. In this study, an ACE-I inhibitory activity of crude protein extract of *L. polychrous* was examined. The crude protein was separated by a dialysis membrane of 10 kDa MWCO and an anion exchange chromatography on Q-Sepharose XL column, consecutively. The fraction with MW <10 kDa with better ACE-I inhibitory activity was further purified by Q-sepharose XL. The active unbound (UQ) fraction contained the highest ACE-I inhibitory activity of $60.93 \pm 9.94\%$ (1.066 μg protein/ml) which is equivalent to 15.46 ± 0.71 nmol Captopril per μg protein extract. The bound protein fractions (BQ1-BQ5) resulted in no protein bands appearing in 15% SDS-PAGE. However, thin layer chromatography (TLC) indicated that these active fractions might contain peptides or very small proteins. Additionally, this mushroom extract had been developed as healthy drink recipes for helping lower blood pressure.

1. Introduction

Hypertension or high blood pressure is a major health problem and has a worldwide prevalence leading to millions of deaths each year. Patients with high blood pressure have a higher risk of diseases such as atherosclerosis, stroke, myocardial infarction and end-stage renal diseases (Liu et al., 2012).

The angiotensin I-converting enzyme (ACE-I, EC. 3.4.15.1), also called dipeptidyl carboxypeptidase I, is an important enzyme in renin angiotensin system (RAS) for blood pressure regulation in our bodies. It catalyzes the transformation of an inactive peptide hormone angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) to a shorter active peptide hormone angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe), which causes the blood pressure to increase (Jang et al., 2011). Therefore, prevention of hypertension could be achieved by inhibiting the ACE-I activity. The medical treatment of hypertension is performed by using the synthetic ACE-I inhibitors such as captopril, enalapril and Lisinopril. However, these synthetic compounds have several side effects including coughing and skin rashes (Je et al., 2004). Peptides with an antihypertensive property have been reported. These peptides are part of a food-derived protein from animal or plant such as casein, whey proteins, chicken, fish, soy, or egg, which have safety advantages over the synthetic compounds (Liu et al., 2013). Among food sources having an antihypertensive property are mushrooms. Several have already been studied for their potential use in antihypertension including *Pleurotus sajor-caju, P. ostreatus, P. cornucopiae, P. nebrodensis, Flammulina velutipes, Tricholoma giganteum, Agaricus bisporus, Poria cocos, Grifola umbellate, G. frondosa, Hypsizygus marmoreus* and *Ganoderma lucidum* (Choi et al., 2001; Lee et al., 2004; Miyazawa et al., 2008; Lau et al., 2012; Jang et al., 2013; Kang et al., 2013; Ansor et al., 2013; Lau et al., 2014).

The mushroom *Lentinus polychrous* is a popular staple in northeastern Thailand. However, there has been no report on the mushroom's ACE-I inhibitory activity. In this study, we investigated this activity in *L. polychrous* and tried to formulate *L. polychrous*-based healthy drinks.

2. Materials and methods

2.1. Mushroom

L. polychrous Lèv. fresh fruiting bodies were purchased from Rujira Mushroom Farm-Ban Hed Thai, Kalasin, Thailand. After purchase, they were stored at 4 °C in a refrigerator until use.

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2.2. Chemicals and reagents

N-Hippuryl-His-Leu hydrate (HHL), angiotensin converting enzyme I (ACE-I) from rabbit lung and ninhydrin solution were obtained from Sigma-Aldrich, USA. Ethyl acetate was purchased from Merck, Germany. Bovine serum albumin was obtained from Fluka, Switzerland. Bio-Rad Protein Assay Dye reagent concentrate was obtained from Bio-Rad, USA. Acrylamide solution (30%) molecular biology grade was obtained from AppliChem Panreac, USA. Other chemicals were of analytical grade.

2.3. Preparation of crude protein

The mushroom samples were washed and cleaned with distilled water. After that, they were homogenized with distilled water in the ratio of 1 g: 3 ml using a blender. The blended mixture was stirred at 4 °C for 1 h and then filtered through a double layer cheesecloth. The clear supernatant was collected as the crude protein extract after centrifugation at 10,000*g*, 4 °C for 30 min and was kept at 4 °C for further investigation.

2.4. Protein determination

The soluble protein content was measured according to the Bradford method (Bradford, 1976). One milliliter of a total reaction mixture consisted of 200 μl of Bio-Rad protein assay reagent and 800 μl of diluted protein extract. After mixing, the mixture was left at room temperature for 5 min before the absorbance was then read at 595 nm using a Vis-spectrophotometer (Thermo spectronic Genesys 20 Model 4001/4 visible range spectrophotometer, USA). Bovine serum albumin (BSA) was used to create a standard curve.

2.5. ACE-I inhibitory assay

ACE-I inhibitory activity assay was modified according to the method of Lee et al. (2004). The total reaction mixture of 350 μl contained 100 μl of sample solution (or inhibitor) mixed with 200 μl of HHL (final concentration 5 mM), which was dissolved in 100 mM sodium borate buffer having a pH of 8.3 and containing 608 mM NaCl. This mixture was then pre-incubated at 37 °C for 5 min. After that, 50 μl of 25 mU/ml of ACE was added to the reaction mixture, which was then subjected to further incubation at 37 °C for 30 min. The reaction was terminated by an addition of 120 μl of 1 M HCl. Hippuric acid (HA) product was extracted with 1 ml of ethyl acetate by vortexing for 1 min and then centrifuging at 4,000g for 15 min. Subsequently, 900 μl of the upper layer of the supernatant was withdrawn, dried out in a boiling water bath for 15 min and re-dissolved in 1.5 ml of deionized water. The formation of HA product was measured at 228 nm by a UV/VIS spectrophotometer (model SPEKOL 1500, Analytik Jena, Germany). The %inhibition value was calculated according to the equation below;

$$\%\text{Inhibition} = \frac{C-(I-IB)}{C} \times 100$$

where C is the absorbance of the reaction containing enzyme and substrate without the sample, I is the absorbance of the reaction containing enzyme, substrate and sample (or inhibitor), IB is the absorbance of the solution containing only sample (or inhibitor) without the enzyme and substrate.

2.6. Partial purification of ACE-I inhibitor

The ACE-I inhibitor protein from *L. polychrous* mushroom was first partially purified by 10 kDa MWCO and then by strong anion exchange column chromatography. The crude protein extract was dialyzed using 10 kDa MWCO dialysis tubing (Thermo Scientific, USA). Both fractions of MW less than 10 kDa (< 10 kDa) and MW larger than 10 kDa (> 10 kDa) were obtained and subjected to ACE-I inhibitory assay. The selected active fraction (< 10 kDa fraction) was then purified by Q-sepharose XL column (5×5 ml, GE Healthcare, Sweden) coupled to AKTAprime plus system (GE Healthcare, Sweden). The column equilibrated with 20 mM Tris-HCl buffer (pH 8.5) was loaded with < 10 kDa fraction at a flow rate of 3 ml/min. After washing out the unbound proteins, the bound proteins were eluted out with a linear gradient of 0–0.5 M NaCl in 20 mM Tris-HCl buffer (pH 8.5) and fractions were collected at 3 ml/tube. The proteins in the fractions were monitored at 280 nm. The fractions were collected and pooled according to peak and subjected to ACE-I inhibitory activity assay.

2.7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE was performed according to the Laemmli method (Laemmli, 1970) using a 15% separating gel and a 5% stacking gel by Mini-Protean® 3 cell (Bio-Rad, USA). The upper and lower parts of the chamber were filled with an electrophoretic buffer containing SDS. The gel was run at a constant voltage of 100 V/gel. The gel was stained with silver staining solution to visualize protein bands.

2.8. Thin layer chromatography (TLC)

To confirm the presence of peptides in the active fractions, samples of each fraction were analyzed by TLC using aluminium sheet coated with a stationary phase of silica gel (Silica gel 60 F254, Merck, Germany) and distilled water was used as a developing solvent. The TLC was sprayed with ninhydrin solution and heated on a hotplate until purple spots appeared.

2.9. Mushroom drink recipes

The fresh fruiting bodies of mushroom *L. polychrous* were used to develop drink recipes accompanied with other herbs for good flavor as shown in Table 1. The mushrooms were cut into very small pieces, combinatively boiled with other herb ingredients for 20 min, left to cool down and then filtered through a mesh and a double layer cheesecloth. Each mushroom drink was then assayed for its ACE-I inhibitory activity.

2.10. Statistical analysis

Data was reported as mean ± standard deviation (S.D.) and statistical analysis was performed with the SPSS version 18 software (SPSS Inc., Chicago, USA). A p value of less than 0.05 was taken as significant.

Table 1
Ingredients of each mushroom drink recipes.

Ingredients (%)	Drink recipes				
	A	B	C	D	E
Fresh mushroom *L. polychrous* Lèv. (10)	✓	✓	✓	✓	✓
Dried bael (0.6)	–	–	✓	–	✓
Dried chrysanthemum (5)	–	–	–	✓	✓
Honey* (6)	–	✓	✓	✓	✓

* Honey was added after the mushroom drink cooled down.