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The analysis of Angiotensin-I Converting Enzyme (ACE) by *maitake* (*Grifrola frondosa*) mycelia

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Abstract

The *Maitake* (*Grifola frondosa*) is useful in treating diseases, specifically hypertension. Research on the *maitake* mycelia′s biological properties, nevertheless, are limited in the literature. This study aimed to (i) produce mushroom biomass adopting submerged fermentation, and (ii) investigate the Angiotensin-I Converting Enzyme inhibitory activity. *Maitake* mycelia′s yield after 14 days of fermentation under controlled conditions (approx. 1.32 g/L) were freeze-dried into powder and later were hydrolysed for analyses of Angiotensin-I Converting Enzyme inhibitory activity. Current results showed that the degree of hydrolysis increased in line with hydrolysis time, as the protein concentration for hydrolysed sample was 283.61 ± 7.14 μg/mL, however, the non-hydrolysed sample resulted in lesser protein content (46.76 ± 1.09 μg/mL). The hydrolysate *maitake* mycelia has higher Angiotensin-I Converting Enzyme inhibitory activity (46.48%) as compared to the non-hydrolysate *maitake* mycelia (20.19 ± 0.17%). This finding suggested *maitake* mycelia hydrolysate can be a source of potential bioactive peptides used in treating hypertension.

1. INTRODUCTION

Hypertension disease is the leading cause of global mortality. Nowadays, consumers prefer natural agro-resources over synthetic drugs as the former has limited side effects and are cheaper (Actis-Goretta *et al*., 2003). Mushrooms, which contain incredible functional and active constituents, can be used as nutraceuticals or functional food ingredients (Miyazawa *et al*., 2008). Furthermore, mushrooms are well-recognised for their therapeutics and medicinal effects in treating various chronic diseases, including hypertension, depression, etc. Their fruiting bodies are highly sought for phytochemicals or active metabolites, for example, phenolics, flavonoids, glucans, bioactive peptides, antioxidants, antidiabetic compounds, to name a few. Current research trend focuses on the search for potential bioactive peptides (Angiotensin-I Converting Enzyme (ACE) inhibitory peptides) that are derived from the mushroom species in treating hypertension and related problems (Kim *et al*., 2004; Kang *et al*., 2013; Farrag *et al*., 2015; Wu *et al*., 2019; Karnchanatat *et al*., 2020)

Maitake (*Grifola frondosa*) is one of the popular cultivated species, which has great market demand for its superior nutritional and medicinal values. Conventional *maitake* cultivation is time-consuming, quality instability, and prone to contamination as it heavily relies on its fruiting bodies development. Unlike the conventional ones, growing mushroom through submerged fermentation has been appraised to produce excellent quality mushroom biomass at a quicker rate. Several recent studies have revealed that the nutritional values of the mushrooms mycelial is comparable to those found in the fruiting bodies (Kim *et al*., 2007; Ulziijargal, & Mau, 2011).

Consuming mushrooms has long been associated with a reduced risk of countless lifestyle-related health problems. However, to the best of our knowledge, information on the biological properties, especially antihypertensive peptides and fibrinolytic level that contained in the *maitake* mycelia are still lacking in the literature. Thus, this study aimed to produce mushroom biomass using submerged fermentation besides examining the ACE inhibitory peptides from the fermented *maitake*.

The outcomes of this study are important as it determines the feasibility to grow *maitake* mycelia that might possess potential biological properties to fulfil local demand on healthy and functional foods shortly.

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2. MATERIALS AND METHODS

2.1. Mushroom stock culture preparation and storage

Commercial cultures of *maitake* were collected from the Mushroom Research Centre (MRC), University of Malaya. The stock culture was maintained and further prepared as seed culture for future analysis using shake-flask fermenter located in the General Chemistry Laboratory, Faculty of Agro-Based Industry, Universiti Malaysia Kelantan, Jeli Campus.

2.2. Culture and mycelium production using submerged fermentation

A 2.0 L stirred-tank fermenter with a working volume of 1.5 L was used. The standard fermentation medium was inoculated with 10% (v/v) of the stock culture and cultivated at 28 °C in the shake-flask fermentor (Aminuddin *et al*., 2007).

2.3. Mycelia growth and determination on biomass and reducing sugar concentration

The sampling of the growth media (10 mL) in the fermenter was done on a daily basis until the mycelia biomass and reducing sugar concentration reached their highest values. The growth rate was measured according to the methods described by Aminuddin *et al*., (2007). The reducing sugar concentration of the supernatants was determined by the dinitrosalicyclic acid (DNS) method of Miller (1959). The harvested mycelia was freeze-dried and kept at 4 °C until further analysis.

2.4. Preparation of *maitake* mycelia hydrolysate

The preparation of mycelia hydrolysate was done according to procedure described by Thanasukhon., (2009) with modifications. The *maitake* mycelia powder (1 g) was suspended in 10 mL of phosphate buffet at pH 8.0 and alcalase was added into the suspension followed by incubation at 50 °C in shaking water bath for 5 hours. The hydrolysis was terminated by heating the sample at 90 °C for 20 min in the water bath, followed by centrifugation (10000 x g), 4 °C for another 30 min. The supernatant was collected and stored at -80 °C until further analysis. Besides, the non-hydrolysed sample was prepared by excluding the use of enzyme throughout the analysis.

2.5. Determination on degree of hydrolysis and protein concentration

2.5.1. Degree of hydrolysis

The degree of hydrolysis (DH) of each sample (hydrolysate and non-hydrolysate mycelia) was determined using the o-phthaldialdehyde (OPA) method described by Nielsen *et al*., (2001) with minor modifications. An amount of 10 µL of the sample was mixed with 3.4 mL of the o-phthaldialdehyde (OPA) reagent, and the mixture was allowed to keep at 25 °C for 2 minutes. Eventually, the absorbance of the mixtures of respective samples was read at 340 nm and the DH was calculated according to given Equation (1).

$$\% \text{ DH} = \frac{\text{Soluble N in TCA 10\% (w/v)}}{\text{Total N in the sample}} \times 100 \quad (1)$$

2.5.2. Protein concentration

The total protein of each sample (hydrolysed and non-hydrolysed) was determined using the bicinchoninic acid (BCA) method. Serial dilutions of bovine serum albumin (BSA) was used as standard. The absorbance was recorded at 675 nm using a spectrophotometer.

2.6. Determination of the biological activities of the *maitake* mycelia

2.6.1. Determination of the ACE-I inhibitory activity

The ACE-I inhibitory activity of the sample was determined according to the method described by Cushman & Cheung (1971). Initially, the sample (0.2 mL) was added to ACE solution (50 µL) and the reaction started by adding 0.2 mL of 5 mmol L^{-1} hippuric histidyl leucine, followed by incubated at 37 °C for 15 min. The reaction was terminated by adding 0.25 mL of 1.0 N hydrochloric acid and then 2.0 mL ethyl acetate to extract the hippuric acid formed by the action of ACE. The mixture was then centrifuged at 3600 xg (2 min) before transferring 1 mL of upper layer into a sterilised microcentrifuge tube. The tube was then heated in water bath at 100 °C for 15 min. The resulting solution was then dissolved in distilled water (3.0 mL) and read using a spectrophotometer (228 nm). The ACE activity was determined by the released of hippuric acid by enzymatic hydrolysis per minute per milligram of tissue.

2.7 Statistical analysis

All analyses were done in triplicate. The data were analysed using t-test. SPSS was used for data analysis (SPSS, Version 17.0).

3. RESULT AND DISCUSSION

Table 1 shows the maitake biomass gained from the technique of submerged fermentation. As can be noticed from Table 1, the yield of maitake biomass was 1.32 ± 0.09 g/L within 14 days of fermentation.

Table 1: The *maitake* biomass (1.32±0.09 g/L) produced via submerged fermentation within 14 days of fermentation

Analysis	Initial (0 day)	After fermentation (14 days)
Yield of biomass (g/L)	0.32 ± 0.01^{b}	1.32 ± 0.09^{a}
pH	5.0 ± 0.10^{a}	4.22 ± 0.15^{ab}
Reducing sugar content (µg/mL)	74.81 ± 0.20^{a}	25.18 ± 0.32^{b}

Mean with different letters within a row were significantly different ($P < 0.05$)

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It is understood that the feasibility of a peptide to exert their health-promoting properties rely on the efficacy of gastrointestinal enzyme in releasing them from protein complexes. The use of alcalase meant to liberate more potent soluble peptides that contain in mycelia. Hence, the availability of potent soluble peptides will determine the functionality of the mushroom mycelia. The DH exhibited an increasing trend over the incubation time (4 hours) throughout the test was shown in Fig 1. It was noted that the DH for the hydrolysed sample reached the highest at 78% after 4 hours of treatment, while the non-hydrolysed sample recorded a steep reduction to 18%. The trend of this finding was similar to those reported by Jimenez-Escrig *et al*., (2010) in search of bioactive peptides derived from okara, as the DH for hydrolysed sample relatively greater than those non-hydrolysed.

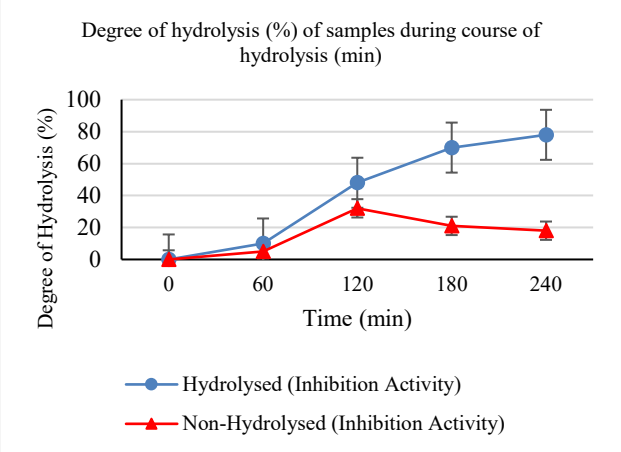

Figure 1: Changes of degree of hydrolysed (%) of samples during hydrolysis (min)

The protein concentration for the hydrolysed sample recorded the highest (283.61 ± 7.14 µg/mL) after 4 hours of hydrolysis (Fig 2). Besides, the hydrolysed sample also showed gradual increment on the protein concentrations with longer time of hydrolysis.

On the contrary, the non-hydrolysed sample contained lesser protein than the hydrolysed sample (46.76 ± 1.09 µg/mL) after hydrolysis completed. Besides, it was observed that protein concentration of the non-hydrolysed samples was inconsistent, as it increased from 45.73 ± 3.20 µg/mL (60 min) to 62.08 ± 2.4 µg/mL (120 min) before dropping to 46.76 ± 1.09 µg/mL (240 min). This indicates that the hydrolysed sample contained more stable potential peptides.

Meanwhile, the hydrolysed sample always showed greater inhibition activity (%) as compared to non-hydrolysed no matter at which concentrations tested (0.5 – 4 mg/mL). For instance, the ACE inhibitory activity of non-hydrolysed sample recorded 28.65 ± 2.09% even at a concentration as low as 2 mg/mL; while the hydrolysed one recorded an approximately of 39.55% of activity when tested at the same concentration (Table 2). This scenario might attributed by the present of potential bioactive peptides that played a significant effect in regulating the conversion of ACE I into ACE II, which eventually control the blood pressure of the biological system. In fact, the use of alcalase during hydrolysis will definitely assist the release of active peptides from their inactive site to cleave to the active site of (Shen *et al*., 2012).

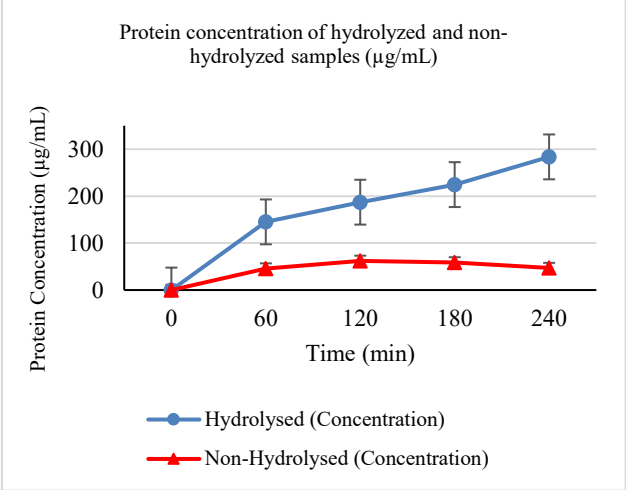

Figure2: Protein concentration of hydrolysed and non-hydrolysed samples (µg/mL)

Table 2: ACE inhibitory activity of *maitake* hydrolysed samples at different concentrations.

Sample (s)	Concentration (mg /mL)				
	0.5	1	2	3	4
Non-hydrolyzed	20.19 ± 0.17[a]	24.63 ±0.98[a]	28.65 ± 2.09[a]	34.10 ±1.4[a]	38.26 ± 1.6[a]
hydrolyzed	29.67 ± 0.22[b]	32.78 ± 1.82[b]	39.55 ± 1.28[b]	43.27 ± 2.05[b]	46.48 ± 2.1[b]

Hydrolysed – Treated with alcalase; Non-hydrolyzed – Control

Mean with different letters within a column were significantly different ($P < 0.05$)

4. CONCLUSION

The study revealed that the protein concentration was correlated with increase degree of hydrolysis (DH). The hydrolysed *maitake* exhibited better ACE inhibitory activity to those non-hydrolysed samples even at lower concentrations. Current findings suggested that hydrolysed *maitake* biomass has the great potential to undergo detailed studies including the isolation and purification of those active peptides that could eventually determine its feasibility to be used as bioactive peptides which can regulate blood pressure in stress management. A rich multiplicity of medical values on *maitake* mycelia has yet to be discovered to date. Special attention can be given to mushroom polysaccharides for future research.

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