# CHAPTER 5

# DISCUSSIONS

## 5.1 Submerged fermentation for mycelia production and protein extraction

In the present study, spent brewer's yeast and brown sugar were the ingredients to a low-cost medium used in the cultivation of mycelia. According to Fang and Zhong, (2002), carbon and nitrogen are the two major nutrient sources that are crucial during mushroom cultivation. Waste products produced from brewery industry, including spent brewer's yeast, has potential as the nitrogen source for cultivating mushrooms (Schildbach *et al.*, 1992). Besides, abundance of nitrogen content, high level of moisture and satisfactory physical properties have positively drawn researchers' attention to adopt the waste product in the cultivation of mushrooms (Noble *et al.*, 2002; Gregori *et al.*, 2008).

Brown sugar which is a product from sucrose and classified as a monosaccharide, was utilised as the carbon supplier during the cultivation. Nonetheless, in situation where production of intracellular polysaccharides and ganoderic acids needs to be enhanced, Tang and Zhong (2002) more preferred lactose as the carbon source.

In general, the optimal pH condition for mycelia growth varies among species and for *G. lucidum*, it favours an acidic environment. The culture medium was well adjusted to pH 5, which apparently lies in the preferable pH value for the mycelia growth, as reported by Yang and Liau (1998). Besides, such acidic condition would reduce the chance for bacterial growth during the culture process.

Introduction of agitation during submerged cultivation was also crucial for obtaining optimal mycelial growth where it enhanced the secretion of extracellular substances as well as maximum oxygen transfer. As suggested by Wagner *et al.* (2003), the agitation speed was performed at 140 rpm since a higher shaking speed might cause detrimental effect in which it attributed to shear stress of the mycelia and led to

decrease in growth rate.

The average dry weight of mycelia biomass obtained in the present study was 496.9 mg/100 mL, slightly lower compared to the previous study done by Yang and Liau (1998) which obtained mycelia yield of 555 mg/100 mL. They performed the cultivation process using medium consisting of malt extract, glucose and yeast extract. This medium was apparently more expensive than the one used in our study. The different type of medium utilized might contribute to the yield discrepancy in these two studies.

According to Chandra and Purkayastha (1977), majority of tropical macrofungi utilized glucose than other carbon sources. This preference was attributed to the fast metabolization of glucose by the fungi in order to produce cellular energy (Garraway and Evans, 1984). However, Jayasinghe *et al.* (2008) found out that the mycelial growth in *G. lucidum* was good when using sucrose instead of glucose.

Prior to protein extraction, the mycelia's strong cell wall was ruptured to ease the release of intracellular proteins. The cell wall disruption process was done on freeze-dried mycelia since in such form the metabolic activity of cellular proteins was low and could minimize proteolysis. The freeze-dried mycelia were crushed using mortar and pestle to lyse the cell wall. Mechanical approach was applied since the method was efficient in releasing high amount of intracellular proteins (Nandakumar and Marten, 2002).

Intracellular proteins of the mycelia were extracted by water due to the interest in extracting water soluble compounds. Utilization of chemical reagent such as methanol for the extraction was avoided as the chemical could destroy sensitive macromolecules including protein (Ker *et al.*, 2011).

## 5.2 *Ganoderma lucidum* mycelia as the source of ACE inhibitors

During submerged fermentation, there were different proteins being produced in the mycelia itself (intracellular proteins) or excreted into the broth culture media (extracellular proteins) (Ker *et al.*, 2011). Accordingly, in the present study, the mycelia biomass was separated from the broth culture. This appeared to be advantageous in the matter of producing a less heterogeneous crude extract endowed with bioactive compounds.

From the preliminary screening result, mycelia crude water extract showed a potent *in vitro* inhibitory activity by reducing 95.50 % of ACE activity at screening concentration of 10 mg/mL (Figure 4.6). While exerting inhibitory strength higher than broth crude water extract, it was suggested that the inhibitory effect might be due to the proteins or peptides that were present in the crude water extract, coincided with that proposed by Kumakura *et al.* (2008). These proteins or peptides with strong anti-ACE effect were produced in intracellular compartment of mycelia and not excreted into the broth culture media. Therefore, strong inhibitory effect on ACE activity was not expressed by extracellular proteins.

Mycelia from *G. lucidum* in the present work showed a higher level of ACE inhibition ($IC_{50}$ = 0.90 mg/mL) when compared with mycelia extract from other medicinal mushrooms. *Ganoderma lucidum* mycelia crude water extract showed approximately 1.82-fold stronger ACE inhibitory activity than *Lyophyllum decastes* mycelia water extract ($IC_{50}$ = 1.637 mg/mL ± 0.057 mg/mL) (Gao *et al.*, 2012). It was suggested that the $IC_{50}$ value varied from one species of mushroom to another with regard to the differences of water extractives obtained.

Protein profiling for *G. lucidum* mycelia was previously performed by Saltarelli *et al.* (2009) using 2D-electrophoresis wherein it was done to compare the protein

contents in two different mycelia isolates. Through a different approach, the present study had developed a protein profile using SDS-PAGE (Figure 4.8). SDS-PAGE profile of the mycelia crude water extract revealed the diversity of intracellular proteins in particular fractions derived from salting out method.

The distinctive assortment of the protein bands suggested the efficiency of ammonium sulphate precipitation in fractionating proteins based on solubility. At present, a number of studies have adopted ammonium sulphate precipitation as the first purification step (Cui and Chisti, 2003; Sun *et al.*, 2003; Du *et al.*, 2007; Li *et al.*, 2009). The employment of salting out method was due to the remarkable characteristics of ammonium sulphate to be admired as a good protein crystallization agent (Dumetz *et al.*, 2007; Burgress, 2008). Salting out was performed at low temperature (4°C) as to minimize protein denaturation and block any enzyme activity.

As presented in Figure 4.8, several intense protein bands were detected in protein fractions 70 % - 100 % which might reflect the lavishness of highly polar proteins in mycelia crude water extract. Regardless of the functions, majority of the proteins appeared in the region of high molecular mass between 37 to 116 kDa. Additionally, there were similar protein bands existed in two or more fractions and possibly would present the same protein. In view of that, it was necessary to pool the fractions with similar protein bands as one protein group.

Based on the inhibitory activity of the protein fractions (Figure 4.9) as well as the statistical analysis, protein fractions A, B and C seemed to exert the strongest inhibitory activity. Subsequently, this result proposed the idea that durable ACE inhibitory proteins might be those of moderately polar proteins.

The ACE inhibitory activity of fractions A, B and C were then compared with the crude water extract. By exhibiting $IC_{50}$ values of 120 and 125 µg/mL respectively,

fractions A (10 % - 40 % salt saturation) and B (50 % - 60 % salt saturation) showed approximately 9-fold stronger ACE inhibitory effect than the crude water extract. On the other hand, fraction C (70 % salt saturation) with the $IC_{50}$ value of 109 µg/mL exerted inhibitory strength of over 10-fold higher than the crude water extract.

Decreased in $IC_{50}$ values following crude water extract's protein fractionation implied that the responsible proteins were isolated and congregated with lesser foreign constituents thus became more effective as ACE inhibitors. On account of the protein fractions were partially pure, further purification step was suggested to improve the ACE inhibitory effect.

### 5.3 Purification of ACE inhibitory proteins by RP-HPLC

Protein fractions A and C were selected to be further purified using RP-HPLC. Based on a claim by Pedroche *et al.* (2004), combining earlier electrophoretic method (SDS-PAGE) with chromatographic method might well result in a better separation of the complex protein mixtures. Most of the reported ACE inhibitory proteins or peptides were discovered through purification by HPLC (Wu and Ding, 2002; Saiga *et al.*, 2008; Rho *et al.*, 2009).

Chromatograms in Figures 4.11 and 4.12 display the separation result acquired at two different wavelengths. It was advantageous and recommended to monitor at multiple wavelengths as it could boost the confidence of protein detection. For both protein fractions, peaks with high absorbance magnitude were noticed when the samples were monitored at 220 nm and these peaks certainly represented the presence of peptide bonds at that particular elution time. Nevertheless, the presence of aromatic amino acid residues could only be detected at a higher wavelength (254 nm).

Six (A1 - A6) and seven (C1 - C7) HPLC peaks were eluted from protein fractions A and C respectively. As shown in Figure 4.13, the upshot of ACE inhibitory evaluation indicated that inhibition on ACE activity was expansively observed in all HPLC eluted peaks at the concentration of 25 µg/mL with different degree of inhibitory strength.

Above all, HPLC peaks C3, C4 and C5 were identified as the most potent ACE inhibitors since they inhibited ACE activity with stupendous strength. The $IC_{50}$ value for C3 (10.0 µg/mL) was the lowest and it demonstrated 10.9-fold inhibitory strength stronger than protein fraction C. C4 (18.0 µg/mL) and C5 (12.5 µg/mL) correspondingly inhibited ACE activity with 6.2-fold and 8.7-fold higher after being purified from protein fraction C.

Improvement of the ACE inhibitory potential due to protein purification had also been proposed for ACE inhibitors identified from other mushrooms including *Grifola frondosa* (Choi *et al.*, 2001), *Tricholoma giganteum* (Lee *et al.*, 2004a) and *Pholiota adiposa* (Koo *et al.*, 2006). Conversely, HPLC eluted peaks from fraction A demonstrated meagre inhibitory rate with less than 50 %. This result suggested that the HPLC eluted peaks required higher concentration than 25 µg/mL in order to inhibit more than 50 % of ACE activity.

The potent HPLC peaks (C3, C4 and C5) were then subjected to SDS-PAGE. Apparently, these HPLC peaks were not relatively pure since there were more than one protein bands observed (Figure 4.15). Thus, it was suggested that the robust ACE inhibitory activity exerted by each of these HPLC peaks could possibly due to the synergistic effect of several proteins. ACE inhibitory peptides/proteins that also portrayed synergistic effect had been reported earlier in fermented milk (Gobbetti *et al.*, 2000), cheese (Gómez-Ruiz, *et al.*, 2002) and tilapia (Raghavan and Kristinsson, 2009).

## 5.4 Identified anti-hypertensive related proteins

Upon characterization of the distinct protein bands from SDS-PAGE profile in Figure 4.15, four proteins were identified to have partial homology with anti-hypertensive related proteins available in ProFound database. Two of the proteins belong to HPLC peak C3 while one protein was identified from HPLC peaks C4 and C5, respectively. The identified anti-hypertensive related proteins were named as cystathionine beta synthase-like protein, DEAD/DEAH box helicase-like protein, paxillin-like protein and alpha beta hydrolase-like protein.

Comparison of molecular weight of the anti-hypertensive related proteins derived from SDS-PAGE gel (Figure 4.15) to those reported in the ProFound results (Table 4.4, 4.5, 4.7 and 4.10) revealed the dissimilarity in the value. It was suggested that the anti-hypertensive related proteins obtained were likely fragments of actual protein available in the database. This might as well explain the case of low percentage of homology matching for the identified proteins.

Three of the remaining protein bands detected in SDS-PAGE gel (Figure 4.15); C3-c, C4-c and C5-b, were identified to have partial homology with hypothetical proteins, whereas C4-b was shown to have partial homology with DNA damage response protein. These proteins were less reported to involve in the blood pressure lowering event.

### 5.4.1 Cystathionine beta synthase-like protein

Cystathionine beta synthase-like protein that has been identified in our study matched 29 % homology of cystathionine beta synthase (CBS). CBS (EC 4.2.1.22) refers to an enzyme which involves in the transulfuration pathway in human body. The presence of CBS in liver and kidney is crucial for the production of hydrogen sulphide

($H_2S$). Endogenous $H_2S$ has been regarded as a therapeutic molecule that plays pivotal roles in the physiologic processes including regulation of the blood pressure (Wang, 2002; Li and Moore, 2007; Laggner *et al.*, 2007).

No study has been reported hitherto on the ACE inhibitory activity of CBS. Nonetheless, there is possibility that CBS could inhibit ACE activity by interfering with the $Zn^{2+}$ in the active centre of ACE. Zinc cofactor is a prominent component for the enzyme catalysis (Ehlers and Riordan, 1991; Wei *et al.*, 1991) and therefore interaction of inhibitor with $Zn^{2+}$ at the active centre could disrupt ACE activity.

Inactivation of ACE resulted in the decrease production of vasoconstrictor substance, angiotensin II. According to Proudfoot *et al.* (2008), CBS has been recognized to possess metal ions binding properties due to the presence of Zn ribbon domain. Based on *in vitro* study done by Treich *et al.* (1991), this Zn ribbon domain was suggested to possess ability to bind zinc *in vivo*.

With regard to the present results, we assumed that the matched homology of CBS-like protein to that of CBS might include protein sequences of Zn ribbon domain. A deep understanding is required to validate whether CBS-like protein inhibits ACE by interacting with $Zn^{2+}$ at the enzyme's active centre. Study on structure-activity relationship is a good approach to justify the postulation as what had been performed by Natesh and co-workers (2003). They had elucidated the inhibitory mechanism of lisinopril on ACE through a three-dimensional analysis. By using certain procedures, they had successfully solved a crystal structure of ACE-lisinopril complex which illustrated carboxyl group of lisinopril bound to the active site's zinc atom and acting as zinc-chelator.

### 5.4.2 DEAD/DEAH box helicase-like protein

The identified DEAD/DEAH box helicase-like protein in this study showed 19 % match sequences with DEAD/DEAH box helicase. DEAD box helicase refers to a group of proteins that are characterized by nine conserved sequence motifs which are the Q-motif, motif I, motif Ia, motif Ib, motif II, motif III, motif IV, motif V and motif VI (Linder, 2006). On the other hand, DEAH box helicase is a related protein family which shares some motifs with DEAD box family but then having little variation in the conserved sequence.

No study has been reported on explicit correlation between DEAD/DEAH box helicase and ACE. Nevertheless, DEAD/DEAH box helicase was suggested to exhibit antihypertensive effect through the regulation of cardiac cellular activities instead of renin angiotensin aldosterone system (RAAS) (Kang *et al.*, 2010; Sahni *et al.*, 2010).

According to Liu and Olson (2002), cardiac helicase activated by MEF2 protein (CHAMP) which belongs to DEAD box proteins, had shown to suppress the growth of cardiomyocyte in cardiac hypertrophy. CHAMP demonstrated antihypertrophic activity by inhibiting proliferation via general cell cycle machinery and was characterized by the up regulation of the cell cycle inhibitor, $p21^{CIP1}$. The inhibitory activity prompted regression of cardiac hypertrophy thus recovered diastolic function and coronary flow reserve (Xiaozhen *et al.*, 2010). Recovery of the blood pressure and flow advantageously contributed to blood pressure lowering effect.

Based on our result, the presence of DEAD/DEAH box helicase-like protein in C3 might contribute to the ACE inhibitory activity and in the same time it might also demonstrate hypotensive effect via cellular mechanism. This synergistic effect may bring considerable success in treating hypertension in the future. A caution of course is that the thorough mechanism of DEAD/DEAH box helicase-like protein is beyond our

meagre comprehension. A more detail investigation on the structural and pharmacological properties is suggested to be carried out.

### 5.4.3 Paxillin-like protein

The third identified anti-hypertensive related protein was paxillin-like protein, which matched 23 % protein sequences with paxillin. In general, paxillin serves primary functions in the signalling pathway and cell motility due to the presence of multi domain bindings (Petit *et al.*, 2000; Bukharova *et al.*, 2005). Since paxillin lacks identifiable enzymatic activity, it is suggested that paxillin plays major role as an adaptor molecule that interacts with regulatory proteins.

No evidence has been provided to clearly vindicate the anti-hypertensive activity of paxillin through ACE inhibition. However, paxillin has been found to associate with the lowering blood pressure ability via regulation of vascular smooth muscle. Positioned at the dense plaques of smooth muscle tissue, paxillin acts as the signalling protein mediated actin cytoskeleton remodelling process (Tang and Anfinogenova, 2008).

Polymerization and depolymerization of the actin filaments resulted in the contraction and relaxation of vascular smooth muscle. Vasodilation of vascular smooth muscle has been understood to trigger blood pressure reduction in hypertensive patients. Thus, paxillin mediated the action of actin cytoskeleton on blood pressure by promoting relaxation of the smooth muscle (Lee *et al.*, 2004b).

Pertaining to our result, paxillin-like protein in C4 might be the significant protein that contributed to the potent anti-ACE activity of the fraction. While exhibiting partial homology with paxillin, we assumed that paxillin-like protein might induce vasodilation of vascular smooth muscle. It is anticipated that this action will be additive

to the ACE inhibitory activity of paxillin-like protein and may possibly provide beneficial response beyond antihypertension treatments described to date. Further studies are needed to resolve these and other possibilities.

### 5.4.4 Alpha beta hydrolase-like protein

By having 18 % protein sequences resembled to alpha beta hydrolase, the identified anti-hypertensive related protein was named as alpha beta hydrolase-like protein. Members of alpha beta hydrolase family range from proteases, lipases, esterases, dehalogenases, peroxidases and epoxide hydrolases. This array of proteins represents the versatile functions of alpha beta hydrolase family (Nardini and Dijkstra, 1999).

With respect to anti-hypertensive properties, there is no study reported earlier on the ability of alpha beta hydrolase proteins in inhibiting ACE activity. However, Sinal and colleagues (2000) have discovered the contribution of alpha beta hydrolase protein in blood pressure regulation through the involvement of soluble epoxide hydrolase (sEH). Their data was in agreement with Yu *et al.* (2000) in which anti-hypertensive effect was observed following sEH inhibition.

Inhibitory activity on sEH resulted in increased epoxyeicosatrienoic acids (EET) level and decreased dihydroxyeicosatrienoic acids (DHET) level. Accordingly, blood pressure lowering event could be explained by the increased release of EET that potentiated vasorelaxant response (Fang *et al.*, 2001). EET acts as endothelium-derived hyperpolarizing factor where it induced vasodilation of vascular smooth muscle. EET concomitantly attenuated inflammation and renal injury (Imig, 2005).

In the present study, alpha beta hydrolase-like protein was expected to exert anti-ACE properties and the detail mechanism of the inhibition still remained unclear.

It is speculated that alpha beta hydrolase-like protein might also restore blood pressure level by acting as sEH inhibitor. This is supported by a study that discovered an inhibitor could possess sequence homology with its enzyme in an inhibitory mechanism, as reported by Mancheva *et al.* (1984).

On account that sEH inhibition could reduce inflammation, it is anticipated that alpha beta hydrolase-like protein might provide synergistic effects in hypertensive patients. The understanding of alpha beta hydrolase-like protein action is far from complete and it is critical to perform an in-depth investigation.