CHAPTER 4

RESULTS

4.1 Cultivation of *G. lucidum* mycelia

Ganoderma lucidum mycelia demonstrated good growth on 2 % (w/v) brown sugar and 1 % (w/v) spent brewer's yeast. The mycelia grew longitudinally radial and formed white dense colony in the Petri plate (Figure 4.1). Following 7 days of submerged cultivation, mycelia biomass was separated from broth culture (Figure 4.2). Based on the observation, mycelia biomass developed clumps structure either in the form of small loose mycelia aggregated in dispersed growth or big masses of aggregated mycelia. On the other hand, broth culture appeared as clear, yellowish brown solution (Figure 4.3 and 4.4). The yield of dried weight mycelia obtained from the submerged cultivation was 4.969 g/L ± 0.874 g/L.

4.2 *Ganoderma lucidum* mycelia and broth inhibitory effect on ACE

4.2.1 ACE inhibitory activity of Captopril (Positive control)

Five concentrations of Captopril had been assessed and the ACE inhibitory curve is shown in Figure 4.5. The lowest concentration tested of 1×10^{-11} mg/mL resulted in no inhibitory effect on ACE. The red arrow at the inhibition curve indicates the Captopril concentration which inhibits 50 % of ACE activity under the experimental conditions (IC_{50}). Captopril exhibited IC_{50} value of 1.585 pg/mL or 0.073 nM. One-way ANOVA was performed to compare the effect of Captopril concentrations on the inhibitory activity (Appendix 2, Figure 2.1). It resulted in a significant differences across the five concentrations, $F(4, 10) = 1079.61$, $p < 0.001$. Based on the statistical analysis, the highest inhibitory rate was significantly observed at the concentration of 1×10^{-6} mg/mL which approximately inhibited 95.93 % of ACE activity.

Figure 4.1 : *Ganoderma lucidum* mycelia in Petri plate

Figure 4.2 : Mycelia biomass following separation from broth culture

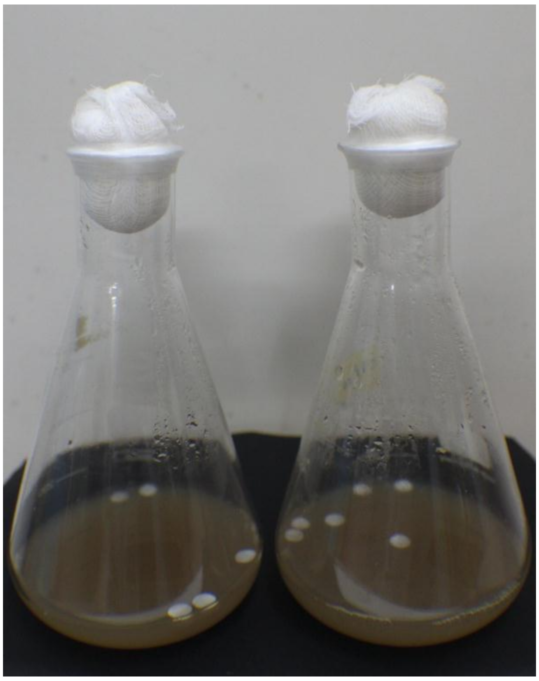


Figure 4.3 : Mycelia plugs at day 0 of cultivation

Figure 4.4 : Mycelia development after 7 days of cultivation

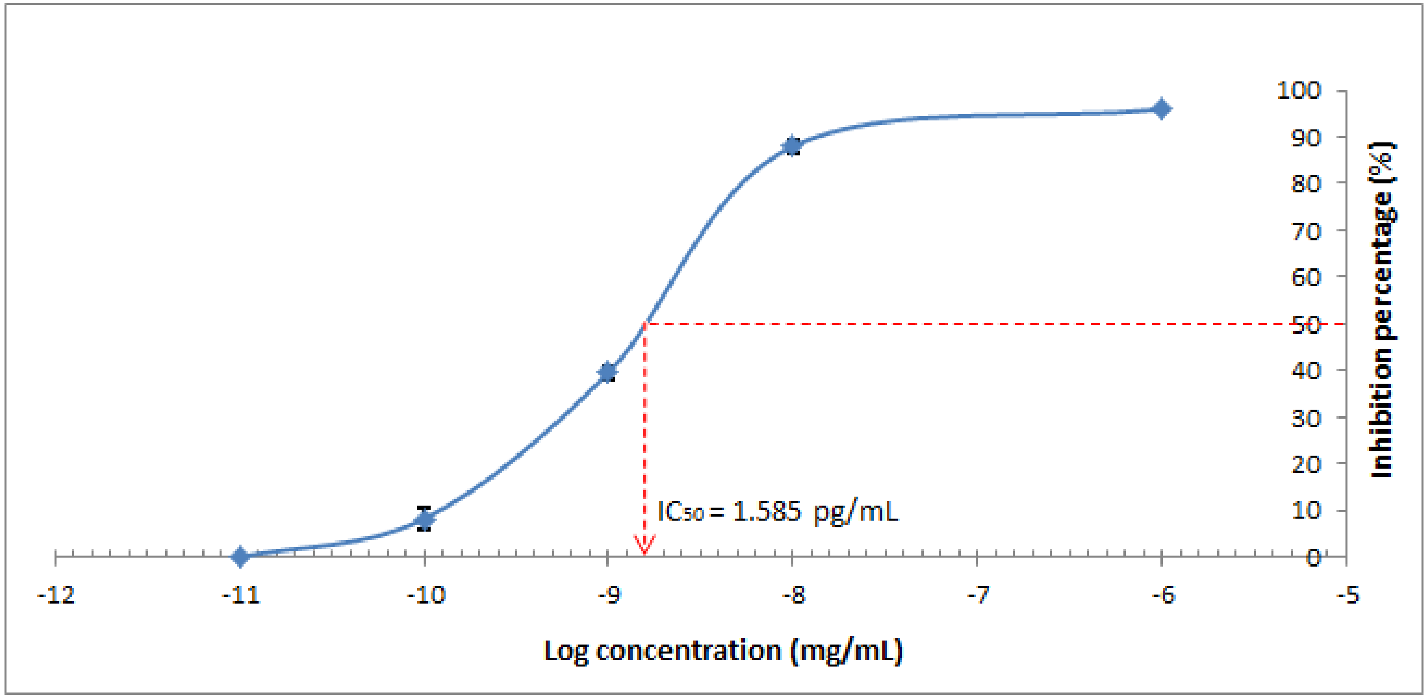


Figure 4.5 : ACE inhibition curve for Captopril

Values are expressed as mean ± SEM of three replicate determinations.

4.2.2 ACE inhibitory activity of mycelia and broth crude water extracts

10 mg/mL of mycelia and broth crude water extracts were compared for inhibitory effect on ACE activity. The results are presented in Figure 4.6. Mycelia showed the uttermost inhibitory rate with 95.50 %, which was more than two times inhibitory potential of broth. There was a statistically significant difference between both extracts as determined by one-way ANOVA (Appendix 2, Figure 2.2). Due to the strong inhibition activity, mycelia crude water extract was selected to be further studied as to investigate the bioactive proteins responsible for the effect. An inhibitory curve for the mycelia is displayed in Figure 4.7. Generally, alteration in sample concentrations apparently caused varied inhibitory effect. Stronger inhibition on ACE activity was observed as the concentration increased from 0 to 10 mg/mL. The red arrow at the inhibition curve indicates the mycelia crude water extract concentration which inhibited 50 % of ACE activity under the experimental conditions (IC_{50}). The IC_{50} value of mycelia crude water extract was determined at 0.90 mg/mL, having a protein content of 1.134 mg/mL.

4.3 SDS-PAGE profile of mycelial proteins

Following salting out process of mycelia crude water extract, ten semi-purified mycelial protein fractions had been collected and labelled according to the final percentage of ammonium sulphate saturation in each step. Figure 4.8 represents the high resolution electrophoresis gel which portrays the protein profile of mycelia crude water extract as well as the semi-purified mycelial proteins. Some of the semi-purified mycelial proteins shared similar protein bands and therefore were grouped together. Pooling and grouping of the fractions brought into the formation of five mycelial protein fractions (A-E).

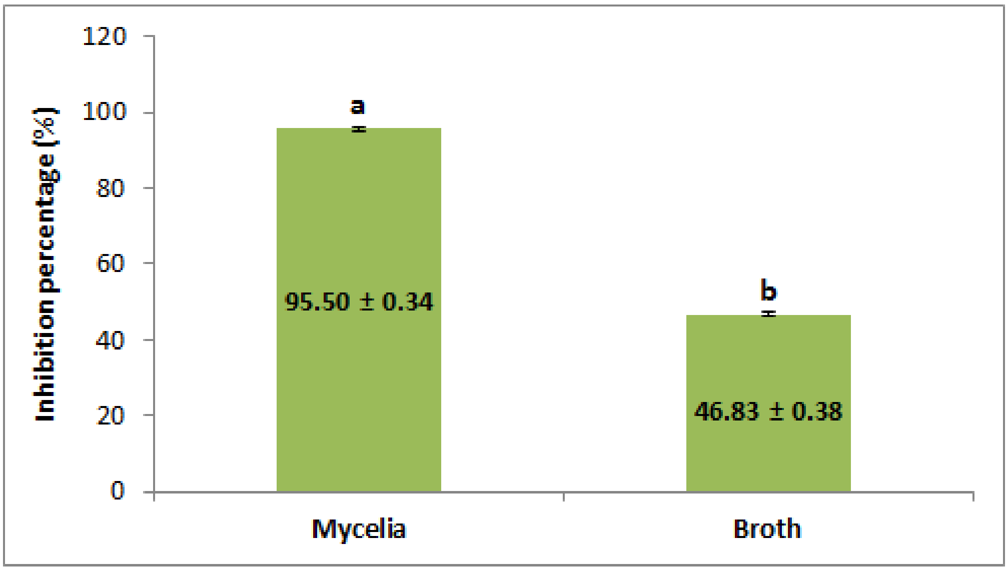


Figure 4.6 : Inhibitory effect by crude water extracts at 10 mg/mL

Values are expressed as mean ± SEM of two replicate determinations. Mean values with different lower case letters (a-b) indicate significant difference at $p < 0.05$.

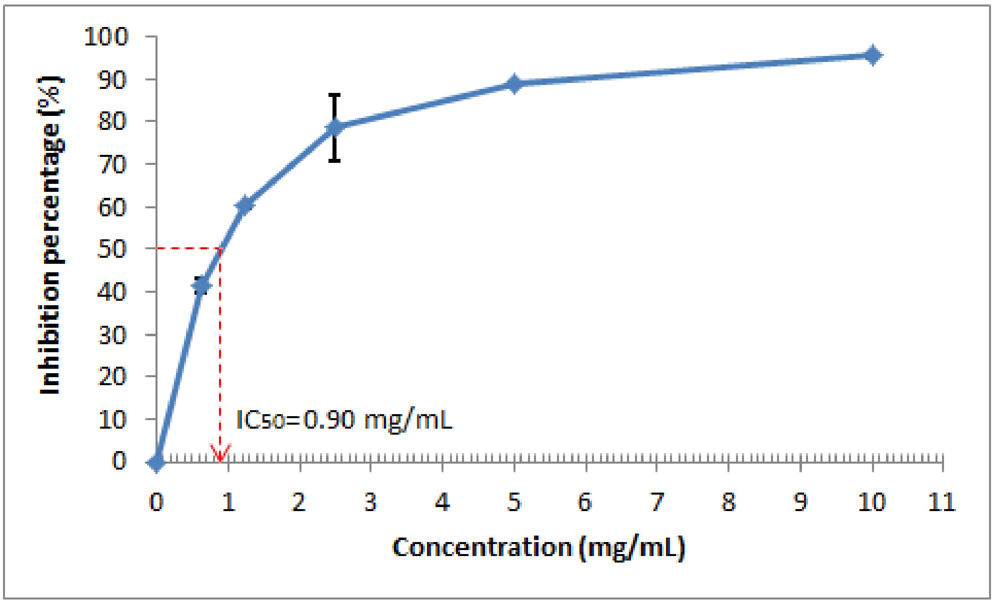


Figure 4.7 : Inhibitory curve for mycelia crude water extract

Values are expressed as mean ± SEM of two replicate determinations.

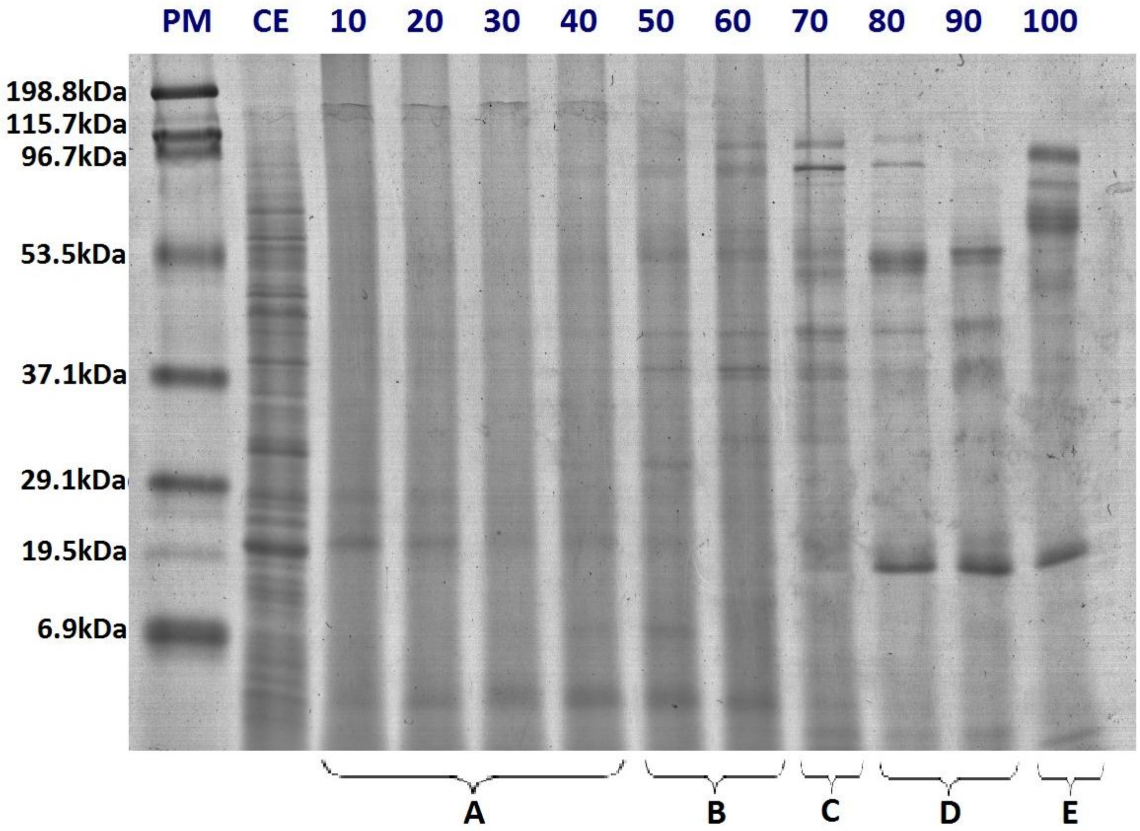


Figure 4.8 : SDS-PAGE profile of mycelial proteins following ammonium sulphate precipitation

The SDS-PAGE gel was stained with Coomassie Brilliant Blue. Lane PM: protein marker, lane CE: mycelia crude water extract (30 µg of crude extract in sample buffer with DTT) and lanes 10-100: semi-purified mycelial proteins (9 µg of semi-purified protein in sample buffer with DTT). A-E are the protein fractions formed upon pooling and grouping of semi-purified mycelial proteins.

4.4 Evaluation of inhibitory activity by mycelial protein fractions A, B, C, D and E

The mycelial protein fractions were subjected to anti-hypertensive assay to evaluate their ACE inhibitory activity. Apparently, all fractions restrained more than 50 % of ACE activity at the concentration of 200 µg/mL except for fraction E, as shown in Figure 4.9. Based on one-way ANOVA test, the preferences for the protein fractions have significant differences (Appendix 2, Figure 2.3). It was revealed that the means for fractions A, B and C were not significantly different since the confidence intervals of the three fractions did overlap at the 0.05 level of significance.

With respect to the insignificant differences of the mean value for fractions A, B and C at the concentration of 200 µg/mL, selection for two candidates to be further studied seemed to be intricate. In view of that, the potential candidate was exclusively chosen according to the IC_{50} value acquired. An inhibitory curve was developed by plotting inhibition percentage obtained against sample concentrations at 0, 25, 50, 100, 200 and 400 µg/mL. Figure 4.10 displays the inhibitory curve for fractions A, B and C. The inhibitory curve for all fractions presented the same pattern. As the protein concentration increased, the inhibition percentage increased. IC_{50} value determined for fractions A, B and C were 120, 125 and 109 µg/mL, respectively. Based on the result, fractions having the lowest two IC_{50} value (A and C) were chosen for a more detail analysis.

4.5 RP-HPLC profile of protein fractions A and C

Purification method by RP-HPLC resulted in the establishment of protein peak profiles for fractions A and C. The profiles depicted the protein elution at two different wavelengths, 220 and 254 nm. Figure 4.11 and 4.12 are the profiles obtained after several optimization steps.

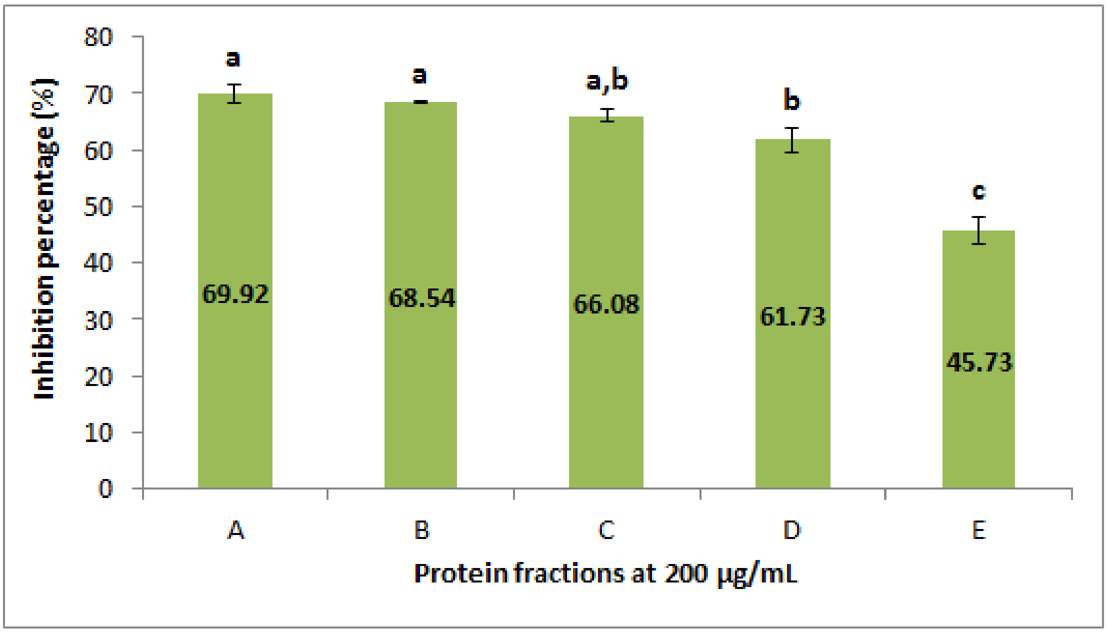


Figure 4.9 : Inhibition percentage of protein fractions at concentration of 200 µg/mL

Values are expressed as mean ± SEM of three replicate determinations.
Mean values in a column with different lower case letters (a-c) indicate significant difference at $p < 0.05$.

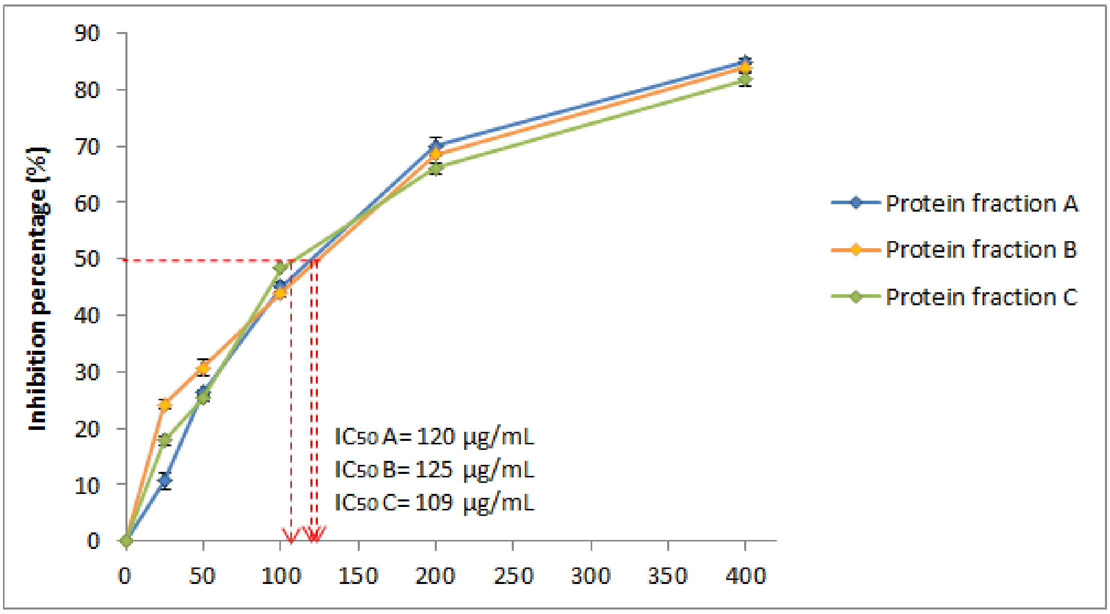


Figure 4.10 : Inhibitory curve for fractions A, B and C

Values are expressed as mean ± SEM of three replicate determinations.

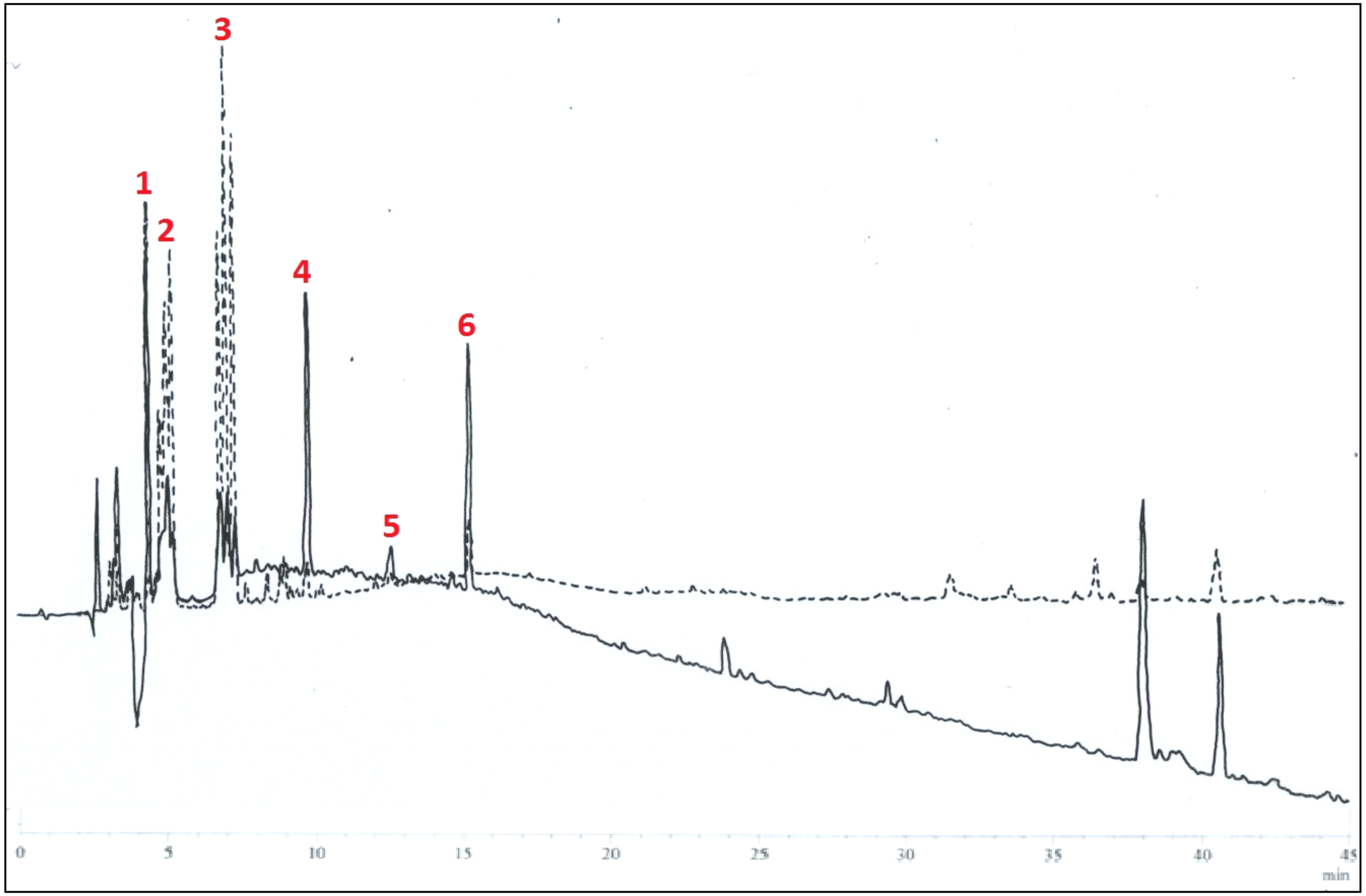


Figure 4.11 : HPLC profile of fraction A at superimposed image

Elution was performed using 5 % - 90 % ACN within 0 to 45 minutes. Number 1-6 indicate the peaks which were collected for further analysis. ——indicates wavelength at 220 nm; ----- indicates wavelength at 254 nm.

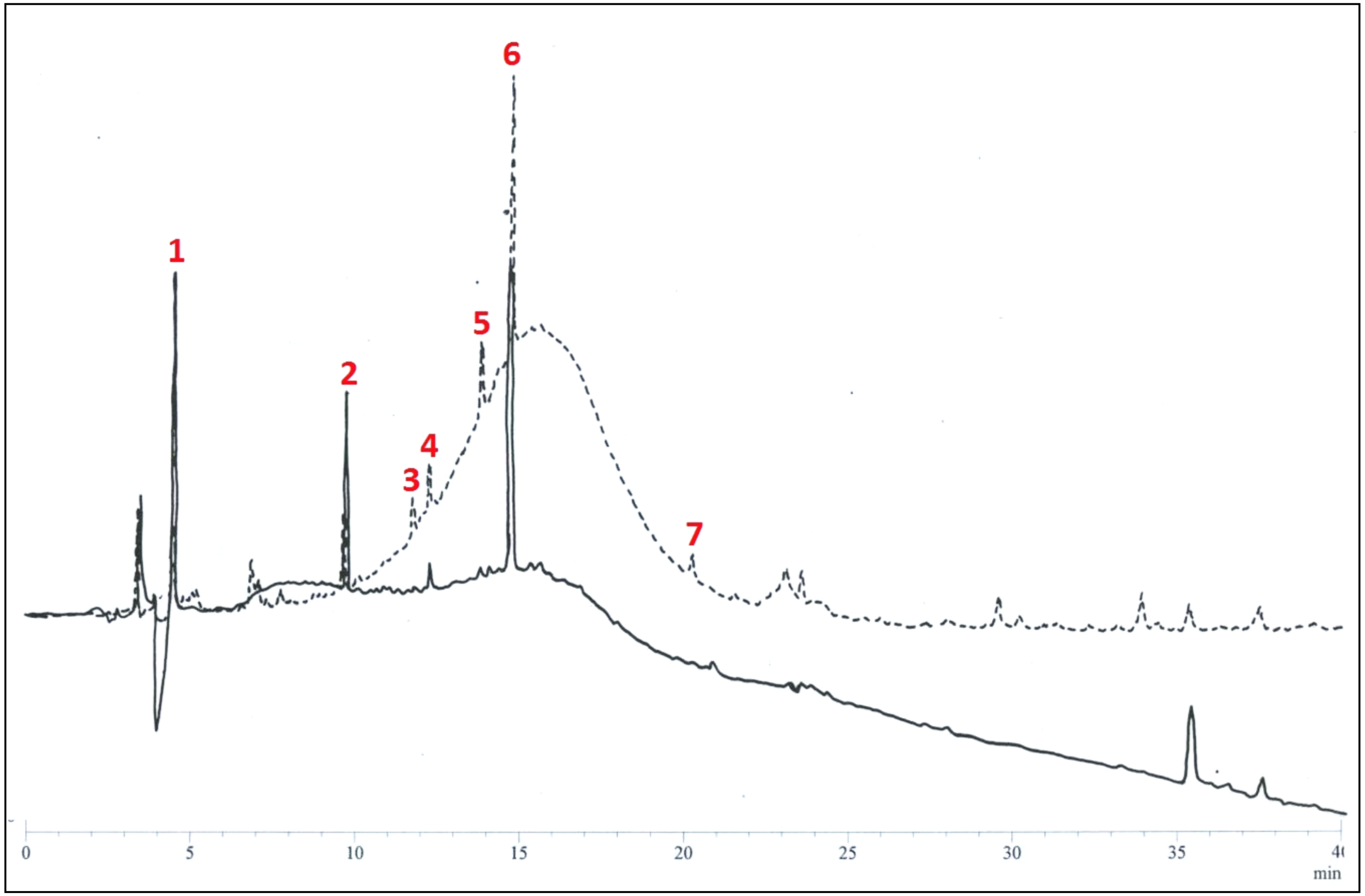


Figure 4.12 : HPLC profile of fraction C at superimposed image

Elution was performed using 5 % - 90 % ACN within 0 to 40 minutes. Number 1-7 indicate the peaks which were collected for further analysis.—— indicates wavelength at 220 nm; ----- indicates wavelength at 254 nm.

Some of the peaks ostensibly expressed in both wavelengths yet having varied milliAU (mAU) magnitude. In point of collecting the peak of interest, profile of both wavelengths for corresponding fraction were superimposed. With reference to Figure 4.11 and 4.12, distinct peaks denoted with red numbering were the peaks of interest and had been collected for anti-ACE evaluation. Significant peaks which appeared after 35 minutes were background peaks and had been disregarded.

4.6 Assessment of HPLC-purified proteins for ACE inhibitory activity

Proteins collected from HPLC were subjected for re-evaluation of inhibitory potential. Eluted peaks that derived from protein fractions A and C were tagged as A1-A6 and C1-C7 respectively. At the concentration of 25 µg/mL, three eluted peaks from fraction C that were C3, C4 and C5 expressed more than 50 % inhibitory activity among all the HPLC peaks evaluated as shown in Figure 4.13. The HPLC peaks preferences were then analyzed with one-way ANOVA and described in Appendix 2, Figure 2.4. The overlapping of confidence intervals for C3, C4 and C5 at the 0.05 level of significance revealed the insignificant different of the mean value for the three HPLC peaks at the concentration of 25 µg/mL.

ACE inhibitory curves were plotted for C3, C4 and C5. The curves comprised of inhibition effect at protein concentrations of 0, 3.125, 6.25, 12.5, 25 and 50 µg/mL. According to the curves (Figure 4.14), the three HPLC peaks appeared to share similar inhibitory pattern. Evidently, there was a boost in the inhibition effect on ACE as the concentration of protein was raised up to 50 µg/mL. IC_{50} value for each HPLC peaks had been identified. By exhibiting IC_{50} value of 10.0 µg/mL, C3 turned up to be the strongest ACE inhibitor followed by C5 (12.5 µg/mL) and C4 (18.0 µg/mL).

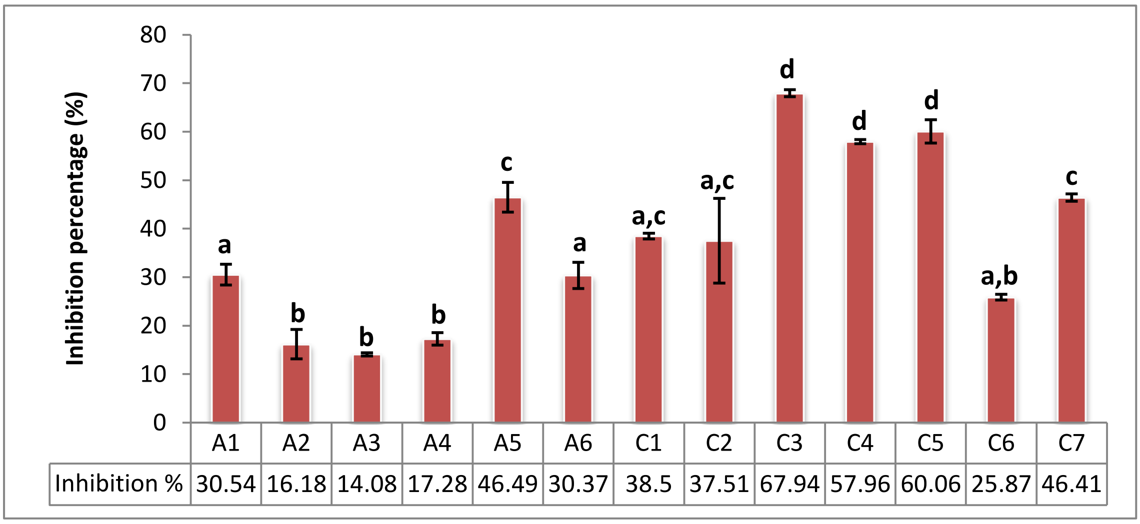


Figure 4.13 : Inhibition percentage of HPLC eluted peaks at 25 µg/mL

Values are expressed as mean ± SEM of two replicate determinations.
Mean values with different lower case letters (a-d) indicate significant difference at $p < 0.05$.

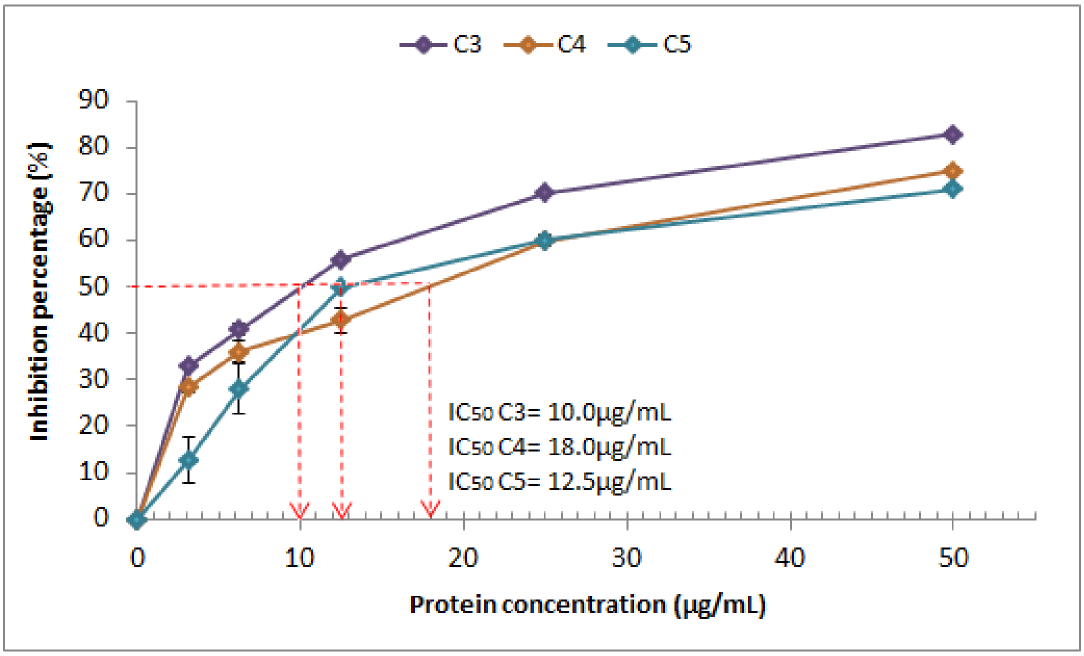


Figure 4.14 : Inhibitory curve for C3, C4 and C5

Values are expressed as mean ± SEM of three replicate determinations.

4.7 SDS-PAGE profile of potent anti-ACE proteins eluted from HPLC

Protein profiling of the potent anti-ACE proteins was performed by SDS-PAGE and is shown in Figure 4.15. Eight protein bands were detected distinctly and are marked in red boxes. Prior to molecular weight determination, a standard curve of the protein markers was developed as presented in Figure 4.16. The plotting of log molecular weight (MW) versus R_f value displayed a non-linear curve represented by the red line. Semi-logarithmic plot is the accepted model for molecular weight determination. A best fit trend line represented by dashed, was introduced into the plot. Subsequently, the molecular weight attained for the unknown proteins is shown in Table 4.1.

4.8 Characterization and identification of potential anti-hypertensive proteins

4.8.1 Data obtained from Mascot program

The protein bands which had been detected previously from SDS-PAGE profile were extracted out from the gel and analysed by MALDI-TOF/TOF MS. Results derived from Mascot program which consisted of the top three protein hits for each unknown protein are shown in Appendix 3. Table 4.2 shows the matched peptide/s reported together with the mass and score obtained. Peptides' masses which did not have match from the database are indicated as 'unmatched peptides' and shown in Table 4.3. The low score attained for every sample spot was not significant at the 0.05 level of confidence thus revealed that the unknown proteins were less identical to the proteins in the database of Mascot program.

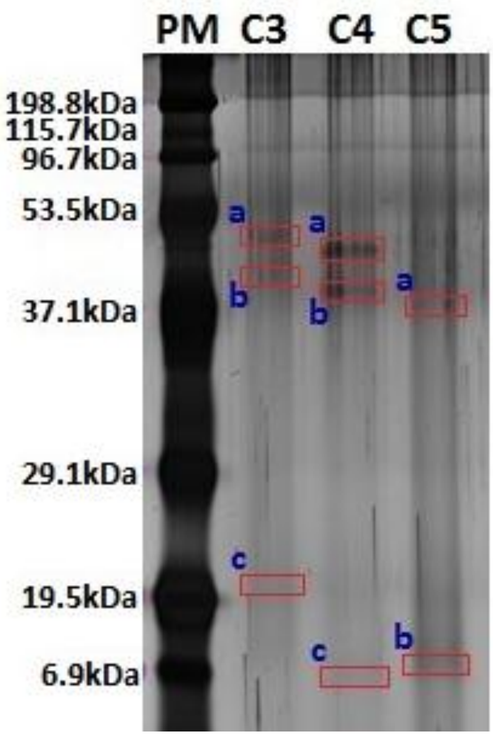


Figure 4.15 : SDS-PAGE profile of anti-ACE fractions

The SDS-PAGE gel was stained with silver nitrate.
Lane PM: protein marker and lanes C3-C5: HPLC-purified proteins (4 µg purified protein in sample buffer with DTT). Red boxes denoted with lower case letter (a-c) indicate the protein bands detected.

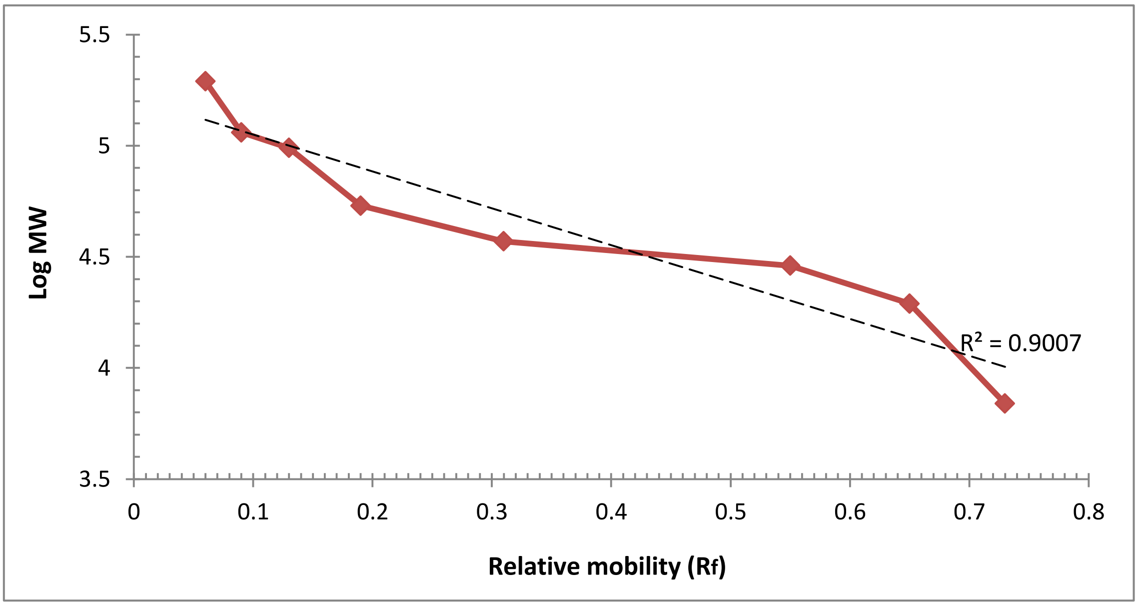


Figure 4.16 : Standard curve for protein molecular weight determination

Table 4.1 : Molecular weight determined for the unknown protein bands

Protein band	Log MW	R_f value	Estimated molecular weight (kDa)
C3-a	4.69	0.22	49.0
C3-b	4.64	0.26	43.7
C3-c	4.31	0.64	20.4
C4-a	4.67	0.24	46.8
C4-b	4.61	0.28	40.7
C4-c	3.76	0.74	5.8
C5-a	4.57	0.31	37.1
C5-b	3.84	0.73	6.9

Table 4.2 : Mascot result of the top three protein hits for each protein band

Protein band	Rank	Putative protein	Matched peptide/s	Mass	Score
C3-a	1	Metallothionein- *Neurospora crassa*	• M.GDCGCSGASSCNCGSGCSCSNCGSK.	2364	12
	2	Histone H4.1 - *Ashbya gossypii* (Yeast) (*Eremothecium gossypii*)	• K.RISGLIYEDVR.A • K.ILRDNIQGITKPAIR.R	11331	11
	3	Inner membrane protein COX18, mitochondrial precursor (Cytochrome c oxidase assembly protein 18)	• R.KVVQSVPPVVK.L • K.VVQSVPPVVKLR.L • K.NSVLPLVQVPLWVTMSMGLRK.L + Oxidation (M)	34941	9
C3-b	1	GrpE protein homolog, mitochondrial precursor - *Neurospora crassa*	• R.TAAPQLRSAAR.W • R.VLRPAQVGVVK.N • K.MTESILLSTLK.K • R.ALCSGARVAAQRPIASQIFQMQAAR.T + Carbamidomethyl (C); Oxidation (M)	26811	17
	2	40S ribosomal protein S21 - *Aspergillus fumigatus* (*Sartorya fumigata*)	• R.IIKANDHASVQISIAK.V • -.MENEKGEIVDLYVPR.K	10003	12
	3	Histone H4.1 - *Ashbya gossypii* (Yeast) (*Eremothecium gossypii*)	• K.RISGLIYEDVR.A • K.ILRDNIQGITKPAIR.R	11331	11

Table 4.2, continue.

Protein band	Rank	Putative protein	Matched peptide/s	Mass	Score
C3-c	1	Metallothionein - *Neurospora crassa*	• M.GDCGCSGASSCNCGSGCSCSNCGSK.-	2364	12
	2	40S ribosomal protein S9 (S7) - *Podospora anserina*	• K.SIHHARVLIR.Q • R.ELLTLDEKDPK.R • K.LDYVLALKAEDFLER.R	21762	11
	3	Mitochondria fission 1 protein - *Kluyveromyces lactis* (Yeast) (*Candida sphaerica*)	• K.EDQMLGVKLLTDIYK.E • K.GIALISAGIAIGATTIGLLIR.G	17527	9
C4-a	1	Metallothionein - *Neurospora crassa*	• M.GDCGCSGASSCNCGSGCSCSNCGSK.-	2364	12
	2	DASH complex subunit DAD3 (Outer kinetochore protein DAD3) - *Kluyveromyces lactis* (Yeast) (*Candida*)	• R.SVLDKYR.F • K.ISLVSTLLK.G	9810	11
	3	Histone H2A - *Ustilago maydis* (Smut fungus)	• R.IHRLLR.K • R.IIPRHLQLAIR.N	14191	9
C4-b	1	Histone H4.1 - *Ashbya gossypii* (Yeast) (*Eremothecium gossypii*)	• K.RISGLIYEDVR.A • K.ILRDNIQGITKPAIR.R	11331	13
	2	Metallothionein - *Neurospora crassa*	• M.GDCGCSGASSCNCGSGCSCSNCGSK.-	2364	12
	3	40S ribosomal protein S21 - *Aspergillus fumigatus* (*Sartorya fumigata*)	• R.IIKANDHASVQISIAK.V • -.MENEKGEIVDLYVPR.K	10003	11

Table 4.2, continue.

Protein band	Rank	Putative protein	Matched peptide/s	Mass	Score
C4-c	1	Metallothionein - *Neurospora crassa*	• M.GDCGCSGASSCNCGSGCSCSNCGSK	2364	12
	2	40S ribosomal protein S9 (S7) - *Podospora anserina*	• K.SIHHARVLIR.Q • R.ELLTLDEKDPK.R • K.LDYVLALKAEDFLER.R	21762	12
	3	40S ribosomal protein S21 - *Aspergillus fumigatus* (*Sartorya fumigata*)	• R.IIKANDHASVQISIAK.V • -.MENEKGEIVDLYVPR.K	1003	11
C5-a	1	Metallothionein - *Neurospora crassa*	• M.GDCGCSGASSCNCGSGCSCSNCGSK.-	2364	12
	2	Multiprotein-bridging factor 1 - *Kluyveromyces lactis* (Yeast) (*Candida sphaerica*)	• R.GIPNQQILGK.M • K.NIGEPLGGPKK.- • K.IDVSVGKAIQK.G	16436	12
	3	Histone H2B - *Rosellinia necatrix* (White root-rot fungus)	• K.LAAYNKK.S • K.KPAAKAPVASK.A • R.VATEASKLAAYNK.K	14789	12

Table 4.2, continue.

Protein band	Rank	Putative protein	Matched peptide/s	Mass	Score
C5-b	1	Phosphoglycerate kinase (EC 2.7.2.3) - *Kluyveromyces lactis* (Yeast) (*Candida sphaerica*)	• R.KAFAATVAEAK.T • -.MSLSSKLTVK.D + Oxidation (M) • K.IQLIDNLLDK.V • K.IVLPTDFVIGDK.F • K.YSLAPVADELSR.L • K.ELPGVTFLSNKQ.-	44487	19
	2	Replication factor C subunit 5 (Replication factor C5) (Probable activator 1 subunit 5)	• R.VVVQDLLK.E • K.ELYGPGVEKIK.I • R.CLLVRVAAPTHK.E • K.EICDVLASSAKK.E + Carbamidomethyl (C) • K.VVVINEADHLTR.D • K.VIFHLEAFVAKFMR.I	39895	18
	3	Mitochondrial ribosomal protein VAR1 - *Williopsis mrakii* (Yeast) (*Hansenula mrakii*)	• K.YLIGLSMLFK.G • K.EKLLFGSLSNK.L • K.YLNFDININKK.Y • K.MINNKHTLYYGSLIK.D • K.ITNELLVNKYLIGLSMLFK.G + Oxidation (M)	44348	14

Note: Refer Appendix 3 for actual protein sequences.

Table 4.3 : Mascot result of unmatched peptides for each protein band

Protein band	Rank	No. of unmatched peptides	Masses of unmatched peptides
C3-a	1	64	807.56, 832.65, 842.67, 854.21, 856.68, 864.66, 865.18, 870.71, 877.21, 887.17, 897.59, 973.72, 1033.71, 1037.73, 1045.77, 1060.77, 1065.72, 1074.73, 1090.74, 1106.76, 1107.76, 1109.71, 1141.76, 1157.81, 1165.81, 1179.83, 1193.84, 1201.84, 1234.91, 1265.88, 1277.95, 1300.78, 1302.96, 1307.93, 1320.85, 1323.92, 1329.92, 1365.91, 1383.95, 1390.95, 1476.05, 1488.04, 1639.17, 1658.11, 1708.10, 1717.18, 1792.08, 1795.16, 1838.32, 1994.36, 2211.53, 2224.52, 2225.55, 2239.57, 2247.53, 2265.47, 2283.62, 2284.62, 2297.63, 2299.62, 2319.42, 2384.42, 2705.69, 2807.88
	2	63	807.56, 832.65, 842.67, 854.21, 856.68, 864.66, 865.18, 870.71, 877.21, 887.17, 897.59, 973.72, 1033.71, 1037.73, 1045.77, 1060.77, 1065.72, 1074.73, 1090.74, 1106.76, 1107.76, 1109.71, 1141.76, 1157.81, 1165.81, 1179.83, 1193.84, 1201.84, 1234.91, 1265.88, 1277.95, 1300.78, 1302.96, 1307.93, 1323.92, 1329.92, 1365.91, 1383.95, 1390.95, 1476.05, 1488.04, 1639.17, 1658.11, 1717.18, 1792.08, 1795.16, 1838.32, 1994.36, 2211.53, 2224.52, 2225.55, 2233.53, 2239.57, 2247.53, 2265.47, 2283.62, 2284.62, 2297.63, 2299.62, 2319.42, 2384.42, 2705.69, 2807.88
	3	62	807.56, 832.65, 842.67, 854.21, 856.68, 864.66, 865.18, 870.71, 877.21, 887.17, 897.59, 973.72, 1033.71, 1037.73, 1045.77, 1060.77, 1065.72, 1074.73, 1090.74, 1106.76, 1107.76, 1109.71, 1141.76, 1157.81, 1165.81, 1193.84, 1201.84, 1234.91, 1265.88, 1277.95, 1300.78, 1302.96, 1307.93, 1323.92, 1329.92, 1365.91, 1383.95, 1390.95, 1476.05, 1488.04, 1639.17, 1658.11, 1708.10, 1717.18, 1792.08, 1795.16, 1838.32, 1994.36, 2211.53, 2224.52, 2225.55, 2233.53, 2239.57, 2247.53, 2265.47, 2283.62, 2284.62, 2297.63, 2299.62, 2319.42, 2705.69, 2807.88

Table 4.3, continue.

Protein band	Rank	No. of unmatched peptides	Masses unmatched peptides
C3-b	1	61	807.55, 832.64, 840.65, 842.66, 847.61, 854.21, 855.21, 856.68, 861.22, 864.17, 864.65, 865.18, 868.68, 870.19, 870.70, 871.19, 877.21, 887.16, 897.58, 919.12, 973.71, 1033.71, 1045.76, 1057.75, 1060.77, 1065.71, 1066.71, 1090.73, 1106.74, 1107.75, 1109.71, 1121.79, 1140.78, 1157.81, 1158.80, 1179.82, 1184.83, 1194.81, 1201.84, 1232.83, 1234.91, 1265.87, 1300.77, 1307.92, 1320.83, 1323.91, 1357.95, 1365.89, 1381.90, 1383.94, 1390.94, 1476.05, 1498.04, 1658.10, 1708.09, 1717.17, 1792.06, 2211.52, 2225.53, 2265.45, 2384.40
	2	63	807.55, 832.64, 840.65, 842.66, 847.61, 854.21, 855.21, 856.68, 861.22, 864.17, 864.65, 865.18, 868.68, 870.19, 870.70, 871.19, 877.21, 887.16, 897.58, 919.12, 973.71, 1033.71, 1045.76, 1057.75, 1060.77, 1065.71, 1066.71, 1090.73, 1106.74, 1107.75, 1109.71, 1121.79, 1140.78, 1141.76, 1157.81, 1158.80, 1165.80, 1179.82, 1184.83, 1194.81, 1201.84, 1232.83, 1234.91, 1235.76, 1265.87, 1300.77, 1307.92, 1320.83, 1323.91, 1357.95, 1365.89, 1381.90, 1383.94, 1390.94, 1476.05, 1498.04, 1658.10, 1717.17, 2211.52, 2225.53, 2265.45, 2384.40, 2717.59
	3	62	807.56, 832.65, 842.67, 854.21, 856.68, 864.66, 865.18, 870.71, 877.21, 887.17, 897.59, 973.72, 1033.71, 1037.73, 1045.77, 1060.77, 1065.72, 1074.73, 1090.74, 1106.76, 1107.76, 1109.71, 1141.76, 1157.81, 1165.81, 1193.84, 1201.84, 1234.91, 1265.88, 1277.95, 1300.78, 1302.96, 1307.93, 1323.92, 1329.92, 1365.91, 1383.95, 1390.95, 1476.05, 1488.04, 1639.17, 1658.11, 1708.10, 1717.18, 1792.08, 1795.16, 1838.32, 1994.36, 2211.53, 2224.52, 2225.55, 2233.53, 2239.57, 2247.53, 2265.47, 2283.62, 2284.62, 2297.63, 2299.62, 2319.42, 2705.69, 2807.88

Table 4.3, continue.

Protein band	Rank	No. of unmatched peptides	Masses unmatched peptides
C3-c	1	64	807.55, 840.64, 842.66, 854.21, 856.67, 858.66, 864.65, 865.17, 868.68, 870.70, 877.20, 887.16, 919.11, 973.71, 1033.71, 1037.72, 1045.76, 1060.76, 1090.73, 1106.75, 1109.70, 1139.79, 1157.80, 1165.80, 1179.81, 1193.83, 1201.83, 1234.90, 1265.86, 1277.94, 1300.77, 1302.94, 1307.91, 1329.89, 1365.89, 1383.93, 1390.93, 1435.03, 1476.03, 1488.02, 1639.15, 1658.10, 1708.09, 1717.16, 1766.08, 1795.13, 1839.27, 1941.29, 1994.34, 2210.51, 2211.51, 2223.50, 2225.53, 2247.51, 2265.45, 2279.47, 2283.60, 2284.60, 2297.61, 2298.61, 2299.60, 2306.57, 2338.54, 2384.42
	2	62	807.55, 840.64, 842.66, 854.21, 856.67, 858.66, 864.65, 865.17, 868.68, 870.70, 877.20, 887.16, 919.11, 973.71, 1033.71, 1037.72, 1045.76, 1060.76, 1090.73, 1106.75, 1109.70, 1139.79, 1157.80, 1165.80, 1179.81, 1193.83, 1234.90, 1265.86, 1277.94, 1302.94, 1307.91, 1329.89, 1365.89, 1383.93, 1390.93, 1435.03, 1476.03, 1488.02, 1639.15, 1658.10, 1708.09, 1717.16, 1766.08, 1839.27, 1941.29, 1994.34, 2210.51, 2211.51, 2223.50, 2225.53, 2233.50, 2247.51, 2265.45, 2279.47, 2283.60, 2284.60, 2297.61, 2298.61, 2299.60, 2306.57, 2338.54, 2384.42
	3	63	807.55, 840.64, 842.66, 854.21, 856.67, 858.66, 864.65, 865.17, 868.68, 870.70, 877.20, 887.16, 919.11, 973.71, 1033.71, 1037.72, 1045.76, 1060.76, 1090.73, 1106.75, 1109.70, 1139.79, 1157.80, 1165.80, 1179.81, 1193.83, 1201.83, 1234.90, 1265.86, 1277.94, 1300.77, 1302.94, 1307.91, 1329.89, 1365.89, 1383.93, 1390.93, 1435.03, 1476.03, 1488.02, 1639.15, 1658.10, 1708.09, 1717.16, 1795.13, 1839.27, 1941.29, 2210.51, 2211.51, 2223.50, 2225.53, 2233.50, 2247.51, 2265.45, 2279.47, 2283.60, 2284.60, 2297.61, 2298.61, 2299.60, 2306.57, 2338.54, 2384.42

Table 4.3, continue.

Protein band	Rank	No. of unmatched peptides	Masses unmatched peptides
C4-a	1	64	807.55, 817.63, 832.64, 842.66, 847.60, 856.67, 864.65, 865.17, 868.68, 870.69, 877.20, 880.51, 887.16, 973.71, 1033.71, 1037.72, 1045.76, 1060.76, 1065.71, 1090.73, 1106.75, 1107.74, 1109.69, 1157.80, 1165.80, 1179.81, 1184.82, 1193.83, 1201.83, 1234.90, 1262.83, 1265.86, 1277.93, 1300.77, 1302.95, 1307.91, 1329.89, 1365.89, 1381.90, 1383.93, 1390.93, 1435.02, 1476.03, 1488.01, 1498.03, 1658.09, 1708.08, 1717.15, 1839.26, 1994.33, 2194.57, 2211.51, 2223.53, 2225.52, 2239.54, 2247.50, 2265.45, 2283.59, 2284.59, 2297.60, 2299.60, 2305.59, 2367.68, 2384.39
	2	63	807.55, 817.63, 832.64, 842.66, 847.60, 856.67, 864.65, 865.17, 868.68, 870.69, 877.20, 887.16, 1033.71, 1037.72, 1045.76, 1060.76, 1065.71, 1090.73, 1106.75, 1107.74, 1109.69, 1157.80, 1165.80, 1179.81, 1184.82, 1193.83, 1201.83, 1234.90, 1262.83, 1265.86, 1277.93, 1300.77, 1302.95, 1307.91, 1329.89, 1365.89, 1381.90, 1383.93, 1390.93, 1435.02, 1476.03, 1488.01, 1498.03, 1658.09, 1708.08, 1717.15, 1839.26, 1994.33, 2194.57, 2211.51, 2223.53, 2225.52, 2233.49, 2239.54, 2247.50, 2265.45, 2283.59, 2284.59, 2297.60, 2299.60, 2305.59, 2367.68, 2384.39
	3	63	817.63, 832.64, 842.66, 847.60, 856.67, 864.65, 865.17, 868.68, 870.69, 877.20, 880.51, 887.16, 973.71, 1033.71, 1037.72, 1045.76, 1060.76, 1065.71, 1090.73, 1106.75, 1107.74, 1109.69, 1157.80, 1165.80, 1179.81, 1184.82, 1193.83, 1201.83, 1234.90, 1262.83, 1265.86, 1277.93, 1300.77, 1302.95, 1307.91, 1365.89, 1381.90, 1383.93, 1390.93, 1435.02, 1476.03, 1488.01, 1498.03, 1658.09, 1708.08, 1717.15, 1839.26, 1994.33, 2194.57, 2211.51, 2223.53, 2225.52, 2233.49, 2239.54, 2247.50, 2265.45, 2283.59, 2284.59, 2297.60, 2299.60, 2305.59, 2367.68, 2384.39

Table 4.3, continue.

Protein band	Rank	No. of unmatched peptides	Masses unmatched peptides
C4-b	1	63	807.55, 825.26, 832.64, 842.66, 845.25, 847.60, 855.21, 856.68, 861.23, 864.65, 865.17, 868.68, 870.70, 877.20, 887.16, 897.58, 973.71, 1033.71, 1045.76, 1057.76, 1060.76, 1065.71, 1067.73, 1090.73, 1106.25, 1106.75, 1109.69, 1157.80, 1165.80, 1179.81, 1193.83, 1201.83, 1232.82, 1234.91, 1265.86, 1277.94, 1307.91, 1323.91, 1365.89, 1383.93, 1435.03, 1476.03, 1488.02, 1639.16, 1658.10, 1717.16, 1792.05, 1839.27, 1941.28, 2194.55, 2211.51, 2223.53, 2225.53, 2233.49, 2239.54, 2247.52, 2255.48, 2265.45, 2283.60, 2284.59, 2298.60, 2384.40, 2705.68
	2	64	807.55, 825.26, 832.64, 842.66, 845.25, 847.60, 855.21, 856.68, 861.23, 864.65, 865.17, 868.68, 870.70, 877.20, 887.16, 897.58, 973.71, 1033.71, 1045.76, 1057.76, 1060.76, 1065.71, 1067.73, 1090.73, 1106.25, 1106.75, 1109.69, 1157.80, 1165.80, 1179.81, 1193.83, 1201.83, 1232.82, 1234.91, 1265.86, 1277.94, 1307.91, 1320.83, 1323.91, 1365.89, 1383.93, 1435.03, 1476.03, 1488.02, 1639.16, 1658.10, 1708.08, 1717.16, 1792.05, 1839.27, 1941.28, 2194.55, 2211.51, 2223.53, 2225.53, 2239.54, 2247.52, 2255.48, 2265.45, 2283.60, 2284.59, 2298.60, 2384.40, 2705.68
	3	63	807.55, 825.26, 832.64, 842.66, 845.25, 847.60, 855.21, 856.68, 861.23, 864.65, 865.17, 868.68, 870.70, 877.20, 887.16, 897.58, 973.71, 1033.71, 1045.76, 1057.76, 1060.76, 1065.71, 1067.73, 1090.73, 1106.25, 1106.75, 1109.69, 1157.80, 1165.80, 1179.81, 1193.83, 1201.83, 1232.82, 1234.91, 1265.86, 1277.94, 1307.91, 1320.83, 1323.91, 1365.89, 1383.93, 1435.03, 1476.03, 1488.02, 1639.16, 1658.10, 1717.16, 1839.27, 1941.28, 2194.55, 2211.51, 2223.53, 2225.53, 2233.49, 2239.54, 2247.52, 2255.48, 2265.45, 2283.60, 2284.59, 2298.60, 2384.40, 2705.68

Table 4.3, continue.

Protein band	Rank	No. of unmatched peptides	Masses unmatched peptides
C4-c	1	64	807.55, 825.26, 835.21, 842.66, 854.21, 855.21, 856.67, 861.22, 863.18, 864.17, 865.18, 868.68, 870.18, 870.69, 871.18, 877.20, 887.16, 903.14, 919.11, 920.10, 930.58, 973.71, 1023.71, 1033.70, 1037.72, 1045.75, 1057.75, 1060.76, 1066.69, 1069.73, 1106.74, 1108.19, 1126.76, 1157.80, 1158.79, 1160.77, 1165.79, 1176.75, 1179.81, 1201.84, 1234.90, 1265.85, 1300.77, 1307.91, 1323.90, 1365.88, 1383.93, 1435.03, 1476.03, 1658.08, 1708.08, 1717.14, 1792.04, 1795.12, 2211.49, 2225.51, 2239.50, 2248.51, 2265.42, 2279.45, 2283.56, 2299.58, 2319.36, 2384.37
	2	62	807.55, 825.26, 835.21, 842.66, 854.21, 855.21, 856.67, 861.22, 863.18, 864.17, 865.18, 868.68, 870.18, 870.69, 871.18, 877.20, 887.16, 903.14, 919.11, 920.10, 930.58, 973.71, 1023.71, 1033.70, 1037.72, 1045.75, 1057.75, 1060.76, 1066.69, 1069.73, 1106.74, 1108.19, 1126.76, 1157.80, 1158.79, 1160.77, 1165.79, 1176.75, 1179.81, 1234.90, 1265.85, 1307.91, 1323.90, 1365.88, 1383.93, 1435.03, 1476.03, 1658.08, 1708.08, 1717.14, 1792.04, 2211.49, 2225.51, 2233.48, 2239.50, 2248.51, 2265.42, 2279.45, 2283.56, 2299.58, 2319.36, 2384.37
	3	63	807.55, 825.26, 835.21, 842.66, 854.21, 855.21, 856.67, 861.22, 863.18, 864.17, 865.18, 868.68, 870.18, 870.69, 871.18, 877.20, 887.16, 903.14, 919.11, 920.10, 930.58, 973.71, 1023.71, 1033.70, 1037.72, 1045.75, 1057.75, 1060.76, 1066.69, 1069.73, 1106.74, 1108.19, 1126.76, 1157.80, 1158.79, 1160.77, 1165.79, 1176.75, 1179.81, 1201.84, 1234.90, 1265.85, 1300.77, 1307.91, 1323.90, 1365.88, 1383.93, 1435.03, 1476.03, 1658.08, 1717.14, 1795.12, 2211.49, 2225.51, 2233.48, 2239.50, 2248.51, 2265.42, 2279.45, 2283.56, 2299.58, 2319.36, 2384.37

Table 4.3, continue.

Protein band	Rank	No. of unmatched peptides	Masses unmatched peptides
C5-a	1	64	807.54, 825.24, 832.63, 835.19, 842.65, 847.59, 854.19, 855.19, 856.66, 861.21, 864.15, 864.64, 865.16, 868.67, 870.68, 877.19, 887.15, 893.16, 913.56, 919.09, 920.10, 973.70, 1033.69, 1036.71, 1037.71, 1045.74, 1057.75, 1060.74, 1065.69, 1067.72, 1090.71, 1106.72, 1107.72, 1109.68, 1121.77, 1157.78, 1165.78, 1179.79, 1193.81, 1201.81, 1234.89, 1265.84, 1300.76, 1307.89, 1320.81, 1323.89, 1365.87, 1381.88, 1383.91, 1391.91, 1435.00, 1476.01, 1498.02, 1658.07, 1708.05, 1717.13, 1792.03, 2211.47, 2225.49, 2239.50, 2248.51, 2265.40, 2284.54, 2384.35
	2	62	807.54, 825.24, 832.63, 835.19, 842.65, 847.59, 854.19, 855.19, 856.66, 861.21, 864.15, 864.64, 865.16, 868.67, 870.68, 877.19, 887.15, 893.16, 913.56, 919.09, 920.10, 973.70, 1033.69, 1036.71, 1037.71, 1045.74, 1057.75, 1060.74, 1065.69, 1090.71, 1106.72, 1107.72, 1121.77, 1165.78, 1179.79, 1193.81, 1201.81, 1234.89, 1265.84, 1300.76, 1307.89, 1320.81, 1323.89, 1365.87, 1381.88, 1383.91, 1391.91, 1435.00, 1476.01, 1498.02, 1658.07, 1708.05, 1717.13, 1792.03, 2211.47, 2225.49, 2233.46, 2239.50, 2248.51, 2265.40, 2284.54, 2384.35
	3	62	825.24, 832.63, 835.19, 842.65, 847.59, 854.19, 855.19, 856.66, 861.21, 864.15, 864.64, 865.16, 868.67, 870.68, 877.19, 887.15, 893.16, 913.56, 919.09, 920.10, 973.70, 1033.69, 1036.71, 1037.71, 1045.74, 1057.75, 1060.74, 1065.69, 1090.71, 1106.72, 1107.72, 1109.68, 1121.77, 1157.78, 1165.78, 1179.79, 1193.81, 1201.81, 1234.89, 1265.84, 1300.76, 1307.89, 1320.81, 1323.89, 1381.88, 1383.91, 1391.91, 1435.00, 1476.01, 1498.02, 1658.07, 1708.05, 1717.13, 1792.03, 2211.47, 2225.49, 2233.46, 2239.50, 2248.51, 2265.40, 2284.54, 2384.35

Table 4.3, continue.

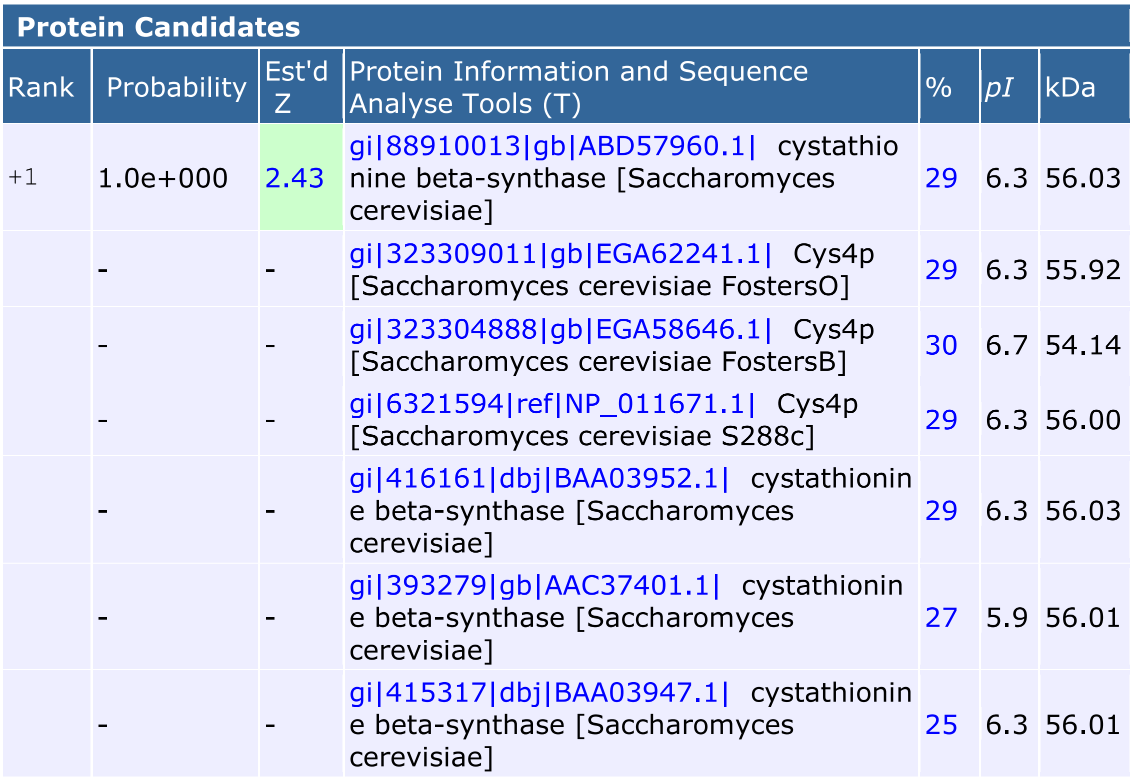
Protein band	Rank	No. of unmatched peptides	Masses unmatched peptides
C5-b	1	59	807.53, 825.23, 832.62, 835.19, 842.64, 847.58, 854.19, 855.19, 856.66, 861.20, 864.63, 865.15, 868.66, 870.16, 870.68, 871.17, 877.18, 887.14, 903.12, 913.55, 973.69, 993.66, 1033.68, 1037.69, 1045.73, 1057.73, 1060.73, 1065.69, 1066.68, 1074.69, 1090.70, 1107.72, 1157.77, 1165.77, 1179.79, 1193.81, 1201.81, 1232.80, 1234.88, 1235.73, 1254.81, 1265.83, 1307.88, 1323.88, 1329.85, 1365.86, 1381.86, 1390.89, 1434.99, 1476.01, 1658.05, 1708.04, 1717.12, 1742.00, 1792.02, 1795.09, 2211.46, 2225.47, 2384.33
	2	59	807.53, 825.23, 832.62, 835.19, 842.64, 847.58, 854.19, 855.19, 856.66, 861.20, 864.63, 865.15, 868.66, 870.16, 870.68, 871.17, 877.18, 887.14, 903.12, 973.69, 993.66, 1033.68, 1037.69, 1045.73, 1057.73, 1060.73, 1065.69, 1066.68, 1074.69, 1090.70, 1106.71, 1107.72, 1109.68, 1157.77, 1165.77, 1179.79, 1184.79, 1193.81, 1201.81, 1234.88, 1235.73, 1254.81, 1265.83, 1316.74, 1323.88, 1329.85, 1332.79, 1381.86, 1390.89, 1434.99, 1476.01, 1658.05, 1717.12, 1742.00, 1792.02, 1795.09, 2211.46, 2225.47, 2384.33
	3	60	807.53, 825.23, 832.62, 835.19, 842.64, 847.58, 854.19, 855.19, 856.66, 861.20, 864.63, 865.15, 868.66, 870.16, 870.68, 871.17, 877.18, 887.14, 903.12, 913.55, 973.69, 993.66, 1033.68, 1037.69, 1045.73, 1057.73, 1060.73, 1065.69, 1066.68, 1074.69, 1090.70, 1106.71, 1107.72, 1109.68, 1157.77, 1165.77, 1179.79, 1193.81, 1201.81, 1232.80, 1234.88, 1254.81, 1265.83, 1307.88, 1316.74, 1320.80, 1323.88, 1329.85, 1332.79, 1365.86, 1390.89, 1434.99, 1476.01, 1658.05, 1708.04, 1717.12, 1742.00, 1792.02, 2211.46, 2384.33

4.8.2 Data obtained from ProFound program

An alternative peptide mass fingerprinting (PMF) program had been implemented to identify the unknown proteins since the results provided by Mascot were less significant. Results derived from ProFound were presented in a different way than Mascot with respect to the differences of algorithms used. In search for an identical protein, the unmatched peptides from Mascot results were integrated in ProFound program. The result was in the form of a list of protein candidates ranked from 1 to 10 together with probability, estimated Z score (Est'd Z), percentage of coverage (%), protein pI as well as protein mass (kDa). The best matched protein candidate was ranked in number 1 and shown on top of the list. The probability obtained referred to the value calculated according to Bayesian probability. Est'd Z reflects the quality of the search result in which it compatible to a corresponding percentile in a random match population. Tables 4.4-4.11 describe the best matched protein candidate for each of the unknown protein.

Results from ProFound revealed the probability value of 1.0e+000 for each of the best matched protein candidate. Identification search for C3-a, C3-b, C4-a, C4-b, C4-c and C5-a resulted in a high estimated Z score values of 2.43, whereas C5-b and C3-c demonstrated estimated Z score value of 1.71 and 1.14 respectively. With regard to Table 4.12, it is indicated that Z scores of 2.43 and 1.71 correlated to a false-positive rate of 1 % and 5 % correspondingly. The search result for C3-c seemed to generate a high false-positive rate which was more than 10 % and therefore the result was disregarded. Following literatures review of matched protein candidates, several anti-hypertensive related proteins had been successfully identified among the unknown proteins and the results are presented in Table 4.13.

Table 4.4 : Protein candidates for C3-a using the unmatched peptides of 2nd ranked protein hit from Mascot

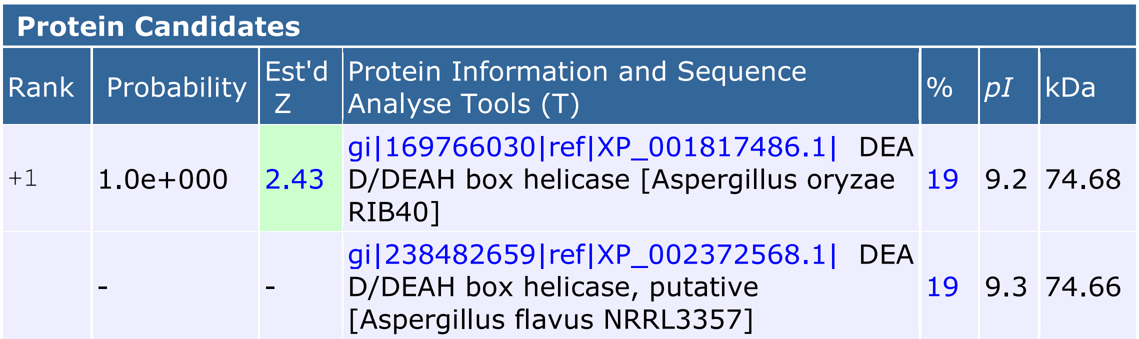


Protein Candidates

Rank	Probability	Est'd Z	Protein Information and Sequence Analyse Tools (T)	%	*pI*	kDa
+1	1.0e+000	2.43	gi|88910013|gb|ABD57960.1| cystathionine beta-synthase [Saccharomyces cerevisiae]	29	6.3	56.03
	-	-	gi|323309011|gb|EGA62241.1| Cys4p [Saccharomyces cerevisiae FostersO]	29	6.3	55.92
	-	-	gi|323304888|gb|EGA58646.1| Cys4p [Saccharomyces cerevisiae FostersB]	30	6.7	54.14
	-	-	gi|6321594|ref|NP_011671.1| Cys4p [Saccharomyces cerevisiae S288c]	29	6.3	56.00
	-	-	gi|416161|dbj|BAA03952.1| cystathionine beta-synthase [Saccharomyces cerevisiae]	29	6.3	56.03
	-	-	gi|393279|gb|AAC37401.1| cystathionine beta-synthase [Saccharomyces cerevisiae]	27	5.9	56.01
	-	-	gi|415317|dbj|BAA03947.1| cystathionine beta-synthase [Saccharomyces cerevisiae]	25	6.3	56.01

The '+' sign of rank 1 indicates the presence of more than one protein candidate with highly similar protein sequences.

Table 4.5 : Protein candidates for C3-b using the unmatched peptides of 2nd ranked protein hit from Mascot



Protein Candidates

Rank	Probability	Est'd Z	Protein Information and Sequence Analyse Tools (T)	%	*pI*	kDa
+1	1.0e+000	2.43	gi|169766030|ref|XP_001817486.1| DEAD/DEAH box helicase [Aspergillus oryzae RIB40]	19	9.2	74.68
	-	-	gi|238482659|ref|XP_002372568.1| DEAD/DEAH box helicase, putative [Aspergillus flavus NRRL3357]	19	9.3	74.66

The '+' sign of rank 1 indicates the presence of more than one protein candidate with highly similar protein sequences.

Table 4.6 : Protein candidate for C3-c using the unmatched peptides of 2^{nd} ranked protein hit from Mascot

Protein Candidate

Rank	Probability	Est'd Z	Protein Information and Sequence Analyse Tools (T)	%	*pI*	kDa
1	1.0e+000	1.14	gi|302697779|ref|XP_003038568.1| hypothetical protein SCHCODRAFT_46853 [Schizophyllum commune H4-8]	46	9.1	32.92

Table 4.7 : Protein candidates for C4-a using the unmatched peptides of 3^{rd} ranked protein hit from Mascot

Protein Candidates

Rank	Probability	Est'd Z	Protein Information and Sequence Analyse Tools (T)	%	*pI*	kDa
+1	1.0e+000	2.43	gi|486577|emb|CAA82169.1| unnamed protein product [Saccharomyces cerevisiae]	23	5.1	79.43
	-	-	gi|330443666|ref|NP_013016.2| Pxl1p [Saccharomyces cerevisiae S288c]	23	5.1	79.42
	-	-	gi|323304064|gb|EGA57843.1| Pxl1p [Saccharomyces cerevisiae FostersB]	21	5.1	79.17

The '+' sign of rank 1 indicates the presence of more than one protein candidate with highly similar protein sequences.

Table 4.8 : Protein candidates for C4-b using the unmatched peptides of 3^{rd} ranked protein hit from Mascot

Protein Candidates

Rank	Probability	Est'd Z	Protein Information and Sequence Analyse Tools (T)	%	*pI*	kDa
+1	1.0e+000	2.43	gi|159122956|gb|EDP48076.1| DNA damage response protein RcaA [Aspergillus fumigatus A1163]	18	6.7	79.20
	-	-	gi|146324578|ref|XP_746804.2| DNA damage response protein RcaA [Aspergillus fumigatus Af293]	18	6.6	79.23

The '+' sign of rank 1 indicates the presence of more than one protein candidate with highly similar protein sequences.

Table 4.9 : Protein candidates for C4-c using the unmatched peptides of 2nd ranked protein hit from Mascot

Protein Candidates						
Rank	Probability	Est'd Z	Protein Information and Sequence Analyse Tools (T)	%	*pI*	kDa
+1	1.0e+000	2.43	gi|303320681|ref|XP_003070340.1| hypothetical protein CPC735_035310 [Coccidioides posadasii C735 delta SOWgp]	38	10.0	52.92
	-	-	gi|320041452|gb|EFW23385.1| conserved hypothetical protein [Coccidioides posadasii str. Silveira]	36	10.0	52.88

The '+' sign of rank 1 indicates the presence of more than one protein candidate with highly similar protein sequences.

Table 4.10 : Protein candidates for C5-a using the unmatched peptides of 2nd ranked protein hit from Mascot

Protein Candidates						
Rank	Probability	Est'd Z	Protein Information and Sequence Analyse Tools (T)	%	*pI*	kDa
+1	1.0e+000	2.43	gi|326474542|gb|EGD98551.1| alpha/beta hydrolase [Trichophyton tonsurans CBS 112818]	18	6.7	58.58
	-	-	gi|326478149|gb|EGE02159.1| alpha/beta hydrolase [Trichophyton equinum CBS 127.97]	17	6.8	65.40

The '+' sign of rank 1 indicates the presence of more than one protein candidate with highly similar protein sequences.

Table 4.11 : Protein candidate for C5-b using the unmatched peptides of 3rd ranked protein hit from Mascot

Protein Candidates						
Rank	Probability	Est'd Z	Protein Information and Sequence Analyse Tools (T)	%	*pI*	kDa
1	1.0e+000	1.71	gi|116206110|ref|XP_001228864.1| hypothetical protein CHGG_02348 [Chaetomium globosum CBS 148.51]	30	9.4	48.38

Table 4.12 : List of estimated Z score and corresponding percentile

Estimated Z score	Corresponding percentile
1.282	90.0
1.645	95.0
2.326	99.0
3.090	99.9

Table 4.13 : Identified anti-hypertensive related proteins

Protein band	Anti-hypertensive related protein	Estimated molecular weight (Da)	Estimated Z value
C3-a	Cystathionine beta synthase-like protein	49,000	2.43
C3-b	DEAD/DEAH box helicase-like protein	43,700	2.43
C4-a	Paxillin-like protein	46,800	2.43
C5-a	Alpha beta hydrolase-like protein	37,100	2.43