CHAPTER VI

ISOLATION AND PURIFICATION OF ANTIBACTERIAL PROTEINS AND PEPTIDES FROM HONEY SAMPLES

6.1. Introduction

Proteins and peptides are found as mixture in the nature which makes purifying these compounds challenging. The purification of proteins and peptides as compared to other compounds like acids and volatile is not easy because of their sensitivity and complexity. Antimicrobial proteins and peptides are obtained from a wide variety of sources, foods as well microorganisms. These molecules have attracted much research interest because of their biochemical diversity, broad specificity on anti-bacterial, anti-fungi, anti-viral, anti-protozoan parasites, and even anti-tumoural or wound-healing effects (Zasloff, 2002). Antimicrobial proteins and peptides play key roles in innate immunity. They interact directly with bacteria and kill them (Lauth *et al*., 2002; Zhang *et al*., 2008).

Proteins and peptides are well known for their antimicrobial activity against different harmful microbes and that activity is dependent on their source and protein type (Matsuyam & Natori, 1988; Casteels *et al*., 1989; James *et al*., 1996; Xu *et al*., 2009; Ilyasov *et al*., 2012). For example a novel protein with antibacterial activity was isolated and purified from marine bacterium D2 (James *et al*., 1996). A protein was detected, extracted and purified from the skin of fish *Epinephelus fario* and found to have

antibacterial activity (Zhang *et al*., 2008).

Peptides were extracted from olive flounder and showed to have antibacterial activity against Gram-positive bacteria (Nam *et al*., 2014). Flaxseed proteins were isolated and purified from flaxseed flour with inhibitory activity to pathogenic bacteria (Tehrani *et al*., 2014).

Many reports have been published on detection isolation, purification, and/or antimicrobial activity of proteins and peptides from honeybees (Casteels *et al*., 1989; Xu *et al*., 2009; Ayaad *et al*., 2009; Ilyasov *et al*., 2012; Ortiz-Vázquez *et al*., 2013), but reports on protein and/or peptides from honey are very limited and mostly focused only on detection of proteins and amino acid composition (Alvarez *et al*., 2010). The antibacterial peptides (AMP) are very important to immune system of the bees and serve as the main weapon for bees in the defence against harmful attacks by pathogens (Hoffmann *et al*., 1999).

Honey is a mixture and source of different components including sugars, acids, volatile compounds, and also contain lower amounts enzymes, proteins and peptides; all those contents make the detection, isolation and purification of proteins and peptides are very complicated. Honey has been suggested to be alternative antimicrobial agent to the presently used antibiotics, especially against *S. aureus*. Antimicrobial peptides from natural sources could be used to replace the normal commercial antibiotics due to their broad ability to kill the microbes with low toxicity and reduce resistance development by the bacteria (Nam *et al*., 2014). The honey samples used in this study were confirmed to have antibacterial activity as presented in CHAPTERS III and IV. Therefore, the aim of

this chapter was to isolate and purify proteins and/or peptides and ascertain that these peptides have the antibacterial activity.

6.2. Materials and methods

6.2.1. Protein Extraction from Honey Samples

Three different methods were used to precipitate the protein from honey, two of them were the same in steps but different in solvents acetone and ethanol, and the third one is ammonium sulphate precipitation method. Using acetone and ethanol as follows where 30 mL of solvent (acetone/ethanol) was added to diluted honey sample 10 mL : 5 ml of deionized water and well mixed. All the samples were kept at room temperature overnight and then the precipitated protein was collected and dried at 45 °C (BINDER, Germany) to remove the acetone. All the samples were freeze dried (LABCONCO, USA) and kept at -80 °C for further study. Ammonium sulphate precipitation method was used by adding a 60 g of ammonium sulphate to 100 mL of diluted honey solution (50% with deionized water). Then, the solution was kept overnight at 4 °C and the results were detected as precipitation in the solution (Imdakim *et al.*, 2015).

6.2.2. Determination of the Protein Content using Bradford Method

The method of Bradford (1976) was followed with some modifications as explained in CHAPTER V, Section 5.2.3.

6.2.3. Determination of Peptide Content using OPA (O-phthalaldehyde) Assay

The OPA reagent was prepared following the method described by Church *et al.* (1983) with some modifications as shown in CHAPTER V, Section 5.2.4.

6.2.4. Determination of Amino Acid Profile of Extracted Proteins

Amino acid composition for precipitated honey samples protein was carried out was at UNIPEQ SDN. BHD, UKM-MTDC Technology Center, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor. All honey samples were hydrolyzed with 5 ml of 6 N HCL at 110^{o}C for 24 h and the hydrolysate was filtered through a 0.45 µm cellulose acetate membrane filter. The amino acid composition was carried out using Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) with auto sampler, AccQ Tag column (3.9 × 150 mm, 5 µl). The samples were submitted to automatic pre-column derivatization AccQ Fluor Reagents with a combination of amino acid standard, hydrolysate (Standrad H, Pierce). The mobile face was 100 ml AccQ Tag Eluent A (Concentrate) with 1000 ml of distilled water filtered through a 0.45 µm cellulose acetate membrane and AccQ Tag Eluent B or 60% acetonitrile. The chromatographic column temperature was set at 36^{o}C with a flow rate of 1 ml/min. The detection by Fluorescence Detector (Waters 2475) was set at 250/395 nm (E_{λ}/E_{m}). This experiment was done in duplicate and mean with standard deviation were calculated.

6.2.5. Sodium Dodecyle Sulphate (SDS-PAGE) for Extracted Freeze Dried Proteins

SDS-PAGE for precipitated proteins was carried out by the discontinuous buffer system as described by Laemmli (1970) with modifications. Electrophoresis was carried out at a constant voltage of 120 V for 150 min using 16% polyacrylamide gel, under the denaturing conditions. The gels were calibrated with known molecular weight marker (high and low ranges: 250, 200, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa). Protein bands were visualized by Commassie Blue dye staining. Calculations were determined by regression analysis using the manufacturer's procedure.

6.2.6. Reverse Phase High Performance Liquid Chromatography (RP-HPLC) of Extracted Protein

Semi-preparative RP-HPLC analysis was carried out to fractionate the acetone precipitated proteins following the method of Quirós *et al*. (2005) with modifications. The honey precipitated proteins were freeze dried, dissolved in 1 ml mobile phase A and injected into a semi-preparative C18 RP-HPLC column (9.40 mm × 250 mm, 5-µm particles, Agilent Technologies, Santa Clara, CA, USA). The sample injection volume and concentration were 500 µL and 400 mg of freeze dried powder per ml. Mobile phase (A) was deionized water containing 0.1% (v/v) TFA and B was acetonitrile containing 0.1% (v/v) TFA. Elution was set at 0-10 min, 100% A; 10-60 min, 0-100% B. The flow rate was 4 mL per min and the detection wavelength was set at (205 and 280 nm). The fractions were collected in 6 ml vials then transferred to deep freeze at -80 °C using 50 mL Eppendrof tubes to remove mobile phase A and B. Each fraction was freeze-dried in

two stages; each stage for 48 h to fully freeze-dried the sample. The fractions were then dissolved in 500 µl sterilized deionised water, filtered using 0.2 µl syringe filter (Minisart RC 4, Sartorius stedim, Germany) and tested against target bacteria in 96 wells microtitter plates.

6.2.7. Antibacterial Activity of RP-HPLC Fractions using Microtiter Plates Assay

The method of Magnusson and Schnurer (2001) was followed with some modifications as described in Chapter III, Section 3.2.8.

6.2.8. Peptide Content of RP-HPLC Fractions using OPA (O-phthalaldehyde) Assay

The OPA reagent was prepared essentially as described by Church *et al*. (1983) with some modifications as shown in CHAPTER V, Section 5.2.4.

6.2.9. Sodium Dodecyle Sulphate (SDS-PAGE) for Freeze Dried Sephadex G-50 Fractions

SDS-PAGE for freeze dried Sephadex fractions was carried out by the discontinuous buffer system as described by Laemmili (1970) with some modifications as shown in section 6.1.5, CHAPTER V.

6.2.10. Statistical Analyses

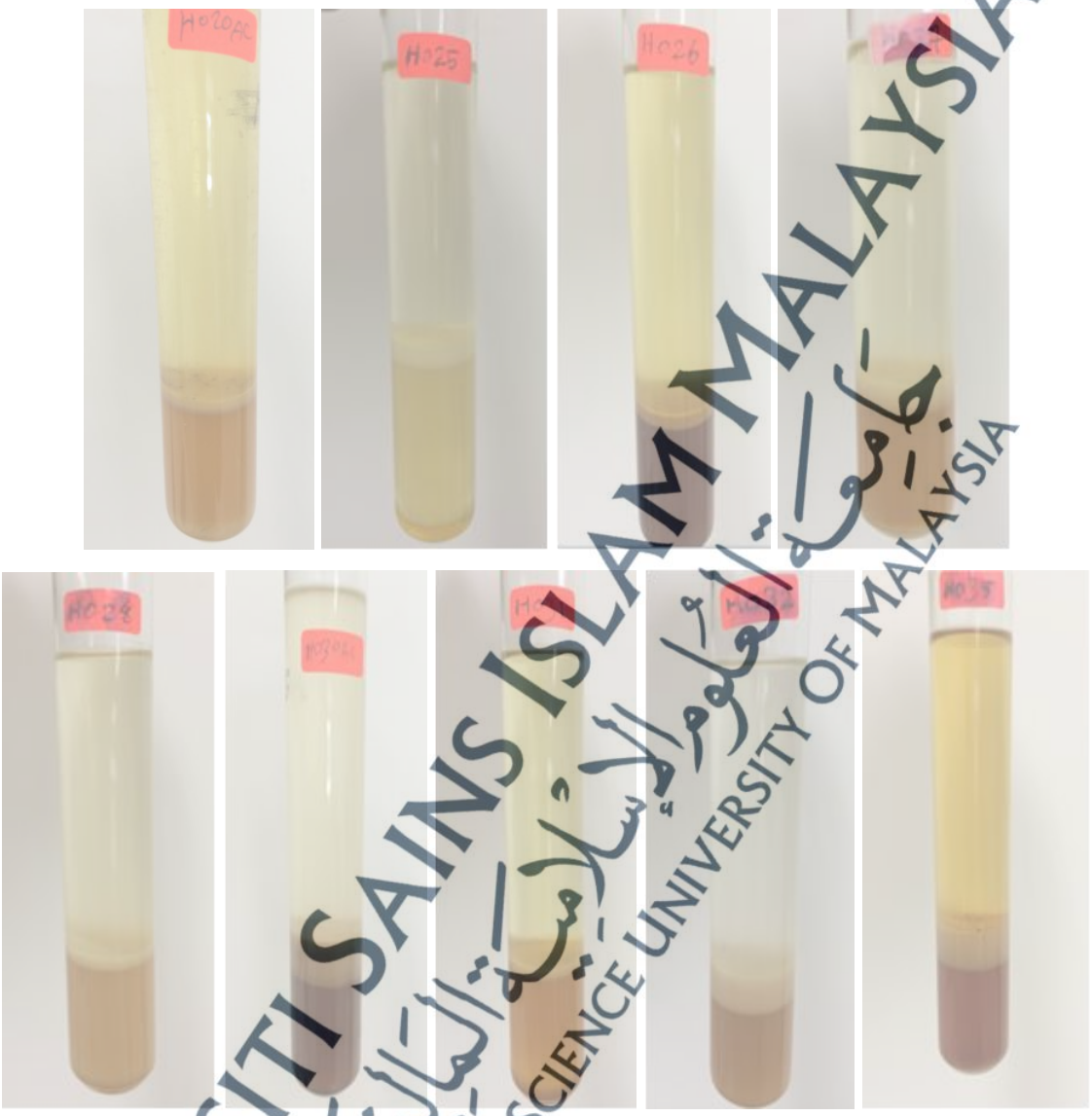
Mean, standard division, percentage of inhibition and ANOVA analysis were carried out to determine statistical differences ($p<0.05$) using Minitab 16.

6.3. Results

6.3.1. Protein Extraction from Honey Samples

Protein in honey samples was successfully precipitated using acetone but not ammonium sulphate or ethanol (Figure 3). Using 2:1 (10 mL : 5 mL) (acetone:honey 50% diluted) produced protein layer as shown in Figure 6. The amount of precipitation from samples H027 (Manuka honey), H030 (Acacia honey), H032 (Acacia honey) and H035 (Manuka honey) were higher than the other samples, while precipitation in samples H020 (Hannon honey), H026 (Tualang honey) and H031 (Acacia honey) were very low even after 48 h of incubation.

Figure 3: Extracted protein from honey samples using acetone precipitation



6.3.2. Protein Content of Freeze Dried Acetone Precipitated Fraction using Bradford Method

The protein content from precipitated honey proteins ranged from 1.14 to 1.579 mg/ml (Table 27). The highest protein content was from H026 (Tualang honey) and H031

(Acacia honey) with concentration of 1.579 mg/ml. Samples H028 (Kharroob Honey) and H027 (Manuka Honey) showed the lowest protein content (1.140 and 1.290 mg/ml, respectively) as shown in Table 28.

Table 27: Absorbance reading at 595 nm of Bradford standard (BSA) using Bradford method

No. of standard	Concentration of BSA (mg/ml)	Absorbance at 595 nm
1	0.0	0
2	0.2	0.097
3	0.4	0.217
4	0.6	0.308
5	0.8	0.467
6	1.0	0.531
7	1.2	0.609

Table 28: Absorbance of honey samples and protein concentration using standard formula (Y=0.5259x+0.0029)

Sample code	Absorbance at 595 nm	Concentration of protein (mg/ml)
H025	0.811	1.537
H026	0.833	1.579
H027	0.681	1.290
H028	0.602	1.140
H031	0.833	1.579
H032	0.719	1.362
H035	0.779	1.476

6.3.3. Peptide Content of Acetone extracted proteins using OPA (O-phthalaldehyde) Method

The glutathione standard was linearly correlated with the concentration used with R^2= 0.9915 as shown in Table 29 and Figure 36 (Appendix 3). The peptide content of precipitated protein samples was calculated using the standard formula (Y=0.2924x+0.01) and the peptide content varies from 0 to 3.32 mM/ml equivalent to 0 to 1.019 mg/ml (Table 30). Sample H026 (Tualang honey) contain significantly higher peptide content than other honey samples. The peptide content of sample H026 (Tualang honey) was 3.32 mM/ml (1.019 mg/ml), followed by sample H032 (Acacia honey) with content of 1.93 mM/ml (0.593 mg/ml). The sample H035 (Manuka honey) showed the lowest concentration of peptide which was 0.06 mM/ml (0.018 mg/ml). Peptide was not detected in samples H027 (Manuka honey) and H031 (Acacia honey).

Table 29: Absorbance at of Glutathione standard using OPA assay

No. of standard	Glutathione concentration (mg/ml)	Absorbance
1	0.0	0.000
2	0.2	0.036
3	0.4	0.102
4	0.6	0.177
5	0.8	0.215
6	1.0	0.287

Table 30: Absorbance of honey samples and peptide concentration using standard formula (Y=0.2924x+0.01)

Honey sample code	Absorbance at 340 nm	Concentration of peptide (mM/ml)	Concentration of peptide (mg/ml)
H020	0.00	0.00	0.000
H025	0.36	1.29	0.396
H026	0.96	3.32	1.019
H027	0.00	0.00	0.000
H028	0.22	0.78	0.239
H030	0.935	3.23	0.991
H031	0.00	0.00	0.000
H032	0.55	1.93	0.593
H035	0.01	0.06	0.018

6.3.4. Determination of Amino Acid Profile of Extracted Proteins

The amino acid profiles of the extracted protein varied with honey samples (Tables 31 and 32). The total amino acid concentration in decreasing order was Acacia honey (H030, 2.670 ± 0.580), Manuka honey (H027, 2.170 ± 0.080), Alseder honey (H025, 2.165 ± 0.841), Kharoob honey (H028, 1.830±0.280), Acacia honey (H032, 1.670 ± 0.270) Tualang honey (H026, 1.610 ± 0.170), Manuka honey (H035, 1.450 ± 0.290), Hannon honey (H020, 1.440 ± 0.045) and Acacia honey (H031, 1.290±0.027). Amino acids alanine (Ala), proline (Pro), lysine (Lys), isoleucine (Ile) and leucine (Leu) was detected present in all precipitated honey protein samples. Samples H026 (Tualang honey) and H032 (Acacia honey) had comparable amounts of total amino acids which were 1.610 ± 0.170 and 1.670 ± 0.270, respectively. The type of essential amino acids was not equally distributed among all honey samples. The number of essential amino acids varies and range from eight in H030 (Acacia honey), seven in H025 (Alseder honey), seven in H028 (Kharoob honey), seven in H027 (Manuka honey), seven in H035 (Manuka honey), six in H026 (Tualang honey), five in H032 (Acacia honey) five in H020 (Hannon honey) and three in H031 (Acacia honey). Glutamic acid (Glu), valine (Val), leucine (Leu) and phenylalanime (Phe) were most abundant amino acids found in sample H027 (Manuka honey) with amounts 0.191 ± 0.114, 0.056 ± 0.003, 0.086±0.006 and 0.122 ± 0.004, respectively.

Table 31: Amino acid composition of precipitated proteins from honey samples*

	Honey samples				
Amino acid	**H020**	**H025**	**H026**	**H027**	**H028**
Hydroxypoline	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
Aspartic acid (Asp)	0.000±0.000	0.074±0.059	0.000±0.000	0.000±0.000	0.054±0.048
Serine (Ser)	0.052±0.009	0.219±0.237	0.055±0.002	0.127±0.068	0.083±0.026
Glutamic acid (Glu)	0.000±0.000	0.000±0.000	0.000±0.000	0.191±0.114	0.000±0.000
Glycine (Gly)	0.000±0.000	0.366±0.328	0.124±0.004	0.186±0.001	0.167±0.040
Histidine (His)*	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
Ammonia (Nh2)	0.148±0.021	0.190±0.012	0.180±0.065	0.160±0.014	0.157±0.042
Arginine (Arg)*	0.000±0.000	0.000±0.000	0.000±0.000	0.076±0.003	0.010±0.010
Threonine (Thr)*	0.007±0.001	0.043±0.049	0.008±0.000	0.010±0.0003	0.014±0.0004
Alanine (Ala)	0.038±0.003	0.032±0.015	0.012±0.000	0.032±0.020	0.080±0.034
Proline (Pro)	0.039±0.002	0.025±0.017	0.085±0.000	0.046±0.0052	0.079±0.052
α-aminobutyric acid	1.000±0.000	1.000±0.000	1.000±0.000	1.000±0.000	1.000±0.000
Tyrosine (Tyr)	0.000±0.000	0.022±0.025	0.000±0.000	0.020±0.003	0.000±0.000
Valine (Val)*	0.029±0.003	0.050±0.032	0.026±0.0004	0.056±0.003	0.035±0.003
Methionine (Met)*	0.000±0.000	0.005±0.005	0.000±0.000	0.000±0.000	0.000±0.000
Lysine (Lys)*	0.020±0.001	0.030±0.300	0.012±0.006	0.016±0.001	0.013±0.009
Isoleucine (Ile)*	0.033±0.0003	0.023±0.016	0.066±0.000	0.045±0.006	0.062±0.036
Leucine (Leu)*	0.033±0.003	0.048±0.019	0.029±0.004	0.086±0.006	0.037±0.004
Phenylalanime (Phe)*	0.037±0.0005	0.032±0.017	0.097±0.003	0.122±0.004	0.032±0.001
Total amino acid	1.440±0.045	2.165±0.841	1.610±0.170	2.170±0.080	1.830±0.280

*Results are stated as mean ± standard deviation (sd)
*The amino acids with star are essential amino acids

Table 32: Amino acid composition of precipitated proteins from honey samples*

	Honey samples			
Amino acid	**H030**	**H031**	**H032**	**H035**
Hydroxypoline	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
Aspartic acid (Asp)	0.506±0.590	0.000±0.000	0.155±0.004	0.000±0.000
Serine (Ser)	0.117±0.034	0.000±0.000	0.037±0.034	0.034±0.049
Glutamic acid (Glu)	0.135±0.008	0.000±0.000	0.063±0.063	0.000±0.000
Glycine (Gly)	0.209±0.040	0.000±0.000	0.000±0.000	0.000±0.000
Histidine (His)*	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
Ammonia (Nh2)	0.154±0.002	0.079±0.011	0.157±0.010	0.151±0.010
Arginine (Arg)*	0.069±0.020	0.000±0.000	0.000±0.000	0.112±0.150
Threonine (Thr)*	0.044±0.013	0.000±0.000	0.000±0.000	0.000±0.000
Alanine (Ala)	0.076±0.040	0.072±0.008	0.051±0.043	0.032±0.015
Proline (Pro)	0.071±0.080	0.046±0.006	0.041±0.028	0.025±0.017
α-aminobutyric acid	1.000±0.000	1.000±0.000	1.000±0.000	1.000±0.000
Tyrosine (Tyr)	0.023±0.006	0.000±0.000	0.000±0.000	0.000±0.000
Valine (Val)*	0.053±0.009	0.000±0.000	0.028±0.016	0.016±0.023
Methionine (Met)*	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
Lysine (Lys)*	0.011±0.005	0.022±0.006	0.021±0.002	0.025±0.016
Isoleucine (Ile)*	0.065±0.038	0.047±0.006	0.044±0.036	0.023±0.016
Leucine (Leu)*	0.063±0.007	0.029±0.001	0.034±0.028	0.020±0.029
Phenylalanine (Phe)*	0.072±0.005	0.000±0.000	0.036±0.025	0.009±0.013
Total amino acid	2.670±0.580	1.290±0.027	1.670±0.270	1.450±0.290

*Results are stated as mean ± standard deviation (sd)

*The amino acids with star are essential amino acids

6.3.5. Protein Profile for Freeze Dried Extracted Proteins

The presence of proteins in freeze dried extracted protein samples were confirmed using SDS-PAGE (Table 33 and Figures 4 to 6). Both protein bands and smears of different molecular weight 9 to 116.5 KDa were detected (Table 34 and Figures 4 to 6). The highest number of protein bands was eight from sample H025 (Alseder honey) with molecular weight from 9.80 to 116.50 KDa, followed by four bands from sample H032 (Acacia honey) with molecular weight from 9.00 and 83.30 KDa and three bands were obtained from sample H026 (Tualang honey) with molecular weight of 10.00 to 90.60 KDa. In contrast the extracted protein from samples H027 (Manuka honey) and H028 (Kharoob honey) showed only two bands (83.30-102.70), while samples H031 (Acacia honey) and H035 (Manuka honey) showed only one protein band for each (86.90 and 90.60 KDa).

Table 33: Molecular weight of the marker used with R_f calculations

Band number	Molecular weight	Log of Molecular weight	Migration distance of unknown protein (cm)	R_f
1	10	1.00	7.50	0.85
2	15	1.18	7.00	0.80
3	20	1.30	6.00	0.68
4	25	1.40	4.90	0.57
5	37	1.57	4.30	0.48
6	50	1.70	3.40	0.39
7	75	1.76	2.90	0.33
8	100	2.00	2.30	0.26
9	150	2.18	2.10	0.24
10	250	2.40	1.70	0.19

Table 34: Approximate molecular weight of the bands of tested honey samples

No.	Honey sample	Number of bands	Range of molecular weight (kDa)
1	H025	8	9.80 to116.50
2	H026	3	10.00-57.10-90.60
3	H027	2	86.90 - 02.70
4	H028	2	83.30-102.70
5	H031	1	86.90
6	H032	4	9.00-83.30
7	H035	1	90.60

Figure 4: Freeze-dried precipitated proteins from honey samples using SDS-Page 16% gel with 10 to 250 kDa marker

Figure 5: Freeze-dried precipitated proteins from honey samples using SDS-Page12/4% gel with 10 to 250 kDa Marker

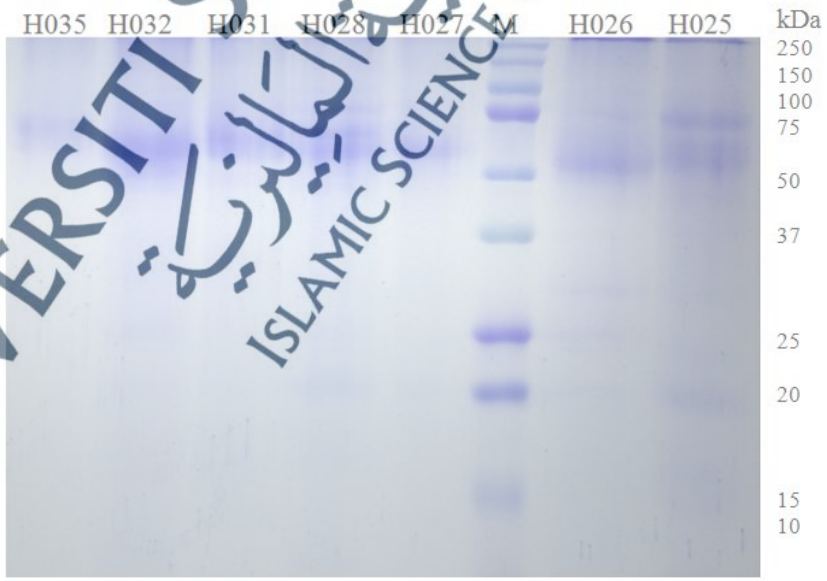
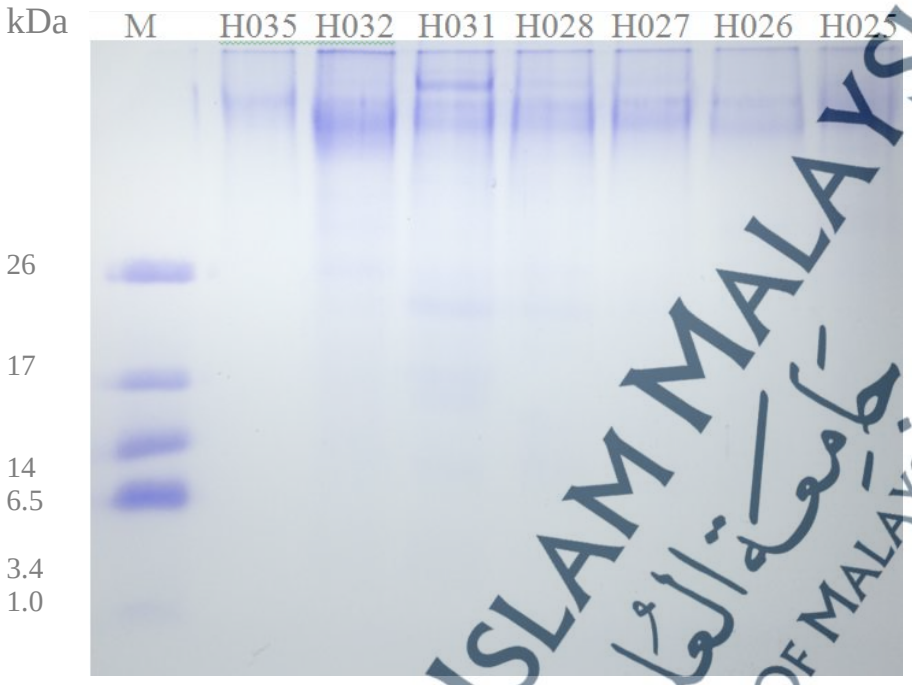


Figure 6: Freeze-dried precipitated proteins from honey samples using SDS-Page by 16/10% gel with 1 to 26 kDa Marker



6.3.6. RP-HPLC of Extracted Proteins

Five precipitated protein samples were selected for further characterization. The protein from honey samples H025, H026, H028, H032 and H035 were selected based on their peptide content (Table 30). The RP-HPLC method was successful in separating the fractions and the profile was variable among samples (Figures 7, 8, 9, 10 and 11). The first peptide fraction appeared after 10 min followed by other peaks until 60 min elution time. The samples contain a mixture of peptides as indicated by the number of peaks in the chromatogram. Sample H026 (Tualang honey) and H032 (Acacia honey) showed peptide peaks at 10 to 50 min, H025 (Alseder honey) showed peptides peaks at 10 to 40 min, samples H028 (Kharoob honey) and H035 (Manuka honey) were between 10 to 60 min of fractionation time and the peaks were not well separated (Figures 9 and 11). The

peptide fractions of H025, H026 and H032 tended to be well separated under the operating conditions used (Figures 7, 8 and 10).

Figure 7: RP-HPLC fractionation graph of sample H025 extracted protein

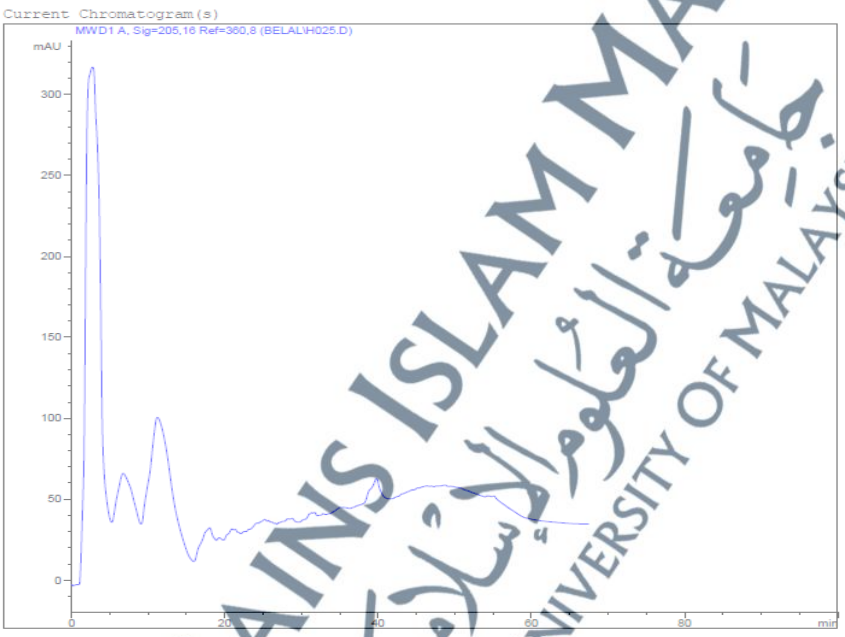


Figure 8: RP-HPLC fractionation graph of sample H026 extracted protein

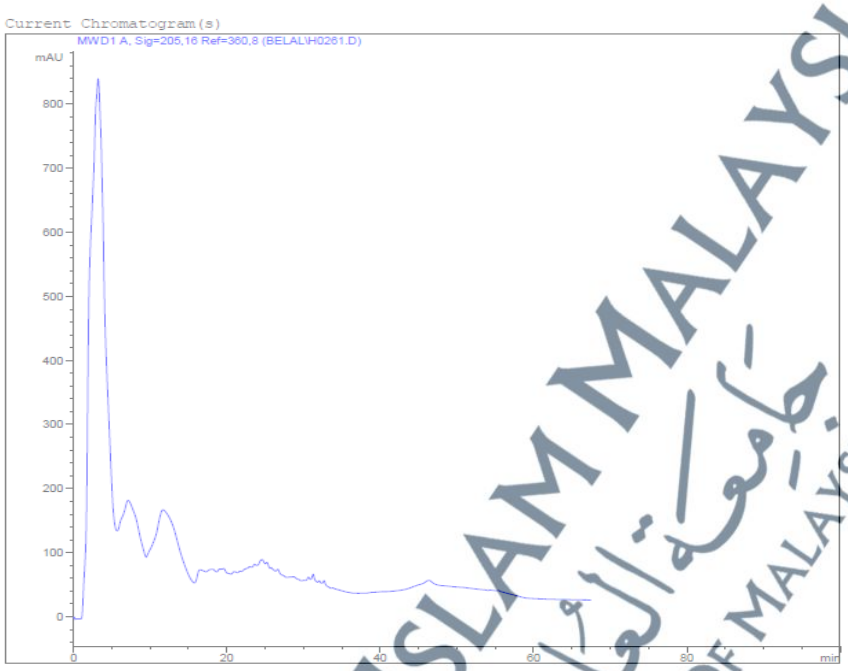


Figure 9: RP-HPLC fractionation graph of sample H028 extracted protein

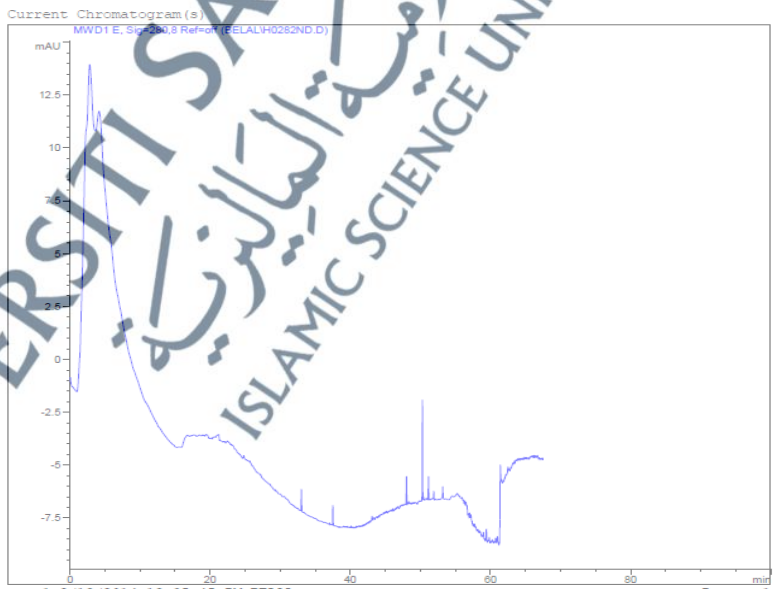


Figure 10: RP-HPLC fractionation graph of sample H032 extracted protein

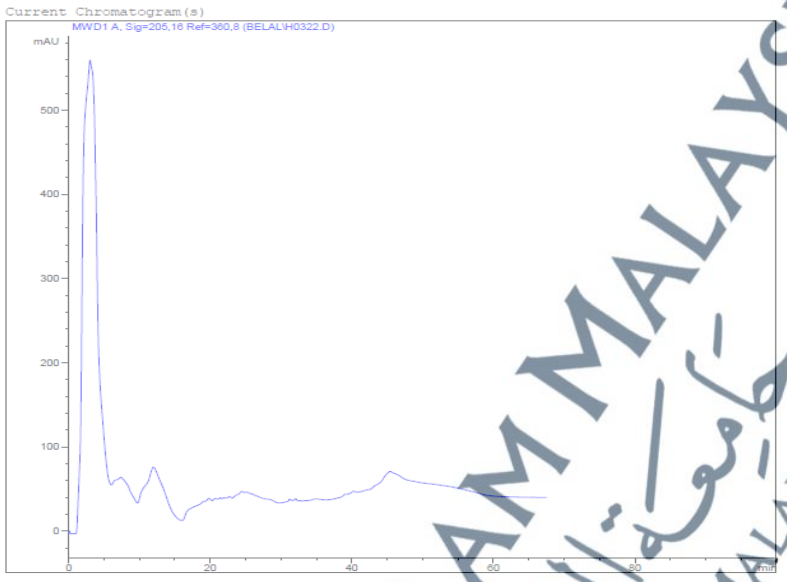


Figure 11: RP-HPLC Fractionation graph of sample H035 extracted protein

6.3.7. Antibacterial Activity of RP-HPLC Fractions from Extracted Honey Proteins using Microtiter Plates Assay

Evaluation on the antibacterial activity of RP-HPLC peptide fractions indicated that some fractions showed no inhibitory activity while others showed total inhibition of *S. aureus* (Table 35). Fraction F4 from samples H025 (Alseder honey) and H035 (Manuka honey) presented the highest percentage of inhibition (100.00 %), followed by fractions F2 and F3 from H025 and H035 (Alseder and Manuka honey), respectively, and F2 and F4 from H032 (Acacia honey) (>98.00%). The lowest percentage of inhibition was obtained from fraction F10 (21.22 %) from sample H026 (Tualang honey). Fraction F3 from H032 (Acacia honey) and fractions F2 and F3 from H026 (Tualang hone) showed antibacterial activity with 97.92 %, 97.69 % and 96.41 %, respectively. Fractions F14 and F18 from H035 (Manuka honey) did not show antibacterial activity (0.00 %).

Table 35: Percentage inhibition of RP-HPLC fractions against *S. aureus* using microtiter plates assay

RP-HPLC Fractions	Percentage inhibition (%) of RP-HPLC fractions				
	H025	**H026**	**H028**	**H032**	**H035**
F1	73.84	65.08	68.40	95.59	86.97
F2	98.96	97.69	97.04	98.31	98.05
F3	98.71	96.41	98.25	97.92	98.51
F4	100.0	79.92	82.76	98.18	100.0
F5	73.58	76.59	88.68	80.54	76.00
F6	59.79	70.97	70.75	77.43	72.80
F7	52.70	69.56	69.97	76.65	59.77
F8	54.63	61.38	65.10	69.89	61.82
F9	55.41	50.76	70.93	58.95	63.08
F10	55.41	21.22	69.62	66.14	70.51
F11	53.09	49.48	82.68	71.59	63.65
F12	49.74	47.69	61.61	34.76	57.71
F13	55.79	55.24	69.62	43.32	37.71
F14	55.28	47.31	71.27	48.63	00.00
F15	60.18	47.57	68.84	44.87	60.91
F16	61.46	43.98	65.10	42.93	59.88
F17	61.08	46.16	68.32	46.17	59.31
F18	60.95	31.45	66.40	45.52	00.00
F19	61.08	42.96	69.27	51.10	59.77
F20	57.98	47.31	68.23	49.80	55.42
F21	24.35	45.14	67.36	53.43	54.62
F22	52.06	46.16	66.66	51.10	58.85
F23	48.32	53.06	46.64	49.93	58.40
F24	54.51	45.01	66.92	51.49	64.34
F25	52.70	45.52	65.01	50.71	63.88

6.3.8. Peptide Content of RP-HPLC Fractions using OPA (O-phthalaldehyde) Assay

The glutathione standard curve was straight line with R^2= 0.9915 as shown in Figure 37 (Appendix 3). The peptide concentration assay showed the variation of peptide contents among the fractions (Figures 12, 13, 14 and 15). The peptide content of RP-HPLC fractions of honey extracted proteins ranged from 0.0009 to 1.004 mg/ml. The highest

peptide content was shown by fraction F7 from sample H032 (Acacia honey), while the lowest peptide content was shown by fraction F21 from sample H025 (Alseder honey). Fractions F3, F4 and F5 from sample H026 (Tualang honey) showed peptide content of 0.957, 0.850 and 0.608 mg/ml, respectively. Fractions F2 and F3 from sample H028 (Kharoob honey) contained peptide but in lower amounts, 0.144 and 0.128 mg/ml, respectively.

Figure 12: Peptide concentration of RP-HPLC fractions of the extracted proteins from honey sample H025 using standard formula (y= 0.2924x - 0.01)

Figure 13: Peptide concentration of RP-HPLC fractions of the extracted proteins from honey sample H026 using standard formula (y= 0.2924x - 0.01)

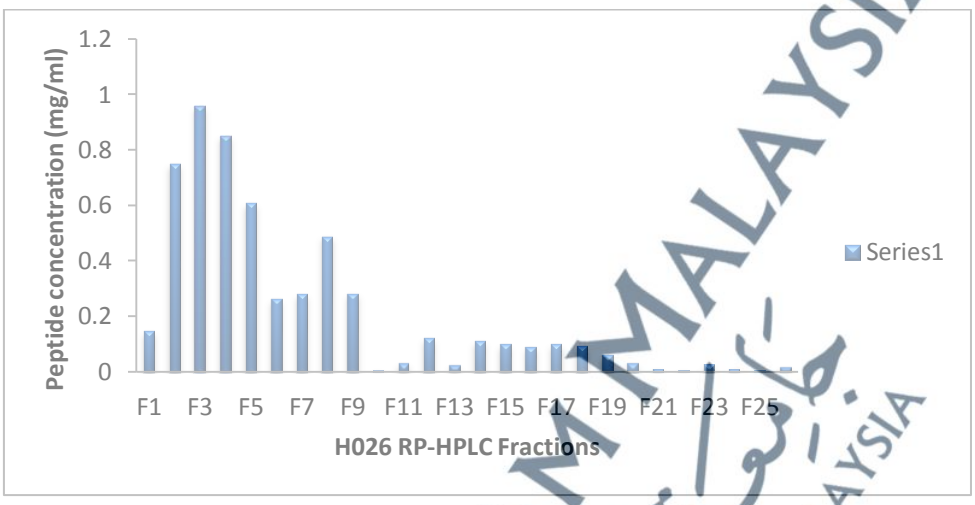


Figure 14: Peptide concentration of RP-HPLC fractions of the extracted proteins from honey sample H028 using standard formula (y= 0.2924x - 0.01)

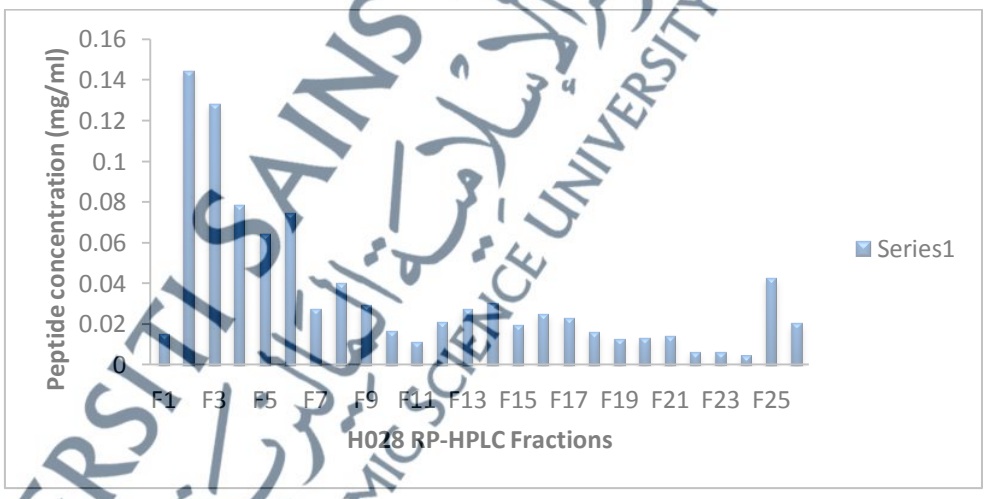
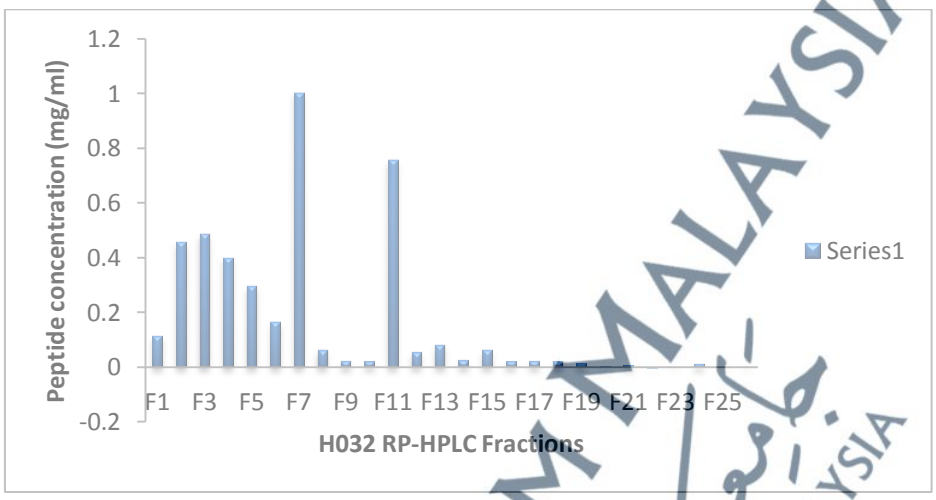


Figure 15: Peptide concentration of RP-HPLC fractions of the extracted proteins from honey sample H032 using standard formula (y= 0.2924x - 0.01)



6.4. Discussion

There are limited studies on the extraction, purification and identification of protein from honey, which could be because of the low amount of proteins in honey (0.4 to 0.5 %) (NHB, 2003; James *et al*., 2009; Buba *et al*., 2013), or the difficulty of extracting the proteins from high osmolarity and the complex structure of honey. The ammonium sulphate precipitation is one of the most recognized methods used to extract or isolate the proteins from different sources (Zhao *et al*., 2013; Imdakim *et al*., 2015), but in the current study it was not suitable for honey protein extraction. Based on previous studies ammonium sulphate was used to extract proteins from sources such as milk and bacteria metabolite which are rich in proteins (Imdakim *et al*., 2015). Honey protein was precipitated using ethanol and acetone and it was observed that acetone could precipitate honey proteins (Figure 3) but not ethanol. However, Alvarez (2010) successfully used ethanol, methanol and propanol to extract the proteins from honey samples.

The proteins content in freeze dried extracted samples varied from 1.140 to 1.579 mg/ml based on the honey sample as determined by Bradford method (Table 28). Tualang honey (H026) and Acacia honey (H030) samples showed the highest concentration of protein (1.579 mg/ml), while Kharoob honey (H028) showed the lowest (1.140 mg/ml) concentration compared to the other studied samples. Alvarez (2010) checked the protein content using the bicinchoninic Acid (BCA) method of the extracted proteins from honey samples and found that the protein concentration ranged from 96.2 to 3344.6 µg/ml. The higher value for protein was obtained in this study, which could be due to the different methods used or related to the type of honey samples.

In fact, the chemical composition of honey depends on origin, plants, floral, season and bee. It was observed that not all honey contained peptides; peptides was not detected in samples H020 (Hannon honey), H027 (Manuka honey) and H031 (Acacia honey) but present in variable amounts in other honey analysed (Table 30). Sample H026 (Tualang honey) showed the high peptide content (1.019 mg/ml) and the lowest content was at 0.018 mg/ml from sample H035 (Manuka honey) compared to the other honey samples extracted proteins. Acacia honeys H030 and H032 showed peptide content of 0.991 and 0.593, respectively. To date most published work (Alvarez, 2010; Chua *et al*., 2013; Chua *et al*., 2014) detected the presence of proteins by SDS-PAGE without looking into peptides. This study, however, further characterized the amino acid composition of the peptides from the extracted protein.

Amino acids are the compounds that proteins and peptides are built from, the number and type of amino acids is responsible for the final structure and functional properties of the

protein/peptide. Reports on amino acid composition of honey were relatively few except those repoted by Cotte *et al*. (2004); Von der Ohe *et al*. (1991) and Iglesias *et al*. (2006) and, none from honey obtained in Malaysian and Libya.

The amino acid profile for all precipitated proteins from honey showed alanine (Ala), proline (Pro), lysine (Lys), isoleucine (Ile) and leucine (Leu) were present in all honey samples studied (Table 31 & 32). However, hydroxypoline and histidine (His) were the only amino acids that were not detected in all precipitated proteins of honey samples. Methionine (Met) was not discovered in all honey samples except in sample H025 (Alseder honey) with amount of 0.005 ± 0.005 mg/100 g. The highest composition of amino acids was detected in sample H030 (Acacia honey, 2.670 ± 0.580 mg/100g) compared to the other checked protein samples. Comparable amounts of total amino acids (610 ± 0.170 and 1.670 ± 0.270 mg/100 g) were shown by protein samples from Tualang honey (H026) and Acacia honey (H032). Acacia honey (H030) contained the highest amounts of aspartic acid and tyrosine, while H025 (Alseder honey) was the richest in serine, glycine, methionine and lysine. The number of essential amino acids in extracted proteins of honey varies between samples between three to eight.

However, the amino acid composition of honey is reported variable depending on sources. The amino acid profile of 192 samples of seven floral types of Serbian honey (acacia, sunflower, linden, rape, giant goldenrod, buckwheat and basil) was analysed and found that the most abundant amino acids were proline, phenylalanine, alanine, arginine and threonine reported by Keckes *et al*. (2013). Study in Turkey by Silici and Karaman (2014) of 25 different honey samples concluded that aspartic acid, lysine, and arginine

were the major amino acids detected in rhododendron honeys while lysine, arginine, and histidine were found in honeydew honeys. Very low amounts of amino acids were detected in honey and ranges from 34.19 to 1 83.16 µg/100g (Cotte *et al*., 2004; Iglesias *et al*., 2006; Alvarez, 2010). Among the amino acids detected, proline is the most abundant amino acid in the honey samples studied similar to that observed by Iglesias *et al*. 2006 and Von der Ohe *et al*. 1991. Proline levels correspond to about 33% of the total free amino acid composition. Proline is mainly derived from bees' secretions.

The molecular weight of the proteins/peptides presence in honey samples were in the range of 116.5 to 20.00 to KDa as determined using SDS-PAGE, and some smears were detected at molecular weight range of <20 to 9 KDa (Table 34 and Figures 4 to 6). Sample H025 (Alseder honey) showed 8 clear bands, while sample H032 (Acacia honey) recorded 4 clear bands which ranged from 9.00 to 116.5 and 9.00 to 83.30 kDa, respectively. Sample H026 (Tualang honey) showed lower number of bands which was 3 and has a molecular weight of 10.00, 57.10 and 90.60 kDa compared to samples H025 and H032. Alvarez (2010) carried protein profile for eleven honey samples (without protein extraction) using SDS-PAGE and found that the molecular weight of proteins ranged from 130 to 25 KDa. This further indicates that honey contains different types of proteins.

The peptides present in samples H025, H026, H028, H032 and H035 (Table 30) were further fractionated using RP-HPLC (Figures 7 to 11) to ascertain the antibacterial properties of the peptide fractions. Protein samples from H025 (Alseder honey), H026 (Tualang honey) and H032 (Acacia honey) tended to give clearer and more separated

peaks (Figures 7, 8 and 10). The peptides were eluted between 10 to 40 min indicating that these peptides are amphipathic and eluted with the mixture of eluent A and B.

For the purpose of evaluating the antibacterial activity of the RP-HPLC fractions, *S. aureus* was used as the target bacteria. A total of 45 fractions were collected but fractions 2 to 25 showed antibacterial activity towards target bacteria with percentage of inhibition from 21.22 to 100.00 % (Table 35), and the amount of peptide was in range between 0.0009 and 1.004 mg/ml (Figures 12 to 15). Fraction F4 from sample H025 (Alseder honey) and H035 (Manuka honey) showed total inhibition of the target bacteria; the peptide content was 0.098 and 0.40 mg/ml, respectively. Fractions F2 to F8 of protein samples H026 (Tualang honey) contain high amounts of peptides (0.263 to 0.957 mg/ml) and high percentage of inhibition against target bacteria (61.38 to 97.69 %). Fractions F2 to F8 from sample H032 (acacia honey) showed high antibacterial activity of 69.39 to 98.31 % with range of peptide from 0.14 to 0.02 mg/ml. All 45 fractions from sample H035 (Manuka honey) did not show any peptide content, but variable antibacterial activity (37.71 to 100.00%). Other compounds like methylglyoxal has been reported to contribute to the antibacterial activity of Manuka honey (Weigel *et al*., 2004; Adams *et al*., 2008; Kwakman & Zaat, 2012). Peptide concentration and the antibacterial activity assays showed the variation among the fractions depends on the source of sample.

6.5. Conclusion

Proteins present in honey samples were successfully extracted using acetone and contain

varied amount of both essential and non-essential amino acids depending on the source of honey. The RP-HPLC fractions of honey protein extracts from all honey samples showed antibacterial activity against *S. aureus* except F14 and F18 from Manuka honey.