CHAPTER VII

PURIFICATION AND IDENTIFICATION OF EXTRACTED HONEY PROTEINS

7.1. Introduction

Honey contains a variety of different compounds and not all these compounds are well known or identified. Such compounds are proteins and peptides and known to be present in bees (Casteels *et al*., 1989; Engel, 1999; Chernysh *et al*., 1999; Xu *et al*., 2009; Aronstein *et al*., 2010; Ilyasov *et al*., 2012), honey (Gallardo-Chacon *et al*., 2008; Kwakman *et al*., 2010; Chua *et al*., 2014) and could originate from different sources in the environment where the bees have attached with or from the place of harvest. Proteins are known to impart the nutritional value of a food and may also have other functional properties such as antimicrobial peptides.

Novel peptides with antibacterial activity were isolated from Saudi Arabian honeybees; the polypeptides were effective against range of Gram-positive and Gram-negative bacteria (Ayaad *et al*., 2009). Different peptides in the gene families of abaecin, defensin, apidaecin, and hymenoptaecin were isolated and purified from Asiatic honeybee and, reported that the Asiatic honeybee peptides have more variable antimicrobial activity than western honeybee against pathogenic bacteria, especially hymenoptaecin (Xu *et al*., 2009). Definsin is one of the peptides that are present in honeybee and it is well known

for its ability to inhibit the growth of harmful microorganisms to the bees and humans including bacteria and fungi (Casteels-Josson *et al*., 1994; Mandrioli *et al*., 2003; Bulet & Stocklin, 2005; Saltykova *et al*., 2010; Ilyasov *et al*., 2012).

There are several methods to purify proteins and peptides isolated from different sources. RP-HPLC is one of the protein/peptides purification methods and it is effective and well known for fractionation of proteins. However, this method is known to cause changes in structure and functionality of the proteins or the peptides fractions by the mobile phase used such as acetonitrile, methanol or others. The fractions collected normally contained low amount and lower activity because of the low injection volume of sample used. The column characteristics also could affect the nature of proteins or peptides (Aguilar, 2004).

Sephadex column chromatography is often used in the purification of protein and peptide from different sources such as peptide from bacteria, from honeybee and honeybee venom (Kemeny *et al*., 1983; Gauldie *et al*., 1976; Srisuparbh *et al*., 2003; Zhao *et al*., 2013). There are several sizes of Sephadex column chromatography (G-10, G-15, G-25, G-50, G-75, G-100 and G-200), which depend on the expected molecular weights of the proteins or peptides that need to be purified and fractionated (Porath & Flodin, 1959; Janson, 1987; Zhao *et al*., 2013). However, work on using Sephadex to purify proteins/peptides from honey has not been reported.

In the previous Chapter (VI) the antibacterial activity of the RP-HPLC peptide fractions was evaluated. The peptide bands from these fractions was not detectable using SDS-PAGE to determine their molecular weight and their sequence. Therefore, this Chapter reports the fractionation of honey protein extracts using Sephadex G-50. The fractions

were evaluated for the antibacterial activity, peptide content and sequencing using LC-MS-MS.

7.2. Materials and Methods

7.2.1. Gel Filtration using Sephadex Column Chromatography (G-50 Fine)

Sepahadex G-50 Fine (SIGMA) column was prepared according to the manufacturer instructions.

7.2.1.1. Sephadex and column preparation: Sephadex G-50 (5 gm) were mixed with 60 ml of phosphate buffer (0.2 M) pH 7 and heated at 95°C for 1h and kept at 4 °C for 12 h. Then the suspension was de gassed using sonnicator (HWASHIN Technology, South Korea) for 15 min at room temperature. The chromatography column with size of 1 × 55 cm was acid washed several times using hydrochloric acid (HCl) to remove any attached substances, and small amount of glass wool was placed at the bottom of the column. The Sephadex suspension was carefully loaded to the column using 5 ml pipet (Eppendorf), and the level was checked using water level monitor.

7.2.1.2 Column volume calculation: column volume (ml) = 3.14× (radius of the column (cm))^2× the column length (cm).

7.2.1.3 Sample preparation: freeze dried extracted proteins used was 3% of the column volume. Freeze dried extracted proteins (200 mg) was mixed in 1.5 ml of phosphate buffer (0.2 M, pH 7), then vortexed and gently loaded to the column at the wall without

making air bubbles.

7.2.1.4 Void volume calculation: The void volume was 30% of the column volume.

The samples fractions were collected using fraction collector/manually every 10 min. The absorbance and protein content were measured using Nanophotometer (IMPLEN, Germany) at OD_{280} nm and the first fraction was used as blank. The collected fractions were freeze dried and kept at -80 freezer (New Brunswick Scientific, England) for further study.

7.2.2. Determination of Antibacterial Activity of Sephadex Fractions using Microtiter Plates Assay

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

7.2.3. Determination of Peptide Content of Sephadex Fractions 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.

7.2.4. 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 and Chapter V.

7.2.5. Sequencing of Antimicrobial Sephadex Fractions

Peptides were extracted according to standard techniques following the method of Bringans *et al*. (2008). Peptides were analysed by electrospray ionisation mass spectrometry using the Agilent 1260 Infinity HPLC system [Agilent] coupled to an Agilent 6540 mass spectrometer [Agilent] (Agilent Technologies, Santa Clara, CA, USA). The fractions were digested with pepsin enzyme and tryptic peptides were loaded onto a C18 column 300 SB, 5 µm [Agilent] and separated with a linear gradient of water/acetonitrile/0.1% formic acid (v/v). Spectra were analysed to identify proteins of interest using Mascot sequence matching software [Matrix Science] with Ludwig NR database. The experiments were performed using the facilities of 1st base, Malaysia.

7.2.6. Statistical Analyses

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

7.3. Results

7.3.1. Gel Filtration using Sephadex Column Chromatography (G-50 Fine) for Freeze Dried Extracted Proteins

Elution profile of honey proteins using Sephadex column chromatography (G-50 Fine) produced two well separated peaks from all honey peptide samples. The highest peak over protein samples was obtained from H026 with absorbance of 1.379 at 280 nm with protein amount of 1.956 mg/ml, followed by H035 for the second peak with absorbance of 0.714 at 280 nm and protein concentration of 0.972 mg/ml. While the lowest peak was obtained from H025 with absorbance of 0.294 at 280 nm and protein amount of 0.454 mg/ml. H032 precipitated protein (Acacia honey) showed peak with absorbance of 0.476 at 280 nm and protein concentration of 0.69 mg/ml. Fractions number of 12 fractions was obtained from both H025 and H032 (Alseder honey and Acacia hone, respectively), 11 fractions from H026 (Tualang honey), 14 fractions from H028 (Kharoob honey), 9 fractions from H035 (Manuka honey), as shown in Table 36 and Figures 16 to 20. The elution time of the fractions were different for different honey and was started from 30 to 140 min as shown in Table 36.

Table 36: Freeze dried protein fractions using Sephadex G-50 Fine with elution time, highest absorbance at OD_{280} nm and protein content

Honey sample	Number of fractions	Elution time/ min	Highest peak Abs. (280 nm)	Highest peak protein content (mg/ml)
H025	12	30 to 140	0.294	0.454
H026	11	40 to 140	1.379	1.956
H028	14	30 to 160	0.497	0.740
H032	12	40 to 150	0.476	0.690
H035	9	40 to 140	0.714	0.972

Figure 16: Elution time, absorbance and protein concentration of H025 honey protein fractions. Two peaks eluted between 30 and 140 min, 12 fractions were collected

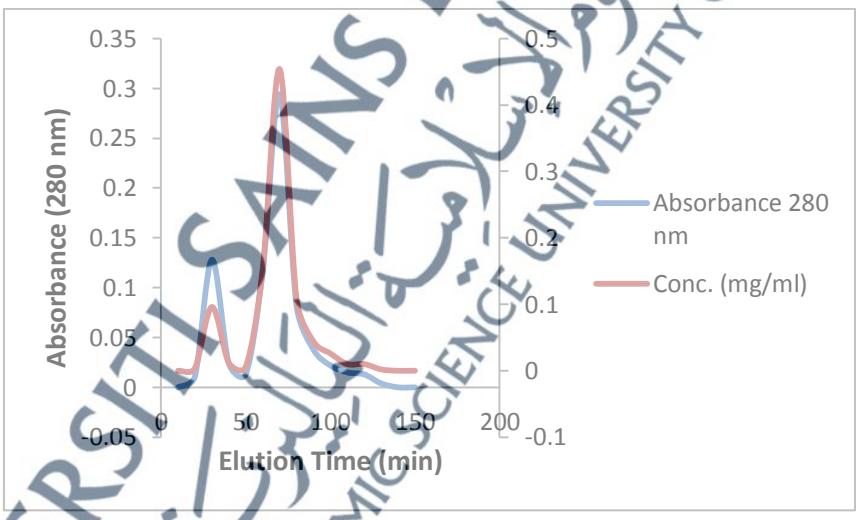


Figure 17: Elution time, absorbance and protein concentration of H026 honey protein fractions. Two peaks eluted between 40 and 140 min, 11 fractions were collected

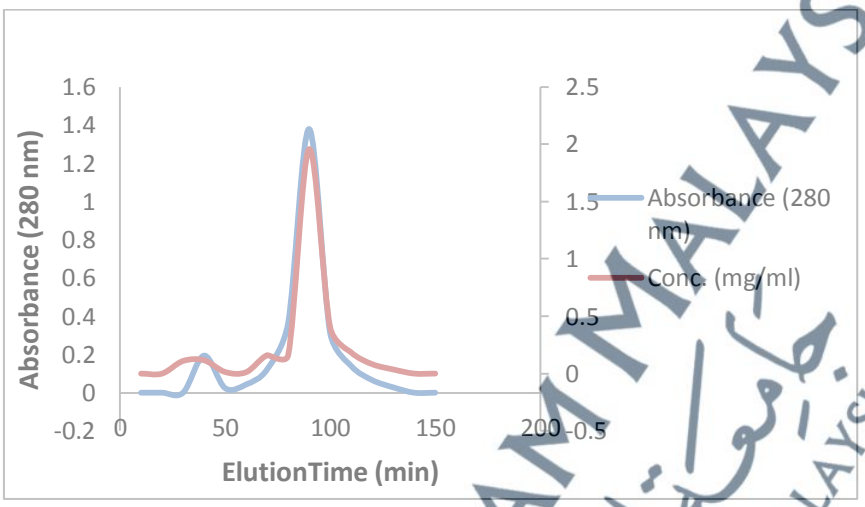


Figure 18: Elution time, absorbance and protein concentration of H028 honey protein fractions. Two peaks eluted between 30 and 160 min, 14 fractions were collected

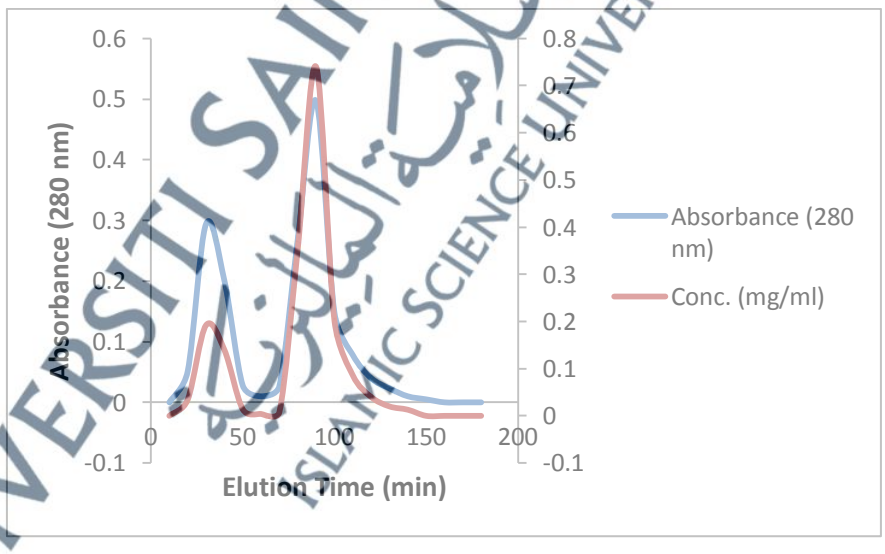


Figure 19: Elution time, absorbance and protein concentration of H032 honey protein fractions. Two peaks eluted between 40 and 150 min, 12 fractions were collected

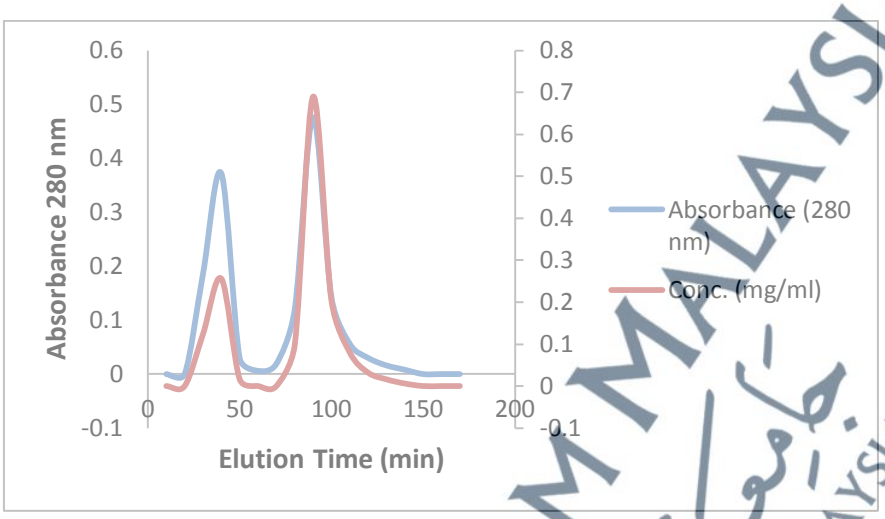
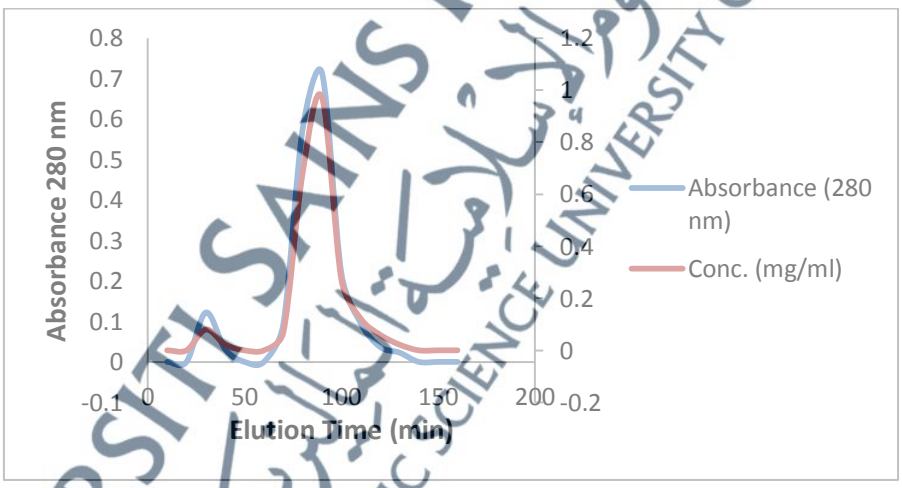


Figure 20: Elution time, absorbance and protein concentration of H035 honey protein fractions. Two peaks eluted between 40 and 140 min, 9 fractions were collected



7.3.2. Antibacterial Activity of Sephadex Fractions using Microtiter Plates Assay

The freeze dried Sephadex fractions of extracted honey proteins showed antibacterial activity against *S. aureus* that ranged from 19.52 to 92.39 % as shown in Table 37. Good antibacterial activity ranging from 68.63 to 92.3 % inhibition was observed for fraction

F6 from sample H028 (Kharoob honey) 92.39%, fraction F5 from sample H025 (Alseder honey) 90.00 %, fraction F6 from samples H032 (Acacia honey) 84.79 and F6 from H026 (Tualang honey) 68.63 %. The lowest percentage of inhibition was shown by fraction F14 from sample H025 (19.52 %) while fractions F9, F10 and F14 from sample H035 (Manuka honey) did not show any antibacterial activity (0.00 %) against target bacteria *S. aureus*.

Table 37: Percentage inhibition of protein Sephadex fractions against *S. aureus* using microtiter plates assay*

	Percentage inhibition (%) of protein Sephadex fractions				
Fractions	**H025**	**H026**	**H028**	**H032**	**H035**
F3	62.26	-	63.90	-	-
F4	58.49	50.34	65.82	55.13	71.09
F5	90.00	24.24	67.46	71.78	75.20
F6	86.02	68.63	92.39	84.79	-
F7	60.27	60.20	21.78	78.83	-
F8	83.63	60.61	86.16	72.67	29.58
F9	83.15	30.13	54.38	42.87	00.00
F10	63.69	41.02	36.98	37.26	00.00
F11	53.21	48.76	48.49	00.00	35.61
F12	34.58	48.01	58.63	39.45	36.84
F13	50.34	26.64	73.83	70.82	04.38
F14	19.52	56.09	42.94	16.91	00.00
F15	-	-	87.39	61.78	-
F16	-	-	30.06	-	-

*(-) No fraction collected, (0) No activity

7.3.3. Peptide Content of Sephadex Fractions using OPA (O-phthalaldehyde) Method

The peptide content for Sephadex fractions was carried out using glutathione standard which showed straight line with $R^2 = 0.991$ and, the concentration of peptide in fractions was calculated using formula: y= 0.2546x + 0.1044 (Figure 38, Appendix 3). The peptide concentration of Sephadex fractions ranged from 0.035 to 0.937 mg/ml (Figures 21 to 25). The highest content was shown by fraction F10 (0.937 mg/ml) from sample H026 (Tualang honey), followed by fraction F10 (0.450 mg/ml) from sample H032 (Acacia honey). Fraction F14 from H025 sample (Alseder honey) showed the lowest peptide content (0.035 mg/ml) compared to other samples fractions. Fraction F10 from sample H028 (Kharoob honey) showed peptide content of 0.167 mg/ml and fraction F10 from sample H035 (Manuka honey) indicated peptide content of 0.105 mg/ml.

Figure 21: Peptide concentration of Sephadex fractions of the extracted proteins from honey sample H025 using standard formula (y= 0.2546x + 0.1044)

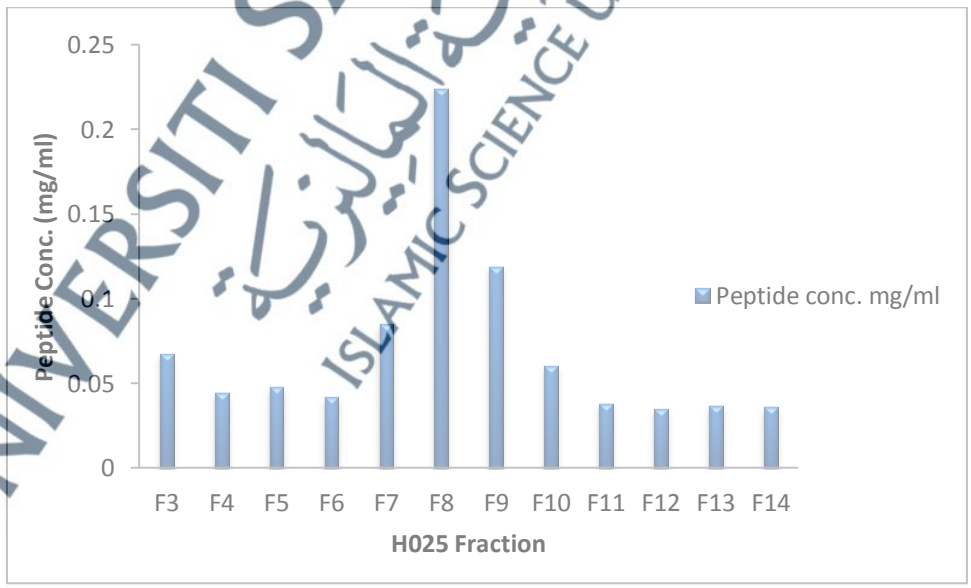


Figure 22: Peptide concentration of Sephadex fractions of the extracted proteins from honey sample H026 using standard formula (y= 0.2546x + 0.1044)

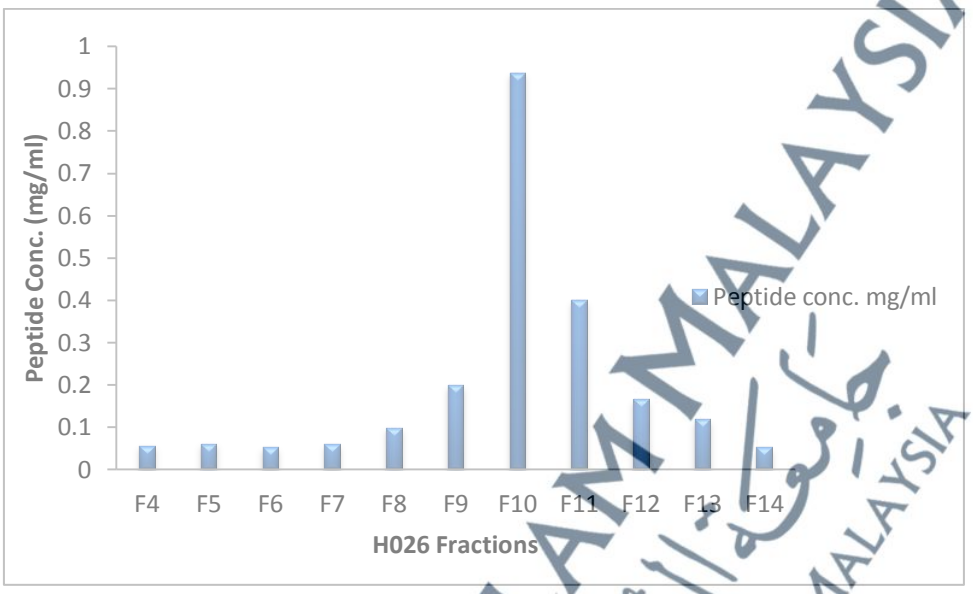


Figure 23: Peptide concentration of Sephadex fractions of the extracted proteins from honey sample H028 using standard formula (y= 0.2546x + 0.1044)

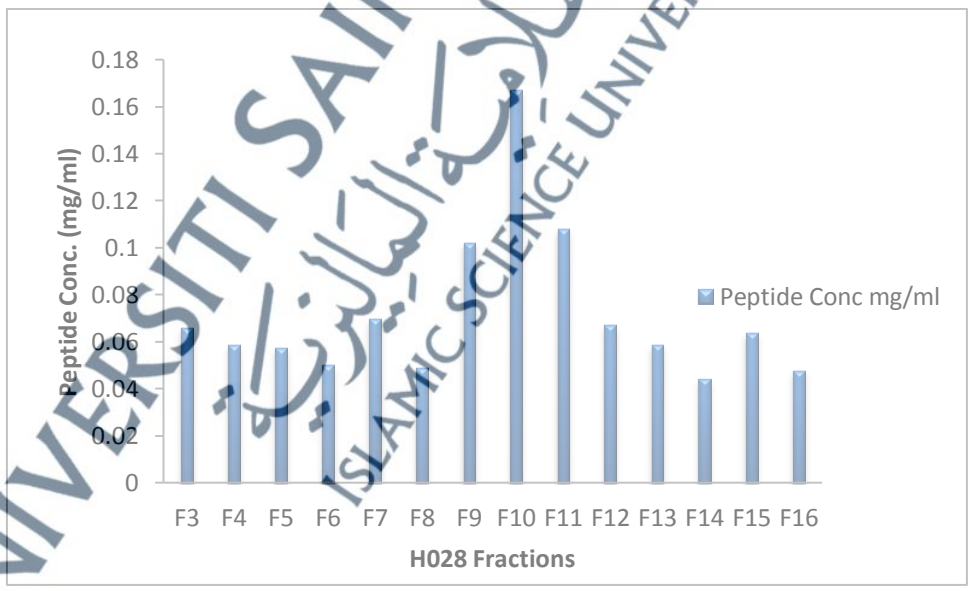


Figure 24: Peptide concentration of Sephadex fractions of the extracted proteins from honey sample H032 using standard formula (y= 0.2546x + 0.1044)

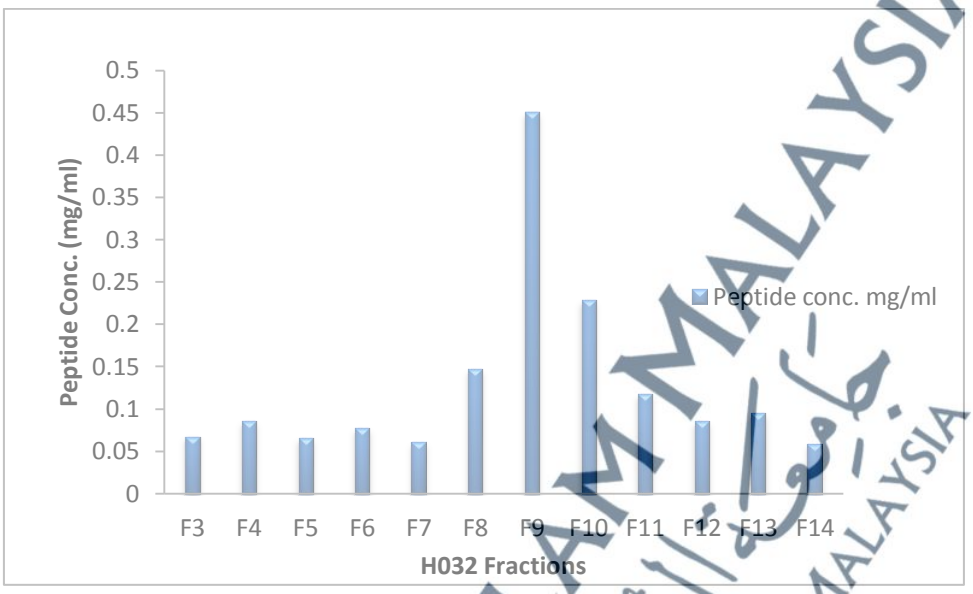
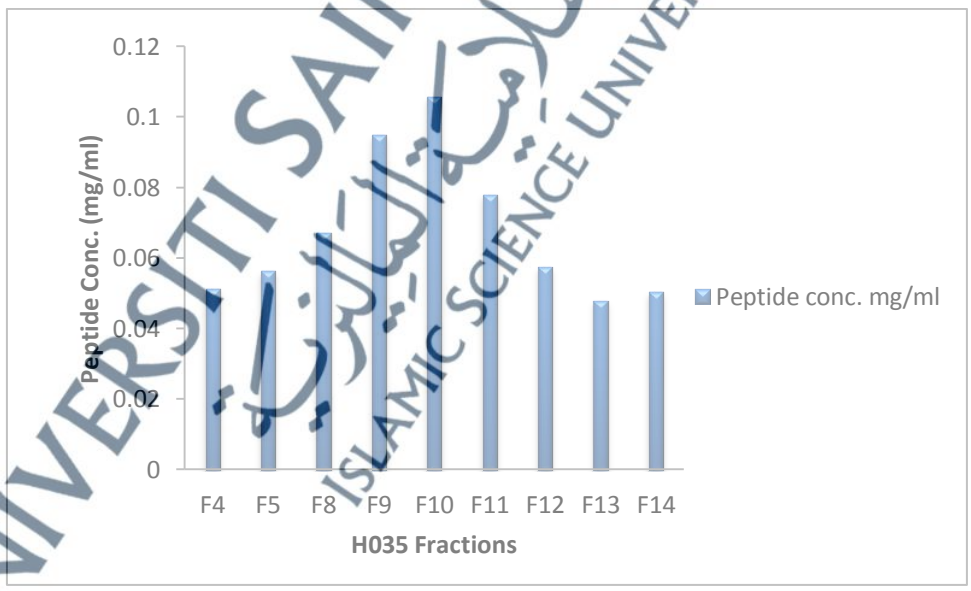


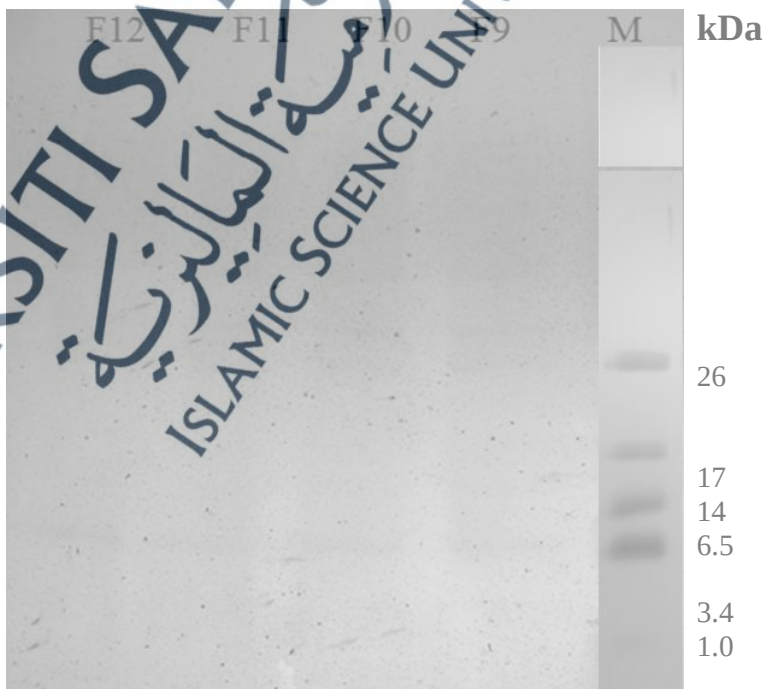
Figure 25: Peptide concentration of Sephadex fractions of the extracted proteins from honey sample H035 using standard formula (y= 0.2546x + 0.1044)



7.3.4. Protein Profile using SDS-PAGE of Sephadex Fractions

Sephadex fractions F9 and F10 from H026 (Tualang honey) and F11 and F12 from H032 (Acacia honey) that showed good antibacterial activity (>60%) against *S. aureus* (Table 37) and peptide content >0.45 mg/mL (Figure 22 and 24) were selected and analysed further for protein profiling by SDS-PAGE (16 %gel) as shown in Figure 26. The molecular weight of the protein bands of Sephadex fractions ranged from 1 to 30 KDa from all fractions. Similar pattern at molecular weight of 26 to 1 KDa were obtained Fractions F9 and F10 from sample H026 (Tualang honey), and both expressed four bands and two groups of smears. Three bands at molecular weight range of 30 to 1 KDa and smears at less than 1 KDa were obtained from Sephadex fractions F11 and F12 from sample H032 (Acacia honey).

Figure 26: Protein bands of fractions F9 and F10 from H026, and F11 and F12 from H032 using SDS-PAGE. M: is the marker used



7.3.5. Sequencing of Antimicrobial Sephadex Fractions

The dried bands and smears using SDS-PAGE of Sephadex fractions F9 and F11 from samples H026 and H032, respectively, were analysed for peptide sequencing. A total of 33 peptides were identified in fraction 9 of protein sample H026 (Tualang honey) (Tables 38 and 39). The molecular weight (MW) of the 34 peptides was varied between 672 to 2962.34 Da. The net charge (NT) for all the peptides was between -2 to +2 except for the peptide AEQNIVCPPGTDLEEYQEK which has +4 NT. The hydrophobicity ratio (HR) of peptides in these fractions ranged from +6.13 to +29.87 Kcal x mol^{-1}. The peptide fragments consisted of 6 residues per peptide to 25 residues per peptide. Number of 14 peptides from Sephadex G-50 fraction F9 of sample H026 were not existed in the data base and considered novel (*De novo*) peptides (Table 41).

Eighteen peptides were obtained from fractions F11 and F12 of sample H032 (Acacia honey) (Table 40). The length of the identified peptides was 6 to 22 residues. Five peptides were cationic (positive charge) and eight peptides were anionic (negative charge), the others were 0 charge. The molecular weight (MW) of the peptide residue was less than 2 KDa for the majority of sequenced peptides and the biggest peptide with MW of 2163 Da was IFASSGESTSGGMDALFASISK. The hydrophobicity ratio (HR) of the identified peptides was from +4.72 to 30.46 Kcal × mol^{-1}. Five novel (*De novo*) peptides were detected from fraction F11 of sample H032 (Table 42).

Table 38: Sequences of peptides identified in fraction 9 of protein sample H026 obtained by Sephadex G-50 Fine with peptides properties and source

No.	Peptide sequence	Residues	Hydrophobicity (Kcal*mol^{-1})	Net Charge	M.W (Da)	Isoelectric Point	Source
1	NIHMNDNVLQTAHENGIVK	19	+23.62	-1	2147	6.48	*Arabidopsis thaliana*
2	FLPGRTTTMGESFEFAAASK	20	+18.33	0	2148	6.25	*Microplitis demolitor*
3	EEGAEMK	7	+22.57	-2	792	4.14	*Amborella trichopoda*
4	CRLQAPSGMK	10	+13.59	+2	1090	9.34	*Harpegnathos saltator*
5	GLIVMTPEIEILGSYILSAK	20	+12.00	-1	2147	4.47	*Apis mellifera*
6	AEQNIVCPPGTDLEEYQEK	19	+29.87	-4	2163	3.86	*Acromyrmex echinatior*
7	RGQVMNFTCSPLDLR	15	+13.42	+1	1737	8.22	*Apis mellifera*
8	AMAHTTLHTLNQCLNILYQ	19	+10.03	0	2185	7.38	*Acromyrmex echinatior*
9	QTTAMGHLVQTGRAFEEGSK	20	+25.21	0	2148	7.19	*Apis mellifera*

Table 39: Sequences of peptides identified in fraction 9 of protein sample H026 obtained by Sephadex G-50 Fine with peptides properties and source

No.	Peptide sequence	Residues	Hydrophobicity (Kcal*mol^{-1})	Net Charge	M.W (Da)	Isoelectric Point	Source
10	IIEFDQYVIDNVGKQINNIIQETVR	25	+22.79	-2	2962	4.42	*Apis cerana*
11	GCPSSCGAK	9	+14.52	+1	808	8.03	*Nasonia vitripennis*
13	AIAETRLSNIQNK	13	+16.83	+1	1457	8.88	*Acromyrmex echinatior*
14	SYHGIPWTSGNLLYTQIGDILRSTLR	26	+12.96	+1	2962	8.88	*Nasonia vitripennis*
15	QTLHYLDTKGQTALVMLSK	19	+17.03	+1	2147	8.69	*Apis mellifera*
16	IFASSGESTSGGMDALFASISK	22	+18.35	-1	2163	4.47	*Acromyrmex echinatior*
17	QFPSPSSGPSA	11	+10.87	0	1061	5.50	*Acromyrmex echinatior*
18	AIDHMCRK	8	+17.17	+1	973	8.22	*Solenopsis invicta*
19	YTDPCTVAGWGR	12	+13.51	0	1325	6.25	*Apis mellifera*
20	TYIIFVEQIYEK	12	+12.03	-1	1545	4.47	*Acromyrmex echinatior*

Table 40: Sequences of peptides identified in fraction 11 of protein sample H032 obtained by Sephadex G-50 Fine. Peptides properties and source

No.	Peptide sequence	Residues	Hydrophobicity (Kcal*mol^{-1})	Net Charge	M.W (Da)	Isoelectric Point	Source
1	TTACAFWNDR	10	+11.88	0	1184	6.25	*Polyrhachis vicina*
2	INLSTLRLIIDQNIYSMR	18	+9.19	+1	2163	8.88	*Nasonia vitripennis*
3	MVVIEQQNDADLENNEASK	19	+30.46	-4	2147	3.86	*Nasonia vitripennis*
4	NIHMNDNVLQTAHENGIVK	19	+23.62	-1	2147	6.48	*Apis mellifera*
5	VADKLNENAFR	11	+19.06	0	1276	6.25	*Harpegnathos saltator*
6	NIHMNDNVLQTAHENGIVK	19	+23.62	-1	2147	6.48	*Apis mellifera*
7	AEQNIVCPPGTDLEEYQEK	19	+29.87	-4	2163	3.86	*Acromyrmex echinatior*
8	LCIDSNANEK	10	+18.24	-1	1106	4.47	*Nasonia vitripennis*
9	QTTAMGHLVQTGRAFEEGSK	20	+25.21	0	2148	7.19	*Harpegnathos saltator*
10	IFASSGESTSGGMDALFASISK	22	+18.35	-1	2163	4.47	*Acromyrmex echinatior*
11	SAMTVFKSAHTLEK	14	+17.79	+1	1549	8.88	*Camponotus floridanus*
12	EVYLFERIDTAVHNDTLK	18	+24.27	-2	2163	4.89	*Harpegnathos saltator*
13	LSWERISSLAD	11	+13.15	-1	1276	4.47	*Zootermopsis nevadensis*

Table 41: Sequences of novel peptides (*De novo*) in fraction 9 of protein sample H026 obtained by Sephadex G-50 Fine

No.	Peptide sequence	Residues	Hydrophobicity (Kcal*mol^{-1})	Net Charge	M.W (Da)	Isoelectric Point	Source
1	IYMIVAR	7	+6.13	+1	865	6.20	*De novo*
2	MGIDNTSK	8	+15.26	0	864	6.25	*De novo*
3	ESNISNKR	8	+17.64	+1	947	8.88	*De novo*
4	ITANIKNHAQ	10	+14.51	+1	1109	9.06	*De novo*
5	TIYVCR	6	+7.65	+1	753	8.22	*De novo*
6	LANTMFEK	8	+12.30	0	953	6.25	*De novo*
7	APCVKR	6	+12.67	+2	672	9.34	*De novo*
8	ESSILALSK	9	+12.59	0	947	6.25	*De novo*
9	NTMPTTKGGR	10	+15.88	+2	1062	11.12	*De novo*
10	MEVTCR	6	+12.44	0	737	6.25	*De novo*
11	IALKNADSDNCMVR	14	+19.43	0	1549	6.25	*De novo*
12	TPPLALGLK	9	+9.13	+1	909	8.88	*De novo*
13	DFDVMK	6	+15.14	-1	753	4.47	*De novo*
14	TSMVELSVQR	10	+12.44	0	1149	6.25	*De novo*

Table 42: Sequences of novel peptides (*De novo*) in fraction 11 of protein sample H032 obtained by Sephadex G-50 Fine

No.	Peptide sequence	Residues	Hydrophobicity (Kcal*mol^{-1})	Net Charge	M.W (Da)	Isoelectric Point	Source
1	LLLVVPVR	8	+4.72	+1	908	10	*De novo*
2	EDMLWK	6	+13.96	-1	820	4.47	*De novo*
3	SFLKAPER	8	+14.28	+1	947	8.88	*De novo*
4	MFTSLAQK	8	+9.05	+1	925	8.88	*De novo*
5	ALSNDFSK	8	+13.65	0	880	6.25	*De novo*

7.4. Discussion

Peptide fractions from Sephadex chromatography is reported to maintain its antibacterial activity. Sephadex G-25 and G-50 were used to purify the bee venom peptides and reported that three new peptides were isolated (Gauldie *et al*., 1976). Similarly, Kemeny *et al*. (1983) reported that hyaluronidase from honeybee venom was purified using Sephadex G-50 and G-100. Recently, Zhao *et al*. (2013) identified antifungal peptide that was isolated from novel *Bacillus* subspecies in honey using Sephadex G-50 column chromatography. Haard and Simpson (2000) reported honey contained peptides with anti browning activity of 600 Da using Sephadex G-15 column chromatography.

Fewer numbers of fractions (6 to 14) were collected using Sephadex G-50 than the 45 fractions obtained from RP-HPLC, but the amount of peptides were relatively higher (0.035 to 0.937 mg/ml) (Figures 16 to 20). Two well separated peaks were obtained from samples H025 (Alseder honey), H028 (Kharoob honey) and H032 (Acacia honey) (Figures 16, 18 and 19).

The peptide content of the Sephadex-G50 fractions were in range of 0.035 to 0.937 mg/ml (Figures 21 to 25), while the antibacterial activity against *S. aureus* was between 19.52 and 92.39 % (Table 37). Generally, the fractions eluted within the first 50 min showed high antibacterial activity against *S. aureus* but with lower peptide content (Table 37 and Figures 21 to 25). In contrast, the later fractions of all samples showed higher peptide content but moderate to low antibacterial activity. However, such relationship is not always true for antimicrobial peptides. The antibacterial activity of the fractions with high peptide content could be explained as not all the peptides that present in these fractions

were able to act as antibacterial agents or some of them have antibacterial activity but low which may due to its concentration in that specific fraction. As for the fractions with high antibacterial activity and low peptides content, that may be related to the ability of the present peptides to inhibit the growth of target bacterial even at very low concentration, or the activity could be associated with other components.

The peptide fractions showing antibacterial activity were subjected to SDS-PAGE to determine their molecular weight. It is known that the low molecular weight proteins or peptide are more possible to have antibacterial or antimicrobial activity against microbes (Muhialdin *et al*., 2015). In this study the molecular weight of Sephadex G-50 fractions of sample H026 (Tualang honey) were ranged from 26 to 1 KDa and for H032 (Acacia honey) were 30 to <1 KDa and these fractions showed good antibacterial activity against *S. aureus* (Figure 26).

Most of the antimicrobial peptides are cationic peptides with 5-50 residues and have positive net charge +2 to +9 (Marshall & Arenas, 2003; Hancock & Brown, 2006). The hydrophobic ratio of 30% and above allows the cationic peptides to have high antimicrobial activity and mainly contributed by tryptophan and/or valine (McPhee & Hancock, 2005; Laverty *et al*., 2011). Recently, Brudzynsk and Sjaarda (2015) isolated glycoproteins from honey with antimicrobial activity and concluded that the antimicrobial activity of honey was correlated with the activity of glycoproteins.

Sequencing of the antibacterial fractions from sample H026 (Tualang honey) revealed 14 of the 34 peptides have positive net charge (cationic), 12 with 0 net charge and the rest have negative net charge (anionic). On the other hand, 18 peptides were detected from

sample H032 (Acacia honey), five of them carry positive net charge (cationic), while 4 with 0 net charge and the rest have negative net charge (anionic). The isoelectric point of the all sequenced peptides were in range of 4.47 to 11.12, which make those peptides stable at variable pH ranges and allow them to be used in different needs.

Based on the peptides sequencing data, the origins of the peptides in honey samples were from many sources namely, the bees, floral, trees or jungle ants. The possible relationship of the peptides sources and honey could be as follows: the bees pass their peptides to the honey as for bees peptides, the bees carry the peptides from the trees and florals to the honey as for trees and florals peptides. Peptides from jungle ants were detected in Tualang honey and acacia honey since these honey were obtained from tropical Malaysian jungles and acacia forest, respectively. The direct association of honey and the ants is that honey is used as their food.

Interestingly, 14 peptides of 33 from fraction 9 of sample H026 (Tualang honey) were not recorded in data base and therefore considered novel (*De novo*) (Table 41). Similarly, five of eighteen peptides of fraction 11 from sample H032 (Acacia honey) were considered novel (*De novo*) (Table 42). The novel peptides detected from Tualang honey were almost triple the novel peptides from Acacia honey. Seven peptides from H026 carried positive net charge (cationic), while three peptides from sample H032 were cationic (positive net charge). The isoelectric point of all these cationic peptides was 8.88 and more with molecular weight range of 672 to 1109 kDa. Considering the pH of honey is 3.3 for Tualang honey and 3.7 for Acacia honey, the peptides will be in the ionic stage. Thus, it is possible that these novel cationic peptides contribute to the antibacterial

activity of the fractions (F9 H026 and F11 H032) and honey samples in general (Tualang honey and Acacia honey).

7.5. Conclusion

The Sephadex G-50 fractions from honey showed peptides with antibacterial activity against *S. aureus*. The molecular weight of peptides was in the range of 30 to 1 kDa. The presence of cationic peptides was in both Tualang and Acacia honeys further supports the contribution of honey peptides to the antibacterial properties.