



Honeybee proteins in Saudi honeys: A shotgun gel-free proteomic study

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ABSTRACT

This study investigated the protein content of Acacia and Ziziphus honey samples from the southwestern region of Saudi Arabia following the shotgun gel-free proteomics. Honey proteins were extracted, digested by trypsin and the trypsin digests were separated and characterized using the LC-ESI-QTOF-MS (SCIEX X500R QTOF). The precursor masses of the trypsin digests were used to identify the proteins through searching the mascot spectral database search engine. Nine protein classes originated from honeybees were identified as follows: 1) Gene expression regulatory proteins, 2) Enzymes, 3) Bee venom proteins, 4) Major Royal Jelly Proteins (MRJP), 5) Immune proteins, 6) Structural proteins, 7) Neuropeptides, 8) Vision protein and 9) Olfactory proteins. This study reported, for the first time, the presence of sixteen honeybee proteins in Acacia and Ziziphus honey samples from the southwestern region of Saudi Arabia. Moreover, this study reported that the honey proteomics can predict the honeybee origin of honey samples.

1. Introduction

Honey is well known to contain proteins with major contributions to the honey quality and biological activities. Honey proteins are originated from three sources; honeybees, nectar or pollens and microbes (fungi and bacteria). Honey proteins originated from honeybees are classified according to their function to variable types including enzymes, major royal jelly, bee venom, regulatory, structural, energy metabolism and immune proteins (Alshareef et al., 2022; Bong et al., 2021; Erban et al., 2019).

Examples of honey enzymes include the diastase, invertase, glucose oxidase, catalase, alpha glucosidase and superoxide dismutase (Alshareef et al., 2022; Bong et al., 2021; Erban et al., 2019). Honey enzymes are responsible for some of the biological activities of honey such as the production of gluconic acid by the glucose oxidase. Gluconic acid is the dominant organic acid in honey and is responsible for the low pH of honey which qualifies the honey to act as antimicrobial. Honeys with high glucose oxidase activity and low catalase activity are

characterized by containing high concentration of hydrogen peroxide which is known by its effectiveness in the treatment of wounds and burns (Alshareef et al., 2022; Brudzynski, 2020). Moreover, enzymes are used as quality parameters to indicate the freshness or storage conditions of honey and to reveal temperature processed honeys. Examples of enzymes used as quality standard for honey include the diastase and invertase. However, invertase is not a global quality parameters for honey as it is adopted only by three European countries, Germany, Belgium and Spain (Bogdanov et al., 1999; Codex Alimentarius, 2022).

Royal jelly proteins are components of the royal jelly that is secreted by the worker bees to feed the larvae and it plays a role the development of queen larvae (Buttstedt et al., 2014). Royal jelly proteins are divided to two types according to their concentration in the royal jelly, the major royal jelly proteins (MRJP) and minor royal jelly proteins. The MRJP are classified to nine classes named as MRJP-1, MRJP-2, ..., MRJP-9. The MRJPs are essential for the growth and development of the honeybee queens and they are of high nutritional value (Botezan et al., 2023). Minor royal jelly proteins are short peptides with antioxidant,

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anticancer, anti-inflammatory and antimicrobial activities such as the apisimin, jelleines (I, II, III, IV) and royalisin (Defensin) (Botezan et al., 2023).

Bee venom (Apitoxin) is produced by bee workers as a defense mechanism against enemies. The bee venom is composed of proteins (mellitin, apamin, secapin and mast cell de-granulating peptide), enzymes (phospholipase A2 and hyaluronidase) and amino acids. Bee venom proteins are famous by their anti-rheumatoid arthritis, anti-cancer and antiviral activities beside their allergic action (Wehbe et al., 2019).

Gene expression of honeybees is regulated by 114 proteins such as elongations factors and homeobox proteins. The regulatory proteins are responsible for the regulation of tissue differentiation and embryogenesis such as the development of the honeybee visual system (Fu et al., 2021).

Structural proteins are those who are found in the structure of cells such as the plasma membrane (Apidermin 2), chromosomes (Histone proteins) and ribosomes (nucleoproteins) (Bong et al., 2021; Erban et al., 2019). Energy metabolism proteins are mostly enzymes involved in the production of energy such as the ATP synthase and cytochrome oxidase (Bong et al., 2021; Erban et al., 2019). Immune proteins of the honeybees are those who are responsible for fighting the invading microorganisms including the defensin, hymenopteacin, apideacin and abaecin (Bong et al., 2021; Erban et al., 2019).

Presence of proteins in honey from plant source is mentioned by some studies. Bong et al. (2021) reported presence of 17 proteins (associated with drought and pathogenesis) from the *Leptospermum scoparium* plant in the famous New Zealand Manuka honey. Mureşan et al. (2022^a) studied the protein content of European honey samples and found that the honey proteins are originated from the honeybees and plants.

The honey protein profile is impacted by the floral origin, honeybee associated factors such as the honeybee species, health and nutrition, geographical or climate condition, honey processing, storage conditions and the techniques used for protein extraction, purification, separation and identification (Bocian et al., 2019; Mureşan et al., 2022^a; Hossain et al., 2023).

Expression of honeybee protein genes in is suggested to be used as markers for honeybee diseases such as the relish defensin genes which are recommended to be used as indicator for deformed wing virus (DWV) and varroa mite. Furthermore, diagnosis of honeybee diseases through measurement of proteins originated from the microbes and the honeybees is proved to be effective by some research articles (Barroso-Arévalo et al., 2019; Cuc et al., 2021; Huang et al., 2021; Revainera et al., 2020).

This study was designed to investigate the protein profile of Acacia and Ziziphus honey samples from the southwestern regions (Asir and Jazan) of Saudi Arabia using the shotgun gel-free analysis of proteomics. However, proteomic analysis using mass spectrometry is divided to types, top down and bottom up protein studies. Top-down proteomic studies the intact proteins while the bottom-up proteomic studies the proteins after digesting them with protease enzymes (Catherman et al., 2014; Jiang et al., 2024). Bottom-up proteomic is famous as shotgun proteomics which is further divided to gel-free or gel-based (Ercan et al., 2023).

2. Material and methods

2.1. Honey samples

Fourteen honey samples were collected from the apiaries of the Honeybee and its products research center at King Khalid University. The apiaries were located in Asir and Jazan regions at the southwestern region of Saudi Arabia. The honey samples were Acacia (7 from Asir Apiary and 3 from Jazan Apiary) and Ziziphus (2 from Asir Apiary and 2 from Jazan Apiary).

Asir region is located at 41–45°E longitude and 17–21°N latitude in the southwestern part of Saudi Arabia. The altitude of Asir region increases gradually from the sea level up to 3000 m at Al-souda Mountains. The mountains of Asir region has a foggy climate which leads to the dominance of coniferous trees and dense forests. The temperature in this region is low (12–35 °C) and the rainfall is more (300 mm mean amount annually) compared to the other regions of Saudi Arabia (DeNicola et al., 2015).

Jazan is a southwestern region of Saudi Arabia located 42° 33' 4" E longitude and 16° 53' 21" N latitude. It is bordered by Asir region on the North and the Red Sea on the West. The Jazan region is the richest agricultural part of Saudi Arabia because it contains different environments such as the agricultural lands, mountains, valleys, deserts and semi deserts. It has a 300 km red sea coast and over 100 islands. The Jazan region is well known by its hot desert climate with an average temperature of 30 °C and a rainfall range between 70 and 270 mm annually (DeNicola et al., 2015; Mahmoud et al., 2014).

2.2. Authentication of the honey samples

The floral origin of the honey samples was confirmed microscopically through determining the pollens percentage according to the method of Louveaux et al. (1978).

To confirm that the honey samples comply to the international and local honey standards, some quality parameters were evaluated including the moisture, electrical conductivity (EC), acidity, sugars (sum of fructose and glucose and sucrose) and hydroxymethylfurfural (HMF). The quality parameters were analyzed following the harmonized methods of the international honey commission (IHC) (International Honey Commission, 2009).

2.3. Total protein concentration

Bradford assay was followed to determine the concentration of proteins in the studied honey samples (Azeredo et al., 2003). Honey samples were 50 % diluted (1 g/2 mL) using distilled water. 100 µL of each honey sample was reacted with 5 mL of Coomassie brilliant blue and the absorbance was measured at 595 nm. In order to determine the protein concentration, a calibration curve was created serially using albumin as standard in the range of (0–800 µg/mL).

2.4. Protein identification

The investigation of the honey proteins was divided to five steps:

- 1- Extraction of proteins
- 2- Denaturation and alkylation of the extracted honey proteins
- 3- Trypsin digestion of the denatured proteins
- 4- LC-ESI-QTOF-MS analysis
- 5- Database search

2.4.1. Extraction of the honey proteins with trichloroacetic acid (TCA)

1 g of each honey samples was dissolved to reach a volume of 5 mL (20 %) using LC-MS grade water. The diluted honey samples were centrifuged at 13000 rpm for 30 min for the purpose of removing the pollens. 20 % TCA was added to the supernatant in the ratio of 1:1 (volume) and centrifuged at 13000 rpm for 30 min at 4 °C. The supernatant was discarded carefully and the pellet was washed in cold acetone. The washed protein pellets were dissolved in urea solution and kept at –20 °C. The urea solution was prepared by dissolving urea and thiourea in 100 mM Tris-HCl buffer to obtain 7 M urea and 2 M thiourea and the pH was adjusted to 8 (Bong et al., 2021).

2.4.2. Denaturation and alkylation of the extracted honey proteins

10 µL of each honey protein extract were diluted to one mL using 10 mM Tris (2-carboxyethyl) phosphine (TCEP) dissolved in 10 mM ammonium bicarbonate as denaturing or reducing solution. The

denatured protein samples were heated in a water bath for one hour at 56 °C. After heating for one hour, 25 µL of 1 M iodoacetamide were added as alkylating agent to block the reactive groups such as the thiol of the cysteine. The mixture of the protein iodoacetamide was kept at room temperature in dark for 30 min. The iodoacetamide reaction was terminated by adding 10 µL of 1 M Dithiothreitol (DTT). The pH of the denatured and alkylated protein samples was adjusted to 8.0 using pH strip papers by adding 25 µL from the ammonium bicarbonate solution so as to make the samples ready for trypsin digestion (Bong et al., 2021).

2.4.3. Trypsin digestion of the denatured honey proteins

A stock solution of Trypsin was prepared by dissolving 0.1 g of the enzyme in 100 mL of 50 mM ammonium bicarbonate solution to have a concentration of 100 mg/100 mL (1 mg/mL or 1 µg/µL). 50 µL of the enzyme solution was added to each denatured protein sample (1:27) and incubated at 37 °C for 12 h. The trypsin reaction was stopped by adding 50 µL of formic acid (10 %) making the digested protein sample ready for injection in the LC-QTOF-ESI-MS system (SCIEX500X-QTOF-SCIEX-USA) (Bong et al., 2021).

2.4.4. LC-ESI-QTOF-MS analysis

The chemical composition of the honey samples was scanned using the LC-MS. The separation of the components was carried out using the HPLC Reverse phase elution (Waters Symmetry LC18 column 250 × 4.6 mm, 5 µm) and the masses were identified by the usage of SCIEX X500R Series Accurate-Mass Quadrupole Time-of Flight (Q-TOF; USA). The Mass Hunter software of Agilent technologies was used to analyse the mass so as to identify the molecular formula. The specifications of the LC-MS system with Agilent 1200 Series Diode Array Detector are (module G1315B; detection type: 1024-element photodiode array; light source: deuterium and tungsten lamps; wavelength range 190–950 nm). The mobile phase was gradient composed of (A) formic acid (0.1 %, v/v); (B) acetonitrile +0.1 % formic acid; gradient (in solvent B): (i) 20 %, from 0 to 20 min, (ii) 95 %, from 20 to 27 min, and (iii) 35 %, at 27–30 min of total run time; flow rate was 0.2 mL/min; and injection volume was 3 L. The ESI parameters were both negative and positive ion modes, mass range 100–1200 m/z, spray voltage 4 kV, gas temperature 325 °C, gas flow 10 L/min, and Nebulizer was 40 psi. According to the manufacturer instructions, tuning and optimization are carried out before any run and on each single day (Alarjani et al., 2021).

2.4.5. Database search

The precursor masses of the trypsin digests were used to carry out search in the Mascot search engine available at (<https://www.matrixscience.com/>). The search was conducted in the *Apis mellifera* proteome of the SWISSPROT. The steps of searching the mascot are presented in (Supplement. 1).

2.5. Statistical analysis

The results of the proteins were analyzed using the independent *t*-test of the statistical package for social sciences (SPSS) version 20.

3. Results and discussion

3.1. Results of the honey samples authentication

The microscopic analysis showed that the honey samples were unifloral Acacia and Ziziphus honey samples with more than 50 % dominants of Acacia and Ziziphus pollens. The honey samples complied with the honey standards of the Codex Alimentarius and the Saudi Food and Drug Authority (SFDA) since the quality parameters values were within their ranges in the two mentioned honey standards (Codex Alimentarius, 2022; Saudi Food and Drug Authority, 2021) (Table.1).

All the studied honey samples complied with the honey standards of Codex Alimentarius and the SFDA.

Table 1

The mean and standard deviation of the studied honey quality parameters.

Quality parameter	Honey samples (14)				Value in standards	
	Acacia (10)		Ziziphus (4)		Codex	SFDA
	Asir (7)	Jazan (3)	Asir (2)	Jazan (2)		
Moisture (%) [*]	16.07 ± 1.96	14.77 ± 0.45	16.25 ± 2.47	17.05 ± 0.07	Not more than 20 %	Not more than 23 %
EC (µS/cm) [*]	652.86 ± 331.09	393.33 ± 240.07	422.50 ± 548.01	595.00 ± 304.06	Not more than 800	Not less than 800
Acidity (meq NaOH/kg) [*]	18.57 ± 6.27	18.33 ± 2.89	32.50 ± 24.75	22.50 ± 17.68	Not more than 50	Not more than 80
Fructose + glucose (%) ^{**}	64.67 ± 4.77	73.40 ± 0.16	63.19 ± 9.54	64.97 ± 2.43	Not less than 60 %	Not less than 45 %
Sucrose % [*]	7.10 ± 2.20	2.86 ± 0.66	5.17 ± 0.00	2.58 ± 0.03	Not more than 5 %	not more than 10 %
HMF (mg/kg) ^{***}	1.53 ± 3.74	8.83 ± 6.83	2.50 ± 0.71	0.65 ± 0.92	Not more than 15 %	Not more than 40

^{*} The parameter depends on the floral origin.

^{**} The sugar concentration depends on the honey type (nectar or honeydew honey).

^{***} The HMF concentration depends on the climate condition is it hot or not.

3.2. Total proteins concentration

The equation of the calibration curve of the albumin was ($y = 0.0011x + 0.0205$) and the coefficient of determination (R^2) was 0.9805.

The mean ± SD, minimum and maximum values of the total protein concentration in the Acacia and Ziziphus honey samples of Asir region were ($667.17 ± 210.36$, 416.0, 1172.0 µg/g) and ($610.67 ± 3.06$, 608.0, 614.0 µg/g), respectively. The protein results of the Acacia and Ziziphus honey samples of Jazan region were ($642.06 ± 186.39$, 345.0, 991.0 µg/g) and ($737.5 ± 280.72$, 539.0, 936.0 µg/g), respectively.

The geographical region significantly affected the total protein concentration of Acacia honey (p -value = 0.044) while the floral origin in Asir and Jazan honey samples had insignificant effect on the total protein concentration.

The ranges of total proteins in Asir and Jazan honey samples irrespective of their floral origins were (416.20–1260.0 µg/g) and (345.0–991.0 µg/g), respectively. The protein content range of the studied honey samples of this study (345.0–1260.0 µg/g) was wider compared to the range reported by Alarjani et al. (2021) (222.54–560.5 µg/g). Azeredo et al. (2003) measured the protein concentration in honey samples of different botanical origin and reported high concentration of proteins in two of their honey samples (2236 and 2212 µg/g).

3.3. The identified proteins in the studied honey samples

This study identified twenty nine proteins in the honey samples from Asir region and twenty four proteins in the Jazan honey samples. The identified proteins were classified to nine groups as follows: 1) Gene expression regulatory proteins, 2) Enzymes, 3) Bee venom proteins, 4) Major Royal Jelly Proteins (MRJP), 5) Immune proteins, 6) Structural proteins, 7) Neuropeptides, 8) Vision protein and 9) Olfactory proteins [Fig. 1]. The olfactory proteins are associated with the Jazan honey samples (Table.2 and Table.3) [Fig. 1].

With regard to the significant score percentage of the identified proteins through their trypsin digest, Chua et al. (2013) stated that the protein score of more than 30 % indicates 95 % confidence of protein identification.

3.3.1. Gene expression regulatory proteins

Seven of the identified proteins were gene expression regulatory proteins. The gene expression involves three major processes; the DNA synthesis (Replication), RNA synthesis (Transcription) and protein synthesis (Translation).

3.3.1.1. Protein Krueppel. The Krueppel protein is a juvenile hormone responsible for the regulation of metamorphosis in insects through the regulation of the gene expression of the metamorphosis related proteins. Metamorphosis is the transformation from bee animal stages egg, larva, pupa and adult (Sun et al., 2022).

The Protein krueppel trypsin digests were FTR and HLR corresponding to the precursor masses of 422.6805, 423.0648, 423.0652, 423.0653, 423.0658, 423.0703, 424.0670, 424.0682, 424.0689, 424.0690, 424.0705, 424.0773, 424.1072, 424.1310, 424.6800, 424.6806, 424.6810, 425.0380, 425.0511, 425.0591, 425.0602, 425.0622, 425.0623, 425.0636, 425.0642, 425.1317, 425.1381, 425.1678, 425.1713, 425.3614, 426.0618, 426.0619, 426.0640, 426.0658, 426.0663, 426.1401, 426.1707 and 426.1768. The krueppel protein contains 74 amino acids and has the pI of (9.08). The protein

Krueppel was detected in some honey samples from Asir region (4; 2 Acacia and 2 Ziziphus) and Jazan region (one Ziziphus). The Score was 30 % which indicates 95 % confidence of protein identification (Chua et al., 2013) (Table.2 and Table.3). It is the first time to report presence of protein Krueppel in honey samples.

3.3.1.2. Elongation factor 1- alpha. The elongation factor 1- alpha is needed for the elongation step of the protein synthesis (Translation).

The precursor masses of the trypsin digest of the elongation factor 1-alpha were 417.0996, 417.2635, 417.3019, 417.3354, 417.3716, 417.8770, 417.9465, 418.1044, 418.1057, 418.2666, 418.2684, 418.3046, 418.3066, 418.3523, 418.3724, 418.8348, 418.8808, 418.8815, 419.0382, 419.1163, 419.1358, 419.2587, 419.2652, 419.2742, 419.2820, 419.2822, 419.2923, 419.3051, 419.3056, 419.3109, 419.3169, 419.3527, 419.3554, 419.3628, 419.3705, 419.3866, 419.3922, 419.3928, 420.0415, 420.0542, 420.0545, 420.1154, 420.1559, 420.1602, 420.2645, 420.2833, 420.2844, 420.2844, 420.3011, 420.3042, 420.3058, 420.3114, 420.3151, 420.3168, 420.3194, 420.3491, 420.3608, 420.7997, 420.7892, 420.8073, 420.8856, 420.8857, 420.8866, 420.8931, 421.0518, 421.0531, 421.2594, 421.2600, 421.2629, 421.2754, 421.2789, 421.2913, 421.2946, 421.2947, 421.3027, 421.3272, 421.3285, 421.3296, 421.3300, 422.3355, 421.3589, 421.8874, 421.8912, 422.1734, 422.1740, 422.2097, 422.2618, 422.2627, 422.3258, 422.3365, 422.3442, 422.3531, 422.7734, 422.8833, 422.8847, 423.1339, 423.2773, 423.3874, 423.3497, 423.7482, 423.8936, 424.0925, 424.1347, 424.2062, 489.1470, 489.1500, 489.2210, 489.3168, 489.3182, 489.3375, 489.3446, 489.8295, 490.1833, 490.1840, 490.1841, 490.2271, 490.2703, 490.2876, 490.3183, 490.3216, 490.3234, 490.6640, 490.8741, 491.1388, 491.1843, 491.1847, 491.1855, 491.1868, 491.2304, 491.2317, 491.2894, 491.8741, 492.1865, 492.1936, 492.2218, 492.4602, 492.4630, 492.4639, 492.6506, 492.6528, 492.9203, 493.2444, 493.2842, 493.4600. The trypsin digest sequences corresponding to the precursor masses were FEK, DMR, ELR, AAEK, TIEK, FAVR, TIEKFEK, and FAVRDMR. The protein contains 461 amino acids with the pI of 9.16 and the highest score was 40 %. The elongation factor 1- alpha was detected in six honey samples from Asir region (one Ziziphus and five Acacia) and two from Jazan region (one Acacia and one Ziziphus) (Table.2 and Table.3).

Presence of protein elongation factors including the elongation factor-1 α in Manuka and other honeys was reported by Bong et al. (2021) and Erban et al. (2019).

3.3.1.3. Prohormone-3. Prohormone-3 of the *Apis mellifera* is a

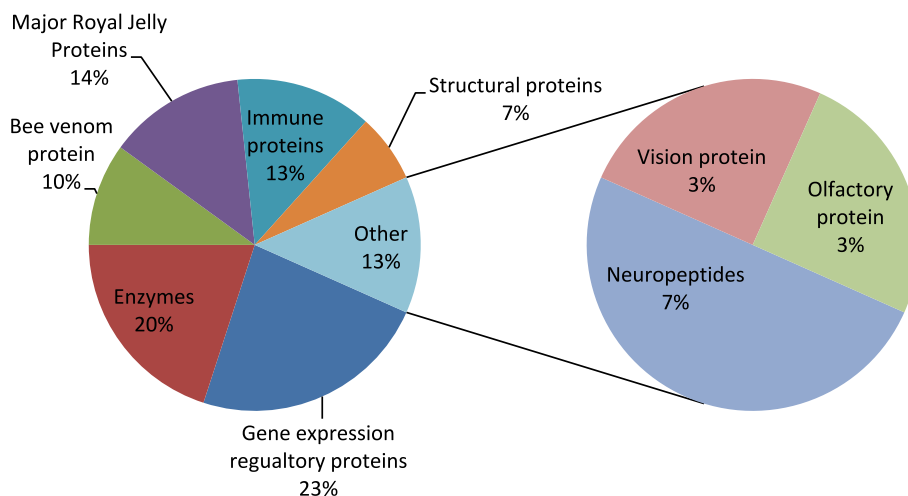


Fig. 1. The classes of the identified honey proteins.

Table 2

The trypsin digest sequences and their precursor proteins from the honey samples from Asir region.

SN	Precursor mass	Searched masses	Trypsin digest sequences	Score %	Honeybee species	Protein name	Proteome ID	pI	Amino acids number	Protein class	Protein function	Honey Sample
1	362.2255 362.9272 362.0763 362.0197 363.2861 364.0791 360.1167 361.1298 361.1539 362.0992 362.1280 362.1579 363.0789 363.9028	14	CIK ADR	24	<i>Apis mellifera</i>	PBAN-type neuropeptide	PBAN_APIME	7.66	195 AA	Neuro- peptide	Muscle contraction	Ziziphus W3 Asir
2	221.9377 222.1146 222.9752 223.0660 223.2066 224.0539 224.0993	7	FG	16	<i>Apis mellifera carnica</i>	Defensin-1	DEF1_APICA	6.28	95 AA	Immune protein	Antibacterial	Ziziphus W3 Asir
3	221.9377 222.1146 222.9752 223.0660 223.2066 224.0539 224.0993	7	FG	14	<i>Apis mellifera</i>	Orcokinin peptides OS	ORCK1_APIME	5.63	147 AA	Neuro-peptide	Hind gut contraction	Ziziphus W3 Asir
4	278.1412 278.8942 279.1946	3	CR	20	<i>Apis mellifera</i>	Secapin-1	SECP1_APIME	10.06	25 AA	Bee venom protein	Antimicrobial	Ziziphus W3 Asir
5	264.1663 264.4832 264.8527 264.9294 265.1067 265.1409 265.1802	7	MN	12	<i>Apis florea</i>	Cytochrome c oxidase subunit	COX2_APIFL	6.19	225 AA	Enzyme	Energy production (Respiratory chain)	Ziziphus W3 Asir
6	439.1274 439.1468 440.1403 440.1554 441.1470 442.1398 439.1089 439.1451 440.1416 440.8052 441.1429 441.3003	12	MYKK	24	<i>Apis mellifera</i>	Waprin-Thr1	WAP_APIME	8.89	110 AA	Bee venom	Antimicrobial	Ziziphus W3 Asir Acacia W5 Asir
7	439.1274 439.1468 440.1403 440.1554 442.1398	5	EYK GPGGR	26	<i>Apis mellifera</i>	Hymenoptaecin	HYTA_APIME	9.44	129 AA	Immune protein	Antibacterial	Ziziphus W3 Asir
8	332.1026 333.0041 333.1009 333.3012 334.1032 335.1298 336.1277 336.1429	8	CGR KGK	28	<i>Apis mellifera</i>	Omega-conotoxin-like protein 1	OCLP1_APIME	8.76	74 AA	Immune protein	Antibacterial Ion channel inhibitor	Ziziphus W3 Asir
9	332.1026 333.0041 333.1009 333.3012 334.1032 335.1298 336.1277 336.1429 476.2425	14	CGR GTR RR HPPK EGDR	47	<i>Apis mellifera</i>	Arginine kinase	KARG_APIME	5.66	355 AA	Enzyme	Energy metabolism	Ziziphus W3 Asir

(continued on next page)

Table 2 (continued)

SN	Precursor mass	Searched masses	Trypsin digest sequences	Score %	Honeybee species	Protein name	Proteome ID	pI	Amino acids number	Protein class	Protein function	Honey Sample
10	423.0653	12	FTR	30	<i>Apis mellifera</i>	Protein krueppel	KRUP_APIME	9.08	74 AA	gene expression regulation	Suppression of metamorphosis (transcription)	Ziziphus W3 and W4 Asir
	423.0703		HLR									
	425.0602											
	426.0619											
	424.0773											
	425.1678											
	426.1707											
	423.0652											
	424.0705											
	424.6806											
	425.0623											
	426.0618											
11	332.1026	8	KGK	23	<i>Apis mellifera</i>	Major royal jelly protein 3 (MRJP 3)	MRJP3_APIME	6.47	544 AA	Royal Jelly Protein	Nutritional wide medicinal value	Ziziphus W3 Asir
	333.0041		TSK									
	333.1009		CGR									
	333.3012		CGR	28	<i>Apis mellifera</i>	Omega-conotoxin-like protein 1	OCLP1_APIME	8.76	74 AA	Immune protein	Antibacterial Ion channel inhibitor	
	334.1032		KGK									
	335.1298											
	336.1277											
	336.1429											
12	430.0829	22	GISR	22	<i>Apis mellifera</i>	Small ribosomal subunit protein eS8	RS8_APIME	10.58	208 AA	Structural Ribosomal protein	Protein synthesis	Ziziphus W4 Asir And Acacia W5 Asir
	430.1857		ATGGK									
	430.1994		RGAK									
	430.9164											
	431.0811											
	431.1891											
	431.9212											
	432.0842											
	432.1988											
	432.8907											
	433.2562											
	434.1157											
	430.0838											
	430.1432											
	430.8892											
	430.9145											
	431.1634											
	431.9173											
	432.0849											
	432.4167											
	432.9240											
	434.0375											
13	431.1634	6	TRR	20	<i>Apis mellifera</i>	Homeobox protein E60	HME60_APIME	10.75	109 AA	Gene regulatory proteins	Regulation of transcription	Ziziphus W4 Asir
	431.9173		SDGR									
	432.0849											
	432.4167											
	432.9240											
	434.0375											
14	403.2296	15	MTR	29	<i>Apis mellifera</i>	Major royal jelly protein 2	MRJP2_APIME	6.83	452 AA	Royal Jelly Protein	Nutritional and wide range of medicinal activity	Ziziphus W4 Asir
	403.2544		AVSK									
	404.1901		MQK									
	404.8214											
	405.9289											
	406.1987											
	406.7847											
	407.1254											
	404.3647											
	405.3594											
	405.4136											
	406.3288											
	406.4182											
	407.3383											
	408.3366											
15	427.1684	3	VIVP	16	<i>Apis mellifera</i>	Secapin-1	SECP1_APIME	10.06	25 AA	Bee venom protein	Antimicrobial, Anti-fibrinolytic and Anti-elastolytic	Ziziphus W4 Asir
	427.1901											
	428.1793											

(continued on next page)

Table 2 (continued)

SN	Precursor mass	Searched masses	Trypsin digest sequences	Score %	Honeybee species	Protein name	Proteome ID	pI	Amino acids number	Protein class	Protein function	Honey Sample
16	228.2331 229.0495 229.1087 229.1415 229.2165 230.0972 231.0667 231.0848 231.1213 232.1290	10	GR IP	30	<i>Apis cerana</i>	Secapin-1	SECP_APICE	8.8	115 AA	Bee venom protein	Antimicrobial, Anti-fibrinolytic and Anti-elasteolytic	Ziziphus W4 Asir
17	420.2645 420.3042 420.3168 421.2947 421.3300 422.2627 422.3258 422.3531 423.3874	9	FEK DMR YIK MDR MNR	19 19	<i>Apis mellifera</i> <i>Apis mellifera</i>	Elongation factor 1-alpha Major royal jelly protein 5	EF1A_APIME MRJP5_APIME	9.16 5.95	461 AA 598 AA	Gene regulatory proteins Royal Jelly Protein	Regulation of translation Nutritional and wide range of medicinal activity	Ziziphus W4 Asir
18	404.3647 405.3594 405.4136 406.3288 406.4182 407.3383 408.3366	7	HPR IFK APFA	34	<i>Apis mellifera</i>	Prohormone-3	PROH3_APIME	7.47	314 AA	Enzyme and regulatory protein	Wide range of functions such as antioxidant and antimicrobial	Ziziphus W4 Asir
19	420.2645 420.3042 420.3168 421.2947 421.3300 422.2627 422.3258 422.3531 423.3874 417.2635 417.3354 417.3716 418.2684 418.3066 418.3724 419.2587 419.2742 419.3109 419.3527 419.3628 419.3922 420.2833 420.3114 421.2600 421.2946 421.3272	26	FEK ELR DMR AAEK	34	<i>Apis mellifera</i>	Elongation factor 1-alpha	EF1A_APIME	9.16	461 AA	Gene regulatory proteins	Regulation of translation	Ziziphus W4 Asir And Acacia W5
20	424.1072 424.1310 425.1317 425.1713 426.1401 426.1768 423.0648 424.0682 425.0511 425.0642 425.3614 426.0663	12	FTR HLR	30	<i>Apis malifera</i>	Protein krueppel (Fragment)	KRUP_APIME	9.08	74 AA	Transcription factor	Suppression of metamorphosis	Acacia W5 Asir
21	426.1401 426.1768 427.1607 428.1811	4	VIVP	17	<i>Apis mellifera</i>	Secapin-1	SECP1_APIME	8.8	25 AA	Bee venom protein	Antimicrobial, Anti-fibrinolytic and Anti-elasteolytic	Acacia W5 Asir
22	439.1089 439.1451 439.8704 440.1416 440.8052	7	HRK	16	<i>Apis mellifera</i>	Histone 4	H4_APIME	11.05	103 AA	Structural protein in DNA (nucleosome)	Regulation of gene expression (replication, transcription and translation)	Acacia W5 Asir

(continued on next page)

Table 2 (continued)

SN	Precursor mass	Searched masses	Trypsin digest sequences	Score %	Honeybee species	Protein name	Proteome ID	pI	Amino acids number	Protein class	Protein function	Honey Sample
23	441.1429	17	ELR DMR AAEK	26	<i>Apis mellifera</i>	Elongation factor 1-alpha	EF1A_APIME	9.16	461 AA	Gene regulatory proteins	Regulation of translation	Acacia W5 Asir
	441.3003											
	417.2635											
	417.3354											
	417.3716											
	418.2684											
	418.3066											
	418.3724											
	419.2587											
	419.2742											
	419.3109											
	419.3527											
	419.3628											
	419.3922											
	420.2833											
420.3114												
421.2600												
421.2946												
421.3272												
24	458.2983	8	TEGR SKPK	26	<i>Apis mellifera</i>	Phospholipase A2	PA2_APIME	7.05	167 AA	Bee venom Enzyme	Hydrolysis of phospho-glycerides in biological membranes	Acacia W5 Asir
	458.3664											
	459.3132											
	459.3359											
	459.3566											
25	460.3111	19	FEK DMR AAEK	24	<i>Apis mellifera</i>	Elongation factor 1-alpha	EF1A_APIME	9.16	461 AA	Gene regulatory proteins	Regulation of translation	Acacia W5 Asir
	460.3458											
	461.3344											
	418.3724											
	419.2652											
	419.2820											
	419.3051											
	419.3169											
	419.3928											
	420.2844											
	420.3608											
	421.2629											
	421.2789											
	422.2618											
	422.3442											
417.9465												
418.3046												
418.8808												
420.3194												
420.8931												
421.3296												
421.3589												
26	488.1644	9	TLAGK MNVK	21	<i>Apis mellifera</i>	Opsin, blue-sensitive	OPSB_APIME	8.53	377 AA	Vision protein	light absorbing proteins	Acacia W6 Asir
	488.8731											
	488.9022											
	489.0431											
	489.1615											
	489.8775											
	490.8693											
	491.1555											
	492.1531											
	492.1531											
27	264.1589	3	MN	12	<i>Apis florea</i>	Cytochrome c oxidase subunit 2	COX2_APIFL	6.19	225 AA	Enzyme	Energy production (respiratory chain)	Acacia W6 Asir
	264.9301											
	265.1392											
28	403.2346	4	R AR RTK	22	<i>Apis mellifera</i>	Homeobox protein H40 (Fragment)	EMS_APIME	10.93	74 AA	Gene regulatory proteins	Regulation of transcription	Acacia W6 Asir
	404.1891											
	404.2096											
	405.1889											
	405.1889											
29	360.9255	9	CIK ADR	24	<i>Apis mellifera</i>	PBAN-type neuropeptides	PBAN_APIME	7.66	195 AA	Neuro- peptide	Muscle contraction	Acacia W6 Asir
	361.1105											
	361.1258											
	361.1547											
	362.1299											
	362.1583											
	363.0963											
	363.1793											
	363.8929											
	363.8929											
30	430.2450	7	GISR ATGGK RGAK	22	<i>Apis mellifera</i>	Small ribosomal subunit protein eS8	RS8_APIME	10.58	208 AA	Structural Ribosomal protein	Protein synthesis	Acacia W6 Asir
	430.9152											
	431.2504											

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Table 2 (continued)

SN	Precursor mass	Searched masses	Trypsin digest sequences	Score %	Honeybee species	Protein name	Proteome ID	pI	Amino acids number	Protein class	Protein function	Honey Sample
31	432.2573	21	TIEK FAVRDMR AAEK	32	<i>Apis mellifera</i>	Elongation factor 1-alpha	EF1A_APIME	9.16	461 AA	Gene regulatory proteins	Regulation of translation	Acacia W6 Asir
	432.2808											
	432.8876											
	433.2838											
	489.1500											
	490.1841											
	490.2703											
	490.3234											
	490.6640											
	491.1855											
	491.2317											
	492.1865											
	492.9203											
	493.2444											
	418.2666											
	418.3523											
	419.2822											
419.3056												
419.3554												
419.3705												
420.3058												
420.3491												
421.2594												
421.2754												
421.3027												
32	696.2591	4	NGDYTK LNNIPK	19	<i>Apis mellifera ligustica</i>	Cystathionine beta-synthase OS	CBS_APIME	6.53	504 AA	Enzyme	synthesis of L-Cysteine	Acacia W7 Asir
	696.2826											
	697.2764											
	698.2802											
33	222.1154	7	FG	16	<i>Apis mellifera carnica</i>	Defensin-1	DEFI_APICA	6.28	95 AA	Immune protein	Antibacterial	Acacia W7 Asir
	223.0973											
	223.1150											
	223.1305											
	223.1700											
34	224.0649	10	GRIP	14	<i>Apis mellifera</i>	Orcokinin peptides	ORCK1_APIME	5.63	147 AA	Neuro-peptide	Hind gut contraction	Acacia W7 Asir
	224.1275											
	228.0535											
	228.2320											
	229.0496											
	229.1425											
	229.2159											
	230.0986											
	231.0815											
	231.1002											
231.1250												
232.1492												
35	459.2948	7	TEGR SKPK	26	<i>Apis mellifera</i>	Phospholipase A2	PA2_APIME	7.05	167 AA	Bee venom Enzyme	Hydrolysis of phospho-glycerides in biological membranes	Acacia W7 Asir
	459.3070											
	461.1764											
	461.2681											
	462.1483											
36	462.2240	17	CIK ADR	29	<i>Apis dorsata</i>	PA2_APIDO	7.11	134 AA	Neuro- peptide	Muscle contraction	Acacia W7 Asir	
	463.0476											
	360.1062											
	361.0696											
	361.1283											
361.1546												
362.1549												
363.0771												
363.8848												
360.2537												
360.3609												
361.0929												
361.2782												
361.2925												
361.3300												
361.3858												
362.3059												
362.3428												
363.2884												
37	360.2537	10	ALR FAK SKK	20	<i>Apis mellifera</i>	Small ribosomal subunit protein eS8	RS8_APIME	10.58	208 AA	Structural Ribosomal protein	Protein synthesis	Acacia W7 Asir
	360.3609											
	361.0929											
	361.2782											

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Table 2 (continued)

SN	Precursor mass	Searched masses	Trypsin digest sequences	Score %	Honeybee species	Protein name	Proteome ID	pI	Amino acids number	Protein class	Protein function	Honey Sample
38	361.2925 361.3300 361.3858 362.3059 362.3428 363.2884 377.2319 377.2911 377.3277 378.2924 378.3310 378.3546 379.3418 380.3389	8	MTK NSR	19	<i>Apis mellifera</i>	Major royal jelly protein 4	MRJP4_APIME	5.89	464 AA	Royal Jelly Protein	Nutritional and wide range of medicinal activity	Acacia W7 Asir
39	430.0836 430.8666 430.9143 431.0795 431.9170 432.0836 432.6075 432.9252 434.1165	9	GISR ATGGK RGAK	22	<i>Apis mellifera</i>	Small ribosomal subunit protein eS8	RS8_APIME	10.58	208 AA	Structural Ribosomal protein	Protein synthesis	Acacia W8 Asir
40	228.2318 229.0497 229.1432 229.1517 230.0527 230.1435 231.0780 231.0967 231.1452 232.1300	10	GRIP	30	<i>Apis cerana</i>	Secapin-1	SECP_APICE	8.8	115 AA	Bee venom protein	Antimicrobial, Anti-fibrinolytic and Anti-elasteolytic	Acacia W8 Asir
41	332.1119 333.1000 334.0966 336.1287	4	CGR KGK	28	<i>Apis mellifera</i>	Omega-conotoxin-like protein 1	OCLP1_APIME	8.76	74 AA	Immune protein	Antibacterial Ion channel inhibitor	Acacia W8 Asir
			CGR GTRRR KGK TSK CGR	27 23		Arginine kinase Major royal jelly protein 3	KARG_APIME MRJP3_APIME	5.66 6.47	355 AA 544 AA	Enzyme Royal Jelly Protein	Energy metabolism Nutritional wide medicinal value	
42	261.1319 328.2051 374.2116 416.2287 433.2851	5	GPRTRR LKR RAKIK	49	<i>Apis mellifera</i>	Homeobox protein E30 (Fragment)	HME30_APIME	10.58	109 AA	Gene regulatory proteins	Regulation of transcription	Acacia W8 Asir
43	419.2923 420.2844 420.3011 421.2913 423.2773 423.3497	6	YIK MDR MNR	19	<i>Apis mellifera</i>	Major royal jelly protein 5	MRJP5_APIME	5.95	598 AA	Royal Jelly Protein	Nutritional and wide range of medicinal activity	Acacia W8 Asir
44	489.3182 489.3375 490.1840 490.3183 491.1847 491.2304 492.2218 492.4602 492.6528 493.2842 419.2923 420.2844 420.3011 421.2913 423.2773 423.3497	16	TIEKFEK FAVRDMR AAEK	40	<i>Apis mellifera</i>	Elongation factor 1-alpha	EF1A_APIME	9.16	461 AA	Gene regulatory proteins	Regulation of translation	Acacia W8 Asir
45	459.2836 459.3551 460.1731 460.2360 461.1731 461.2224	9	TE GR SKPK	29 28 26	<i>Apis dorsata</i> <i>Apis cerana cerana</i> <i>Apis mellifera</i>	Phospholipase A2	PA2_APIDO PA2_APICC PA2_APIME		134 AA 134 AA 167 AA	Bee venom Enzyme	Hydrolysis of phospho-glycerides in biological membranes	Acacia W8 Asir

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Table 2 (continued)

SN	Precursor mass	Searched masses	Trypsin digest sequences	Score %	Honeybee species	Protein name	Proteome ID	pI	Amino acids number	Protein class	Protein function	Honey Sample
		462.1474 462.1770 463.3155										
46	845.6767	3	INKET LK	19	<i>Apis mellifera ligustica</i>	ATP synthase protein 8	ATP8_APILI	10.08	52 AA	Enzyme	Energy production	Acacia W9
47	846.6692 846.6857	24	TIEKFEK FAVRDMR	31	<i>Apis mellifera</i>	Elongation factor 1-alpha	EF1A_APIME	9.16	461 AA	Gene regulatory proteins	Regulation of translation	Acacia W9 Asir
		420.0545 420.1602 420.7892 420.8073 420.8866 421.0531 421.8874 422.1740 422.2097 422.8833 423.8936 424.0925 424.2062 489.1470 489.3168 489.8295 490.1841 490.2271 490.2876 491.1843 491.2894 492.1936 492.4639 493.4600										
48	408.0880	6	HPR IFK	22	<i>Apis mellifera</i>	Prohormone-3	PROH3_APIME	7.47	314 AA	Enzyme and regulatory protein	Wide range of functions such as antioxidant and antimicrobial	Acacia W9 Asir
49	408.1165 409.0840 409.1132 409.2269 410.1167 411.1466	13	YIK MDR MNR	19	<i>Apis mellifera</i>	Major royal jelly protein 5	MRJP5_APIME	5.95	598 AA	Royal Jelly Protein	Nutritional and wide range of medicinal activity	Acacia W9 Asir
		420.0545 420.1602 420.7892 420.8073 420.8866 421.0531 421.8874 422.1740 422.2097 422.8833 423.8936 424.0925 424.2062										
50	361.0990 361.1546 362.0752 362.1562 363.1599 363.9008	6	CIK ADR	24	<i>Apis mellifera</i>	PBAN-type neuropeptides	PBAN_APIME	7.66	195 AA	Neuro- peptide	Muscle contraction	Acacia W9 Asir
51	228.2313 229.0292 229.0498 229.1404 229.2133 231.0645 231.0963 232.0973 232.1484	9	GR IP	30	<i>Apis cerana</i>	Secapin-1	SECP_APICE	8.8	115 AA	Bee venom protein	Antimicrobial, Anti-fibrinolytic and Anti-elasteolytic	Acacia W9 Asir
52	458.2511 459.1704 459.2538 459.2851 460.1736 460.7848 461.1774 462.1492	7	R QR WKK TE GR SKPK TE GR SKPK TE GR SKPK	22 24 23 21	<i>Apis mellifera</i> <i>Apis dorsata</i> <i>Apis cerana cerana</i> <i>Apis mellifera</i>	Homeobox protein H55 (Fragment) Phospholipase A2 Phospholipase A2 Phospholipase A2	SCR_APIME PA2_APIDO PA2_APICC PA2_APIME	10.32 7.11 8.07 7.05	86 AA 134 AA 134 AA 167 AA	Gene regulatory proteins Bee venom Enzyme	Regulation of transcription Hydrolysis of phospho-glycerides in biological membranes	Acacia W9 Asir

(continued on next page)

Table 2 (continued)

SN	Precursor mass	Searched masses	Trypsin digest sequences	Score %	Honeybee species	Protein name	Proteome ID	pI	Amino acids number	Protein class	Protein function	Honey Sample																																																			
53	332.3319 333.1993 333.2245 333.3008 333.3333 334.2711 335.1833 335.2028 336.1330 336.2445 336.2831	11	CGR K GK	28	<i>Apis mellifera</i>	Omega-conotoxin-like protein 1	OCLP1_APIME	8.76	74 AA	Immune protein	Antibacterial Ion channel inhibitor	Acacia W9 Asir																																																			
													K GK T SK C GR T GR GGAK	23 18	Major royal jelly protein 3 Histone H4	MRJP3_APIME H4_APIME	6.47 11.05	544 AA 103 AA	Royal Jelly Protein Structural protein in DNA (nucleosome)	Nutritional wide medicinal value Regulation of gene expression																																											
			54	264.1659 264.8697 264.9286 265.1345 265.1434		5	MN	12	<i>Apis florea</i>	Cytochrome c oxidase subunit 2	COX2_APIFL	6.19									225 AA	Enzyme	Energy production (Respiratory chain)	Acacia W10 Asir																																							
																									55	328.0114 328.0831 328.1759 328.2063 328.5656	5	GPR	15	<i>Apis mellifera</i>	Homeobox protein E30 (Fragment)	HME30_APIME	10.58	109 AA	Gene regulatory proteins	Regulation of transcription	Acacia W10 Asir																										
																																						56	488.1147 488.8096 488.8724 489.0376 489.8748 490.1144 490.1666 490.0479 491.1606 491.8691 492.1520 492.1704	12	TLAG K MNVK	21	<i>Apis mellifera</i>	Opsin, blue-sensitive	OPSB_APIME	8.53	377 AA	Vision protein	light absorbing proteins	Acacia W10 Asir													
																																																			57	430.0837 430.9174 431.0810 432.0805 433.0812	5	GISR ATGGK RGAK	22	<i>Apis mellifera</i>	Small ribosomal subunit protein eS8	RSS_APIME	10.58	208 AA	Structural Ribosomal protein	Protein synthesis	Acacia W10 Asir
			59	428.2431 429.0916 429.1587 431.1766 432.2821 429.2397 429.3192		7	TRR RPR	20	<i>Apis mellifera</i>	Homeobox protein E30 (Fragment)	HME30_APIME	10.58									109 AA	Gene regulatory proteins	Regulation of transcription	Acacia W10 Asir																																							
																									19	Homeobox protein E60 (Fragment)	HME60_APIME	10.75	109 AA																																		
						60	332.2530 332.3310 333.1672 333.2246 333.3028 333.3347 334.2263 335.2266 335.2471 336.2840	10	CGR K GK	28	<i>Apis mellifera</i>	Omega-conotoxin-like protein 1	OCLP1_APIME	8.76	74 AA	Immune protein	Antibacterial Ion channel inhibitor	Acacia W10 Asir																																													
23 18	Major royal jelly protein 3 Arginine kinase	MRJP3_APIME KARG_APIME			6.47 5.66														544 AA 355 AA	Royal Jelly Protein Enzyme	Nutritional wide medicinal value Energy metabolism																																										
								61	376.2569 376.3508 377.2655 377.2912 377.3268 378.3041 378.3303 379.2561 380.2754	9		MTK NSR	19	<i>Apis mellifera</i>	Major royal jelly protein 4	MRJP4_APIME	5.89	464 AA				Royal Jelly Protein	Nutritional and wide range of medicinal activity	Acacia W10 Asir																																							

(continued on next page)

Table 2 (continued)

SN	Precursor mass	Searched masses	Trypsin digest sequences	Score %	Honeybee species	Protein name	Proteome ID	pI	Amino acids number	Protein class	Protein function	Honey Sample
62	430.0844 430.0967 430.1880 430.8845 430.9162 431.1799 431.9192 432.0807 432.2453 432.7763 433.2371 434.1140	12	GISR ATGGK RGAK	22	<i>Apis mellifera</i>	Small ribosomal subunit protein eS8	RS8_APIME	10.58	208 AA	Structural Ribosomal protein	Protein synthesis	Acacia W11 Asir
63	274.1439 275.1285	2	KQ	20	<i>Apis florea</i>	Melittin	MEL_APIFL	11.32	26 AA	Bee venom protein	Hemolytic and antimicrobial	Acacia W11 Asir
64	228.0525 229.0502 230.0975 231.0629 231.1321 232.1252 232.1480	7	GR IP	30	<i>Apis cerana</i>	Secapin	SECP_APICE	8.80	115 AA	Bee venom protein	Antimicrobial, Anti-fibrinolytic and Anti-elasteolytic	Acacia W11 Asir
65	424.0682 425.0380 425.0636 425.1381 426.0640	5	FT R HLR	30	<i>Apis mellifera</i>	Protein krueppel (Fragment) OS	KRUP_APIME	9.08	74 AA	regulation of gene expression	Suppression of metamorphosis at transcription	Acacia W11 Asir
66	332.1032 333.1003 334.0963 336.1261	4	CGR KGK KGK TSK CGR CGR GTR RR	28 23 27	<i>Apis mellifera</i>	Omega-conotoxin-like protein 1 Major royal jelly protein 3 Arginine kinase	OCLP1_APIME MRJP3_APIME KARG_APIME	8.76 6.47 5.66	74 AA 544 AA 355 AA	Immune protein Royal Jelly Protein Enzyme	Antibacterial Ion channel inhibitor Nutritional wide medicinal value Energy metabolism	Acacia W11 Asir
67	437.2368 477.3109 437.2181 437.2983 438.2206 438.4719 438.9999 440.2619	8	MYKK CVRT	37	<i>Apis mellifera</i>	Waprin-Thr1	WAP_APIME	8.89	110 AA	Venom protein	Antimicrobial Inhibits human elastase	Acacia W11 Asir
68	403.1632 404.1895 405.1893	3	RAR RTK	22	<i>Apis mellifera</i>	Homeobox protein H40 (Fragment)	EMS_APIME	10.93	74 AA	Gene regulatory proteins	Regulation of transcription	Acacia W17 Asir
69	417.8770 418.8348 418.8815 419.3866 420.3151 420.8857 421.3285 422.3365	8	FEK DMR AAEK	24	<i>Apis mellifera</i>	Elongation factor 1-alpha	EF1A_APIME	9.16	461 AA	Gene regulatory proteins	Regulation of translation	Acacia W17 Asir
70	261.1319 328.2051 374.2116 416.2287 433.2851 429.1590 431.1169 432.0763 432.6940 433.0729 433.1260	11	GPRTRR RPR LKR RAKIK TRR SDGR LKR RAKIK	59 50	<i>Apis mellifera</i>	Homeobox protein E30 (Fragment) Homeobox protein E60 (Fragment)	HME30_APIME HME60_APIME	10.58 10.75	109 AA 109 AA	Gene regulatory proteins	Regulation of transcription	Acacia W17 Asir

regulatory and neuropeptide that is needed for larval growth (Fritzsche & Hunnekuhl, 2021).

The sequences of trypsin digest of the prohormone-3 were HPR, IFK and APFA. The precursor masses were 404.3647, 405.3594, 405.4136, 406.3288, 406.4182, 407.3383, 408.0880, 408.1165, 408.1194, 408.1442, 408.3366, 409.0831, 409.0840, 409.1132, 409.1147,

409.1596, 409.2269, 410.1151, 410.1167, 410.1477 and 411.1466. The prohormone-3 contains 314 amino acids with 7.47 pI value and 28 % as the highest score. The protein was detected in three honey samples; one from Jazan region (Ziziphus) and two from Asir region (one Ziziphus and one Acacia) (Table.2 and Table.3).

Presence of *Apis mellifera* regulatory proteins in honey samples was

Table 3

The trypsin digest sequences and their precursor proteins from the honey samples of Jazan region.

SN	Precursor mass	Searched masses	Trypsin digest sequences	Score %	Honeybee species	Protein name	Proteome ID	pI	Amino acids number	Protein class	Protein function	Honey Sample
1	298.1250 298.1452 302.1444	3	QRDY	28	<i>Apis mellifera</i>	Omega-conotoxin-like protein 1	OCLP1_APIME	8.76	74 AA	Immune protein	Antibacterial Ion channel inhibitor	Ziziphus W73 /72 jazan
2	302.1444	1	KR	20	<i>Apis dorsata</i>	Melittin	MEL_APIDO	11.10	26 AA	Bee venom	Antimicrobial activity	Ziziphus W73 jazan
					<i>Apis florea</i>	Melittin	MEL_APIFL	11.32	26 AA	Bee venom	antimicrobial activity	Ziziphus W73 jazan
3	328.0071 328.0805 328.1162 329.0050	4	GPR	15	<i>Apis mellifera</i>	Homeobox protein E30 (Fragment)	HME30_APIME	10.58	109 AA	Gene regulatory proteins	Regulation of transcription	Ziziphus W73 jazan
4	422.6805 423.0658 424.0690 424.6800 425.0591 424.0670 425.0623 424.0689 424.6810 425.0622 426.0658	11	FT R HLR	30	<i>Apis mellifera</i>	Protein krueppel	KRUP_APIME	9.08	74 AA	Transcription factor	Suppression of metamorphosis	Ziziphus W73 jazan
5	418.1044 417.0996 417.3019 418.1057 419.0382 419.1163 419.1358 420.0415 489.2210 489.3446 490.1833 490.3216 490.8741 491.1388 491.1868 491.8741 492.4630 492.6506	18	EL R DMR AAEK TIE K FAVR	46	<i>Apis mellifera</i>	Elongation factor 1-alpha	EF1A_APIME	9.16	461 AA	Gene regulatory proteins	Regulation of translation	Ziziphus W73 jazan
6	422.6805 423.0658 424.0690 424.6800 425.0591	5	NY K YLK	18	<i>Apis mellifera</i>	Sensory neuron membrane protein 1	SNMP1_APIME	5.91	520 AA	olfactory protein	Olfactory not restricted to pheromone sensitivity	Ziziphus W73 jazan
7	441.3001 445.1238	2	GKGGK HR K	21	<i>Apis mellifera</i>	Histone H4	H4_APIME	11.05	103 AA	Structural protein	Regulation of gene expression	Ziziphus W73 jazan
8	488.2413 488.8760 489.1807 489.2210 490.1833 490.3216 491.1868 492.4630	8	TLAG K MNVK	21	<i>Apis mellifera</i>	Opsin, blue-sensitive	OPSB_APIME	8.53	377 AA	Vision protein	light absorbing proteins	Ziziphus W73 jazan
9	295.0854	1	FK	17	<i>Apis mellifera</i>	Homeobox protein H40 (Fragment)	EMS_APIME	10.93	74 AA	Gene regulatory proteins	Regulation of transcription	Ziziphus W74 jazan
10	390.1062 392.1046 393.1075 394.1320	4	MSR NKK SK CG	39	<i>Apis mellifera</i>	Allergen Api m 6.03 / Api m 6.04	ALL6_APIME	9.83	92 AA	proteolytic inhibitory protein	Protease inhibitor	Acacia W74 jazan
11	459.1698 459.2743 460.1729 461.1738	4	TEGR SKPK	29 28 26	<i>Apis dorsata</i> <i>Apis cerana</i> <i>Apis mellifera</i>	Phospholipase A2	PA2_APIDO PA2_APICC PA2_APIME	7.11 8.07 7.05	134 AA 134 AA 167	Bee venom protein	Hydrolysis of phosphoglycerides in biological membranes	Acacia W74 jazan

(continued on next page)

Table 3 (continued)

SN	Precursor mass	Searched masses	Trypsin digest sequences	Score %	Honeybee species	Protein name	Proteome ID	pI	Amino acids number	Protein class	Protein function	Honey Sample
12	844.6737 845.6759 846.6798	3	INKET LK	19	<i>Apis mellifera ligustica</i>	ATP synthase protein 8	ATP8_APILI	10.08	52 AA	Enzyme	Energy production	Acacia W74 jazan
13	408.1194 408.1442 409.0831 409.1147 409.1596 410.1151 410.1477	7	HPR IFK	22	<i>Apis mellifera</i>	Prohormone-3	PROH3_APIME	7.47	314 AA	Enzyme and regulatory protein	Wide range of functions such as antioxidant and antimicrobial	Ziziphus W73 jazan
14	362.0880 362.9280 363.0881 363.9319 364.1076	5	CIK ADR	24	<i>Apis mellifera</i>	PBAN-type neuropeptides	PBAN_APIME	7.66	195 AA	Neuropeptide	Muscle contraction	Ziziphus W72 Jazan
15	476.2681 477.3110 478.3126 479.3214	4	HPPK EG DR	19	<i>Apis mellifera</i>	Arginine kinase	KARG_APIME	5.66	355 AA	Enzyme	Energy metabolism	Acacia W66 Jazan
16	439.1414 440.0978 440.1404 441.1432 442.1373	5	MYKK	24	<i>Apis mellifera</i>	Waprin-Thr1	WAP_APIME	8.89	110 AA	Bee venom	Antimicrobial	Acacia W66 Jazan
17	430.0848 430.1967 430.8829 430.9166 431.1836 431.9192 432.0858 433.0834	8	GISR ATGGK RGAK	22	<i>Apis mellifera</i>	Small ribosomal subunit protein eS8	RS8_APIME	10.58	208 AA	Structural Ribosomal protein	Protein synthesis	Ziziphus W72 Jazan
18	673.3089 674.2762 674.2992 674.3106 675.2833 675.2984 676.2770	7	RDAAI k TSKL vk	19	<i>Apis mellifera</i>	Major royal jelly protein 3	MRJP3_APIME	6.47	544 AA	Royal Jelly Protein	Nutritional wide medicinal value	Ziziphus W72 Jazan
19	694.2900 694.8336 695.2858 696.2437 696.3189 697.2311 697.2849	7	YWSIK IRYDK	23	<i>Apis mellifera ligustica</i>	NADH-ubiquinone oxidoreductase chain 1	NU1M_APILI	6.14	305 AA	Enzyme	Energy production Respiratory chain	Ziziphus W72 Jazan
20	428.2495 429.1644 429.1894 429.2383 429.2919 429.3166 431.3496 432.2815	8	TRR RPR	19 20	<i>Apis mellifera</i>	Homeobox protein E60 Homeobox protein E30	HME60_APIME HME30_APIME	10.75 10.58	109 AA	Gene regulatory proteins	Regulation of transcription	Ziziphus W72 Jazan
21	228.0523 228.2315 229.0489 229.1451 229.2143 230.0942 231.0641 231.1004 231.1202 231.2137 232.1339	11	GR IP	30	<i>Apis cerana</i>	Secapin	SECP_APICE	8.8	115 AA	Bee venom protein	Antimicrobial, Anti-fibrinolytic and Anti-elastolytic	Acacia W70 Jazan
22	276.1488 277.1395 278.1502 279.0943	4	MK AW	33	<i>Apis mellifera</i>	Apidermin 2	APID2_APIME	10.43	77 AA	Immune protein Cyclic protein	antimicrobial agent in bees	Acacia W70 Jazan
23	420.0542 420.1154 420.1559 420.7997 420.8856	13	YIK MDR MNR	19	<i>Apis mellifera</i>	Major royal jelly protein 5	MRJP5_APIME	5.95	598 AA	Royal Jelly Protein	Nutritional and wide range of medicinal activity	Acacia W66 Jazan

(continued on next page)

Table 3 (continued)

SN	Precursor mass	Searched masses	Trypsin digest sequences	Score %	Honeybee species	Protein name	Proteome ID	pI	Amino acids number	Protein class	Protein function	Honey Sample
	421.0518		FEK	19		Elongation factor 1-alpha	EF1A_APIME	9.16	461 AA	Gene regulatory proteins	Regulation of translation	
	421.8912		DMR									
	422.1734											
	422.7734											
	422.8847											
	423.1339											
	423.7482											
	424.1347											
24	439.1414	4	EYK	26	<i>Apis mellifera</i>	Hymenoptaecin	HYTA_APIME	9.44	129 AA	Immune protein	Antibacterial activity	Acacia W66 Jazan
	440.0978		GPGR									
	440.1404											
	442.1373											

reported previously in honey samples by Bong et al. (2021). However, the prohormone-3 is not reported namely to be present in honey before.

3.3.1.4. Homeobox E30. It is a DNA binding and a transcription initiation factor that regulates the RNA synthesis (Walldorf et al., 1989). The sequences of the trypsin digest Homeobox E30 were GPRTRR, LKR, RAKIK, GPR, TRRRPR, RPR and RAKIK. The precursor masses of the trypsin digest of the homeobox E30 were 261.1319, 328.0071, 328.0114, 328.0805, 328.0831, 328.1162, 328.1759, 328.2051, 328.2063, 328.5656, 329.0050, 374.2116, 416.2287, 428.2431, 429.0916, 429.1587, 429.2397, 429.3192, 31.1766, 432.2821 and 433.2851. The homeobox E30 is composed of 109 amino acids and the pI of 10.58. It is detected in three Acacia honey samples from Asir region and one Ziziphus honey from Jazan honey with the highest score of 59 % (Table.2 and Table.3).

Homeoboxes proteins are DNA binding proteins associated with the regulation of transcription and protein synthesis. Bong et al. (2021) and Erban et al. (2019) reported presence of DNA binding proteins in honey samples.

3.3.1.5. Homeobox E60. AS the homeobox E30, the Homeobox E60 is a transcription regulating protein that binds the DNA and it is an RNA polymerase specific (Walldorf et al., 1989).

The homeobox E60 is detected in four honey samples from Asir (two Ziziphus and one Acacia) and Jazan (one Ziziphus) with highest score of 50 %. The trypsin digest sequences of the homeobox E60 were TRR, SDGR, LKR, RAKIK, RPR, and TRRRPR. The precursor masses of the trypsin digest of the homeobox E60 were 261.1319, 328.0071, 328.0114, 328.0805, 328.0831, 328.1162, 328.1759, 328.2051, 328.2063, 328.5656, 329.0050, 374.2116, 416.2287, 428.2431, 429.0916, 429.1587, 429.2397, 429.3192, 431.1634, 431.1766, 431.9173, 432.0849, 432.2821 432.4167, 432.9240, 433.2851 and 434.0375. The homeobox E60 contains 109 amino acids with the pI of 10.58 (Table.2 and Table.3).

Manuka honey is reported to contain regulatory proteins that binds the DNA and affects the transcription (Bong et al., 2021; Erban et al., 2019).

3.3.1.6. Homeobox H40. Homeobox H40 is a regulatory protein that participates in the regulation of transcription through binding the DNA (Walldorf et al., 1989).

The amino acid sequences of the trypsin digest of the homeobox H40 were R AR, RTK and FK. The precursor masses of the trypsin digest were 295.0854, 403.1632, 403.2346, 404.1891, 404.1895, 404.2096, 405.1889 and 405.1893. The homeobox H40 contains 74 amino acids and its pI is 10.93. It is detected in three honey samples (two Acacia honeys from Asir and one Ziziphus honey from Jazan) with the highest score of 22 % (Table.2 and Table.3).

Regulatory proteins such as the homeobox H40 are reported to be present in the manuka honey (Bong et al., 2021; Erban et al., 2019).

3.3.1.7. Homeobox H55. Homeobox H55 is a transcription factor that is associated with the determination of cell position in the anterior or posterior axis of the honeybee (Walldorf et al., 1989).

The Homeobox H55 is detected in one Acacia honey sample from Asir region with the score of 22 %. The precursor mass of the homeobox H55 were 458.2511, 459.1704, 459.2538, 459.2851, 460.1736, 460.7848, 461.1774 and 462.1492. The sequences of the trypsin digest were R QR and WKK. The homeobox H55 is composed of 86 amino acids and it has the pI of 10.32 (Table.2 and Table.3).

Similar to the findings of this study, gene regulatory proteins are stated to be present in manuka honey samples (Bong et al., 2021; Erban et al., 2019).

3.3.2. Enzymes

This study detected six enzymes in the studied honey samples. Three enzymes were reported in the honey samples from Asir and Jazan collectively (ATP Synthase, Arginine Kinase and phospholipase A2). Two enzymes were specific to some Asir region honey samples (Cytochrome C Oxidase and Cystathionine beta synthase) while one enzyme was specific for Ziziphus honey from Jazan region (NADH ubiquinone oxidoreductase).

3.3.2.1. ATP synthase. ATP synthase (Complex V of the respiratory chain) is a mitochondrial membrane bound enzyme. It synthesizes ATP from ADP and phosphate in the presence of proton passage through the complex. The ATP synthase has two domains, catalytic domain (extra-membrane or F1) and the membrane domain or proton channel (F0) (Crozier & Crozier, 1992).

The protein digest of the ATP synthase had the amino acid sequences of INKET and LK corresponding to the amino acid sequences of 844.6737, 845.6759, 845.6767, 846.6692, 846.6798 and 846.6857. The ATP synthase contains 52 amino acids with 10.08 pI value. It is detected in two Acacia honey samples (one from Asir region and one from Jazan region) with the score value of 19 %. According to the mascot search, the ATP synthase of the two Acacia honey samples was originated from *Apis mellifera ligustica* (Table.2 and Table.3). The *Apis mellifera ligustica* is not within the honeybees of Saudi Arabia which involves *Apis mellifera jementica*, *Apis mellifera carnica*, *Apis florea* and *Apis mellifera caucasica* (recently imported) (Alqarni et al., 2011).

It is not the first time to report presence of ATPase in honey samples as it is previously stated that honey contains ATP synthase (Bong et al., 2021; Erban et al., 2019).

3.3.2.2. Arginine kinase. Arginine kinase is an enzyme that ensures energy for the visual system of the honeybees. It catalyzes the reaction of Arginine and ATP to produce ADP, Arginine phosphate and energy (Kucharski & Maleszka, 1998).

The precursor masses of the trypsin digest of the Arginine kinase were 332.1026, 332.1032, 332.1119, 332.2530, 332.3310, 333.0041, 333.1000, 333.1003, 333.1009, 333.1672, 333.2246, 333.3012,

333.3028, 333.3347, 334.0966, 334.1032, 334.2263, 335.1298, 335.2266, 335.2471, 336.1261, 336.1277, 336.1287, 336.1429, 336.2840, 476.2425, 476.2681, 477.2787, 477.3110, 477.3111, 478.2590, 478.3126, 478.3139, 479.3214 and 479.3245. The amino acid sequences of the Arginine kinase digest were CGR, GTR, RR, HPPK and EGDR. The Arginine kinase contains 355 amino acids and it has the pI value of 5.66. The Arginine kinase was reported in four honey samples from Asir region (one Ziziphus and three Acacia) and one honey sample from Jazan region (Acacia). The highest score for the Arginine kinase was 47 % (Table.2 and Table.3).

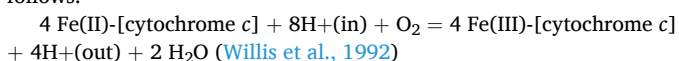
Enzymes are well known to be present in honey with major contribution to the biological and medicinal activities of honey (Alarjani et al., 2022). It is the first time to report the arginine kinase in honey samples.

3.3.2.3. Phospholipase A2. Phospholipase A2 is a bee venom enzyme responsible for the cleavage of the phosphatidyle choline to 1-acyl-sn-glycerol-3-phosphocholine and free fatty acid. The secretion of the phospholipase A2 is directly correlated to the secretion of mellitin (Junior et al., 2010).

The trypsin digest of the phospholipase A2 had the amino acid sequences of TEGR and SKPK. The precursor masses of the phospholipase A2 trypsin digest were 458.2511, 458.2983, 458.3664, 459.1698, 459.1704, 459.2538, 459.2743, 459.2836, 459.2851, 459.2948, 459.3070, 459.3132, 459.3359, 459.3551, 459.3566, 460.1729, 460.1731, 460.1736, 460.2360, 460.3111, 460.3458, 460.7848, 461.1731, 461.1738, 461.1764, 461.1774, 461.2224, 461.2681, 461.3344, 462.1474, 462.1483, 462.1492, 462.1770, 462.2240, 463.0476 and 463.3155. The phospholipase A2 of *Apis dorsata*, *Apis cerana cerana* and *Apis mellifera* had 134, 134 and 167 amino acids with the pI values of 7.11, 8.01 and 7.05, respectively. The phospholipase A2 was reported in 4 Acacia honey samples from Asir region and one Acacia honey from Jazan region with the highest score of 29 % (Table.2 and Table.3).

Bee venom proteins and lipase enzyme existence in honey was reported by Bong et al. (2021) and Erban et al. (2019).

3.3.2.4. Cytochrome C oxidase (COX). COX is a respiratory chain enzyme responsible for the reduction of oxygen to water. 4 electrons are accepted from the cytochrome C and donated to the molecular oxygen to form 2O²⁻. 8 protons are accepted from the mitochondrial intermembrane space. 4 of the 8 protons react with the two oxygen ions to form 2H₂O and the other four protons go back to the intermembrane space as follows:



The trypsin digest of the cytochrome C oxidase had one amino acid sequence (MN) with the following precursor masses 264.1589, 264.1659, 264.1663, 264.4832, 264.8527, 264.8697, 264.9301, 264.9286, 264.9294, 265.1067, 265.1345, 265.1392, 265.1409, 265.1434 and 265.1802. The cytochrome C oxidase was originated from *Apis florea* with 225 amino acids, pI of 6.19 and score of 12 %. It is detected in one Ziziphus honey and two Acacia honeys from Asir region (Table.2 and Table.3).

It is the first time to report that honey contains cytochrome C oxidase. The previous studies detected other energy metabolism enzymes such as the ATP synthase and glucosyl ceramidase (Bong et al., 2021; Erban et al., 2019).

3.3.2.5. Cystathionine beta synthase (CBS). Cystathionine beta-synthase is an enzyme that synthesizes cystathionine from homocystiene and serine. The cystathionine is a precursor for the synthesis of cysteine (Watanabe et al., 2007).

The precursor masses of the trypsin digest of the CBS were 696.2591, 696.2826, 697.2764 and 698.2802. The amino acid sequences of the

CBS trypsin digest were NGDYTK and LNNIPK with 19 % score. The CBS contains 504 amino acids with the pI of 6.53. It is detected in one Acacia honey sample from Asir region (Table.2 and Table.3).

Depending on our literature survey, presence of CBS in honey samples is not detected in honey samples before.

3.3.2.6. NADH-ubiquinone oxidoreductase chain 1. It is a respiratory chain enzyme that catalyzes the oxidation of NADH to NAD⁺ and transfers protons and electron to ubiquinone. The protons move between the mitochondrial intermembrane space to the mitochondrial matrix (5 protons in and 4 protons out). The oxidation of NADH can be summarized as follows: a ubiquinone + 5H⁺(in) + NADH = a ubiquinol + 4H⁺(out) + NAD⁺ (Crozier & Crozier, 1992).

The trypsin digest of the NADH-ubiquinone oxidoreductase was composed of two amino acid sequences, YWSIK and IRYDK. The precursor masses of the trypsin digest were 694.2900, 694.8336, 695.2858, 696.2437, 696.3189, 697.2311, 697.2849. The NADH-ubiquinone oxidoreductase has the pI of 6.14 and contains 305 Amino acids with the score percentage of 23 %. The NADH-ubiquinone oxidoreductase is found in one Ziziphus honey sample from Jazan region by *Apis mellifera ligustica* which reflects that this sample is either an imported honey or mixed with imported honey (Table.2 and Table.3).

Energy metabolism enzymes such as the ATP synthase have been reported previously in honey samples, but not the NADH-ubiquinone oxidoreductase (Bong et al., 2021; Erban et al., 2019).

3.3.3. Bee venom proteins

Bee venom is composed of allergenic proteins that cause allergic reactions (anaphylaxis) or even death. Bee venom contains proteins and enzymes. The proteins include mellitin, vitellogenin, apamin and secapin while the enzymes include hyaluronidase, phospholipase A2, serine proteases and acid phosphatase. Bee venom proteins are known to be present in honey and they are responsible for the allergic symptoms after honey consumption (Bong et al., 2021; Erban et al., 2019).

3.3.3.1. Secapin. Secapin is a bee venom protein with activities of serine protease inhibition and antimicrobial. Moreover, it has the activities of lyase and regulation such as the regulation of the immune system (Meng et al., 2012).

The trypsin digest of the secapin had the amino acid sequences of VIVP and GRIP. The mass precursors of the trypsin digest were 228.0523, 228.0535, 228.2313, 228.2315, 228.2318, 228.2320, 228.2331, 229.0292, 229.0489, 229.0495, 229.0496, 229.0497, 229.0498, 229.1087, 229.1404, 229.1415, 229.1425, 229.1432, 229.1451, 229.1517, 229.2133, 229.2143, 229.2159, 229.2165, 230.0527, 230.0942, 230.0972, 230.0986, 231.0641, 231.0645, 231.0667, 231.0780, 231.0815, 231.0848, 231.0963, 231.0967, 231.1002, 231.1004, 231.1202, 231.1213, 231.2137, 231.1250, 231.1452, 232.0973, 232.1290, 232.1300, 232.1339, 232.1484, 232.1492, 426.1401, 426.1768, 427.1607, 427.1684, 427.1901, 428.1793 and 428.1811. The secapin is originated from *Apis cerana* and *Apis mellifera*. The secapin of *Apis cerana* has 115 amino acid with the pI value of 8.8 while the secapin of *Apis mellifera* has 25 amino acids with 8.8 as pI value. The highest score was reported for the secapin of *Apis cerana* with the percentage of 30 %. The secapin was detected in four honey samples from Asir region (one Ziziphus and three Acacia) and one Acacia honey sample from Jazan region (Table.2 and Table.3).

Secapin was not detected in honey samples before, but the bee venom proteins are stated to be present in variable honey samples (Bong et al., 2021; Erban et al., 2019).

3.3.3.2. Waprin-Thr-1. Waprin is a bee venom protein with antimicrobial activity through the damage of the microbial cell membranes. It inhibits the microbial serine proteases and elastase and it has no hemolytic activity (Lee et al., 2023).

The precursor masses of the trypsin digest of the waprin were 437.2368, 437.2181, 437.2983, 438.2206, 438.4719, 438.9999, 439.1089, 439.1274, 439.1451, 439.1468, 440.1403, 439.1414, 440.0978, 440.1404, 440.1416, 440.1554, 440.2619, 440.8052, 441.1429, 441.1432, 441.1470, 441.3003, 442.1373, 442.1398 and 477.3109. The amino acid sequences of the waprin trypsin digest were MYKK and CVRT with the highest score percentage of 37 %. The waprin was identified in three honey samples from Asir region (one *Ziziphus* and two *Acacia*) and one polyfloral honey from Jazan region. The waprin is composed of 110 amino acids and has the pI of 8.89 (Table.2 and Table.3).

Generally, bee venom proteins are identified in honey samples (Bong et al., 2021; Erban et al., 2019). However, It is the first time to report that honey samples contain the waprin protein.

3.3.3.3. Melitin. Melitin is the major bee venom protein with high hemolytic and antimicrobial activities. It activates the phospholipase A2 of the bee venom (Kreil, 1973).

The trypsin digest of the melitin had the sequences of KR and KQ and they were corresponding to the precursor masses of 302.1444, 274.1439 and 275.1285. The honey samples that contained the melitin were *Acacia* and *Ziziphus* honey samples from Asir and Jazan regions, respectively. The mascot search showed that the honey samples were produced by *Apis florea* and *Apis dorsata*. The melitin of the *Apis florea* and *Apis dorsata* contains 26 amino acids. The pIs of the melitin of *Apis florea* and *Apis dorsata* were 11.32 and 11.1, respectively. The highest score of the melitin was 20 % (Table.2 and Table.3). It is the first time to find melitin in honey samples.

3.3.3.4. Inhibitory protein (allergen *Api m 6.03* / *Api m 6.04*). Allergen *Api m 6.03* / *Api m 6.04* is a bee venom protein that act as a protease inhibitor. Moreover, it has hydrolase and regulatory activities (Kettner et al., 2001).

The Allergen *Api m 6.03* / *Api m 6.04* was detected in a *Ziziphus* honey from Jazan region and its trypsin digest had the amino acid sequences of MSR, NKK and SKCG with the score of 39 %. The precursor masses of its trypsin digest were 390.1062, 392.1046, 393.1075 and 394.1320. The Allergen *Api m 6.03* / *Api m 6.04* has 92 amino acids and the pI of 9.83 (Table.2 and Table.3).

Bee venom proteins are known to be present in honey and they contribute to the allergic consequences after eating honey or meal that contains honey (Bong et al., 2021; Erban et al., 2019).

3.3.4. Major royal jelly proteins (MRJP)

MRJP are secreted by worker honeybees to feed the developing queen larvae. They are of high nutritional values beside other medicinal values such as immune modulatory, hypotensive, hypolipidemic, wound healing, growth promoting, anti-inflammatory, anticancer and antimicrobial. There are nine MRJP named as MRJP-1 to MRJP-9 (Mureşan et al., 2022^b).

3.3.4.1. MRJP-2. MRJP-2 is a highly nutrient protein needed for the development of the queen larvae and for the reproductive ability and immune function of the honeybees and their queens (Feng et al., 2015).

The amino acid sequences of the MRJP-2 trypsin digest were MTR, AVSK and MQK and the precursor masses were 403.2296, 403.2544, 404.1901, 404.8214, 405.9289, 406.1987, 406.7847, 407.1254, 404.3647, 405.3594, 405.4136, 406.3288, 406.4182, 407.3383 and 408.3366. The score of the precursor masses was 29 %. The MRJP-2 contains 452 amino acids and 6.83 as the pI value. The MRJP-2 was detected in one *Ziziphus* sample from Asir region (Table.2 and Table.3).

Some previous studies showed that the MRJP-2 is present in honey samples from different floral and geographical origins (Bong et al., 2021; Mureşan et al., 2022).

3.3.4.2. MRJP-3. MRJP-3 is an RNA binding protein that enhances the bioavailability of RNA in the royal jelly. Availability of RNA in the royal jelly is essential for the synthesis of the royal jelly proteins (Mureşan et al., 2022).

The amino acid sequences of the MRJP-3 trypsin digest were KGG, TSK, CGR, RDAAIk and TSKLVK with the sequences of 332.1026, 332.1119, 332.2530, 332.3310, 332.3319, 333.0041, 333.1009, 333.1993, 333.2245, 333.3008, 333.1000, 333.1672, 333.2246, 333.3012, 333.3028, 333.3333, 333.3347, 334.0966, 334.1032, 334.2263, 334.2711, 335.1298, 335.1833, 335.2028, 335.2266, 335.2471, 336.1277, 336.1287, 336.1330, 336.1429, 336.2445, 336.2831 and 336.2840. The MRJP-3 has 544 amino acids with the pI of 6.47 and the highest score of 23 %. It is detected in 5 honey samples from Asir region (one *Ziziphus* and four *Acacia*) and one *Ziziphus* honey from Jazan region (Table.2 and Table.3).

MRJP-3 was stated to be identified in honey samples from variable floral and geographical origins (Bong et al., 2021; Mureşan et al., 2022).

3.3.4.3. MRJP-4. MRJP-4 is a rich nutrient for the larvae of the honeybee queen is essential for its re-productivity (Mureşan et al., 2022).

The MRJP-4 is found in two *Acacia* honey samples from Asir region and its trypsin digest had the amino acid sequences of MTK and NSR. The precursor masses of the MRJP-4 trypsin digest were 376.2569, 376.3508, 377.2319, 377.2655, 377.2911, 377.2912, 377.3268, 377.3277, 378.2924, 378.3041, 378.3303, 378.3310, 378.3546, 379.2561, 379.3418, 380.2754, 380.3384 and 380.3389. The MRJP-4 has 464 amino acids with the pI of 5.89 and the highest score of 19 (Table.2 and Table.3).

This study is not alone in proving the presence of MRJP-4 in honey samples, some previous studies stated the same finding (Bong et al., 2021; Mureşan et al., 2022).

3.3.4.4. MRJP-5. As a major royal jelly protein, MRJP-5 is needed for the nutrition of honeybee queens larvae and for the reproductivity of the queens (Albert, Bhattacharya, Klaudivy, Schmitzová, & Simúth, 1999).

The MRJP-5 was detected in three honey samples from Asir region (one *Ziziphus* and two *Acacia*) and one *Acacia* honey from Jazan. The Trypsin digest of the MRJP-5 had the amino acid sequences of YIK, MDR and MNR and its precursor masses were 419.2923, 420.0542, 420.0545, 420.1154, 420.1559, 420.1602, 420.2645, 420.2844, 420.3011, 420.3042, 420.3168, 420.7892, 420.7997, 420.8073, 420.8856, 420.8866, 421.0518, 421.0531, 421.2913, 421.2947, 421.3300, 421.8874, 421.8912, 422.1740, 422.2097, 422.1734, 422.2627, 422.3258, 422.3531, 422.7734, 422.8833, 422.8847, 423.1339, 423.2773, 423.3497, 423.3874, 423.7482, 424.0925, 424.1347, 424.2062. The highest score of the detected MRJP-5 was 19 %. It contains 598 amino acids with the pI value of 5.95 (Table.2 and Table.3).

MRJP-5 was identified in honey samples from different floral and geographical origins (Bong et al., 2021; Mureşan et al., 2022).

3.3.5. Immune proteins

Immune proteins are antimicrobial peptides (AMPs) secreted in the hemolymph of the insects in response to pathogens. Antimicrobial peptides are affected by the insect nutrition and stress. The famous honeybee antimicrobial peptides include defensin, abaecin, hymenoptaecin and apidaecins (Daníhlík et al., 2015).

3.3.5.1. Defensin-1. It is an antimicrobial peptide found in the royal jelly and hemolymph. It is also known as royalisin and it has antibacterial activity (anti gram positive bacteria) in low concentration. Defensin-1 is responsible for the social immunity of the honeybees (Ilyasov et al., 2013).

The amino acid sequence of the trypsin digest of defensin-1 was FG corresponding to the precursor masses of 221.9377, 222.1146, 222.1154, 222.9752, 223.0660, 223.2066, 223.0973, 223.1150,

223.1305, 223.1700, 24.0539, 224.0649, 224.0993 and 224.1275 and the highest score was 16 %. The defensin-1 is composed of 95 amino acids and has the pI of 6.28. Defensin-1 is associated with two honey samples from Asir region (Ziziphus and Acacia) (Table.2 and Table.3).

Defensin-1 is known to be one of the major constituents of honey responsible for its antibacterial activity (Bong et al., 2021; Erban et al., 2019).

3.3.5.2. Hymenoptaecin. It is one of the antibacterial peptides of the honeybees (Danilhlk et al., 2015). The trypsin digest of the Hymenoptaecin had the amino acid sequences of EYK and GPGGR and the precursor masses were 439.1274, 439.1414, 439.1468, 440.0978, 440.1403, 440.1404, 440.1554, 442.1373 and 442.1398. It contains 129 amino acids and has the pI of 9.44 and the score of 26 %. The hymenoptaecin was detected in one Ziziphus honey sample from Asir region and one Acacia honey sample from Jazan region (Table.2 and Table.3).

Hymenoptaecin is not stated before to be present in honey. Instead, it is mentioned that hymenoptaecin is one of the honeybee hemolymph antibacterial peptides (Danilhlk et al., 2015). Hymenoptaecin may be used a predictor of honey samples from the Southwestern region of Saudi Arabia.

3.3.5.3. Omega-conotoxin like protein. Omega-conotoxin like protein is an antibacterial peptide that acts through the inhibition of ion channels. Moreover, it has regulatory and transport activities (Kaplan et al., 2007).

The amino acid sequences of the trypsin digest of the omega-conotoxin like protein were CGR, KGK and QRDY. The precursor masses were 298.1250, 298.1452, 302.1444, 332.1026, 332.1032, 332.1119, 332.2530, 332.3310, 332.3319, 333.0041, 333.1009, 333.1000, 333.1672, 333.2246, 333.3012, 334.0966, 333.1003, 333.1993, 333.2245, 333.3008, 333.3028, 333.3333, 333.3347, 334.0963, 334.1032, 334.2263, 334.2711, 335.1298, 335.1833, 335.2028, 335.2266, 335.2471, 336.1261, 336.1277, 336.1287, 336.1330, 336.1429, 336.2445, 336.2831 and 336.2840. The omega-conotoxin like protein contains 74 amino acids with the pI of 8.76 and the highest score of 28 %. It is identified in four honey samples from Asir region (one Ziziphus and three Acacia) and two Ziziphus honey samples from Jazan region (Table.2 and Table.3).

Omega-conotxin like protein is known as a honeybee antibacterial peptide but not as a honey protein (Kaplan et al., 2007). This study found that the omega-conotoxin like protein can be a marker for the honey from the southwestern region of Saudi Arabia.

3.3.5.4. Apidermin 2. Apidermin 2 is a honeybee cuticular protein and has antimicrobial activity. It is a broad spectrum antibacterial as it inhibits the growth of gram positive, gram negative bacteria and fungi. It kills microbes through binding and damaging their cell walls (Kim et al., 2022).

The amino acid sequences of the Apidermin 2 trypsin digest were MK and AW and it had the precursor masses of 276.1488, 277.1395, 278.1502 and 279.0943. Apidermin 2 has 77 amino acids with the pI of 10.43. The Apidermin 2 was identified in one Acacia honey sample from Jazan region with the score of 33 % (Table.2 and Table.3).

Apidermin 2 is not detected in honey samples before. It is mentioned as a promising broad spectrum antibiotic of honeybee origin (Kim et al., 2022).

3.3.6. Structural proteins

Structural proteins which were detected in this study were associated with the structure of ribosomes and DNA.

3.3.6.1. Small ribosomal subunit protein es8. The trypsin digest of the small ribosomal subunit protein es8 had the amino acid sequences of GISR, ATGGK, RGAK, ALR, FAK and SKK. The precursor masses of the

trypsin digest were 360.2537, 360.3609, 361.0929, 361.2782, 361.2925, 361.3300, 361.3858, 362.3059, 362.3428, 363.2884, 364.2800, 364.3330, 430.0829, 430.0836, 430.0837, 430.0838, 430.0844, 430.0848, 430.0967, 430.1432, 430.1857, 430.1880, 430.1967, 430.1994, 430.2450, 430.8666, 430.8829, 430.8845, 430.8892, 430.9143, 430.9145, 430.9152, 430.9162, 430.9164, 430.9166, 430.9174, 431.0795, 431.0810, 431.0811, 431.1634, 431.1799, 431.1836, 431.1891, 431.2504, 431.9170, 431.9173, 431.9192, 431.9192, 431.9212, 432.0805, 432.0807, 432.0836, 432.0842, 432.0849, 432.0858, 432.1988, 432.2453, 432.2573, 432.2808, 432.4167, 432.6075, 432.7763, 432.8876, 432.8907, 432.9240, 432.9252, 433.0812, 433.0834, 433.2371, 433.2562, 433.2838, 434.0375, 434.1140, 434.1157 and 434.1165. The highest score of the investigated precursor masses was 22 %. The small ribosomal subunit protein es8 had 208 amino acids with the pI of 10.58. It was detected in four honey samples from Asir region (one Ziziphus and three Acacia) and in one Ziziphus honey from Jazan region (Table.2 and Table.3).

Structural proteins are found in variable honey samples but not the small ribosomal subunit protein es8 namely (Bong et al., 2021; Erban et al., 2019).

3.3.6.2. Histone-4. Histone 4 is as a major component in the nucleosomes octamer. Nucleosome and DNA forms the chromatin and chromosomes. Histone 4 plays major roles in the regulation of transcription, replication and DNA repair (Jenuwein & Allis, 2001).

The Histone-4 trypsin digest had the amino acid sequences of GKGGK, HRK, TGR and GGAK. The precursor masses of the trypsin digest were 332.3319, 333.1993, 333.2245, 333.3008, 333.3333, 334.2711, 335.1833, 335.2028, 336.1330, 336.2445, 336.2831, 439.1089, 439.1451, 439.8704, 440.1416, 440.8052, 441.1429, 441.3001, 441.3003 and 445.1238. The highest score of the trypsin digest was 22 %. The histone-4 contains 103 amino acids with the pI of 11.05 (Table.2 and Table.3).

This study is not alone in reporting the presence of histone –4 in honey samples as Erban et al. (2019) mentioned that it is found in honey samples from the Czech Republic.

3.3.7. Neuropeptides

Neuropeptides are brain proteins involved in the regulation of animal behavior. There are more than 200 neuropeptides in *Apis mellifera* (Hummon et al., 2006).

3.3.7.1. Pheromone biosynthesis activation neuropeptide (PBAN). It is a neuropeptide and hormone that is found in females and males. In females, it is responsible for the regulation of sex pheromone production while in males, it induces the responsiveness of the males to female sex pheromones (Hummon et al., 2006).

The PBAN neuropeptides was identified in four honey samples from Asir region (one Ziziphus and three Acacia) and in one Ziziphus honey from Jazan region. Its trypsin digest amino acid sequences were CIK and ADR. The precursor masses of the trypsin digest were 360.1062, 360.1167, 360.2537, 360.3609, 360.9255, 361.0696, 361.0929, 361.0990, 361.1105, 361.1258, 361.1283, 361.1298, 361.1539, 361.1546, 361.1546, 361.1547, 361.2782, 361.2925, 361.3300, 361.3858, 362.0197, 362.0752, 362.0763, 362.0880, 362.0992, 362.1280, 362.1299, 362.1549, 362.1562, 362.1579, 362.1583, 362.2255, 362.3059, 362.3428, 362.9272, 362.9280, 363.0771, 363.0789, 363.0881, 363.0963, 363.1599, 363.1793, 363.2861, 363.2884, 363.8848, 363.8929, 363.9008, 363.9028, 363.9319, 364.0791, 364.1076, 364.2800 and 364.3330. The highest score of the mentioned precursor masses and amino acid sequences was 24 %. The PBAN neuropeptide has 195 amino acids with 7.66 as pI value (Table.2 and Table.3).

The PBAN neuropeptide is stated to be a honey constituent before. Its

digestion by the honey enzymes may produce short peptides with biological activities.

3.3.7.2. Orokinin. Orokinin is a neuropeptide and a myopic protein secreted from the brain of the honeybees and induces the frequency of gut muscle contraction (Stangier et al., 1992).

Orokinin was detected in one *Ziziphus* and one *Acacia* honey samples from Asir region and it was identified in any of the Jazan honey samples. The amino acid sequence of the trypsin digest of the orokinin was FG and the precursor masses were 221.9377, 222.1146, 222.1154, 222.9752, 223.0660, 223.0973, 223.1150, 223.1305, 223.1700, 223.2066, 224.0539, 224.0649, 224.0993 and 224.1275. The amino acid sequence of the orokinin trypsin digest scored 14 %. The orokinin has 147 amino acids and the pI of 5.63 (Table.2 and Table.3).

According to the reviewed literature, it is not stated that honey samples contain orokinin. Orokinin digestion products may contribute to their biological and medical values.

3.3.8. Vision protein (opsin blue- sensitive)

The opsin is an apoprotein that acts as visual pigment absorbing light to facilitate vision. It is covalently linked to vitamin A (11- cis- retinal) (Bellingham et al., 1997).

Opsin was identified in two *Acacia* honey samples from Asir region and one *Ziziphus* honey from Jazan region. The trypsin digest amino acid sequences of opsin were TLAGK and MNVK with 21 % score. The precursor masses of the trypsin digest were 488.1147, 488.1644, 488.2413, 488.8096, 488.8724, 488.8731, 488.8760, 488.9022, 489.0376, 489.0431, 489.1615, 489.1807, 489.2210, 489.8748, 489.8775, 490.0479, 490.1144, 490.1666, 490.1833, 490.3216, 490.8693, 491.1555, 491.1606, 491.1868, 491.8691, 492.1520, 492.1531, 492.1704, 492.2201 and 492.4630. The number of amino acids and pI of the opsin protein are 377 and 8.53, respectively (Table.2 and Table.3).

It is not reported before that the opsin is present in honey samples. The opsin blue- sensitive protein digestion may produce short peptides and amino acids which add more biological and medicinal values to honey samples.

3.3.9. Olfactory protein (sensory neuron membrane protein 1)

The sensory neuron membrane protein 1 is an olfactory protein that plays olfactory role not restricted to pheromone sensitivity (Nichols & Vogt, 2008).

The sensory neuron membrane protein 1 was detected in a *Ziziphus* honey sample from Jazan region and the amino acid sequences of its trypsin digest were NY K and YLK with 18 % as score percentage. The precursor masses of the trypsin digest were 422.6805, 423.0658, 424.0690, 424.6800 and 425.0591. The sensory neuron membrane protein 1 is composed of 520 amino acids with the pI value of 5.91 (Table.2 and Table.3).

Presence of The sensory neuron membrane protein 1 in honey is not stated before.

4. Conclusions

For the first time, this study reported nine types of proteins in honey samples from Asir and Jazan regions at the southwestern part of Saudi Arabia as follows: 1) Gene expression regulatory proteins (Protein Krueppel, Elongation factor – 1 alpha, Prohormone-3, and Homeoboxes E30, E60, H40 and H55), 2) Enzymes (ATP synthase, Arginine kinase, Cytochrome C oxidase, NADH Ubiquinone oxidoreductase and cystathionine beta synthase), 3) Bee venom proteins (Mellitin, Secapin, Waprin Thr-1, Allergen and Phospholipase A2), 4) Major Royal Jelly Proteins (MRJP-2, MRJP-3, MRJP-4 and MRJP-5), 5) Antimicrobial or immune peptides (Defensin-1, Hymenoptaecin, Apidermin-2 and Omega Conotoxin like protein-1), 6) Structural proteins (Histone-4 and small

ribosomal subunit es8), 7) Neuropeptides (PBAN-type neuropeptide and Orokinin), 8) Vision proteins (Opsin blue sensitive) and 9) Olfactory proteins (Sensory neuron membrane protein-1).

Moreover, this study reported the presence of 16 proteins in honey which are not discovered before including protein krueppel, prohormone-3, Arginine Kinase, COX, CBS, Secapin, Waprin Thr-1, Mellitin, Hymenoptaecin, Omega-conotoxin, Apidermin-2, Small ribosomal subunit es8, PBAN- type neuropeptide, Orokinin, Opsin blue sensitive and Sensory neuron membrane protein-1.

This study reported presence of proteins in some of studied honey samples produced by honeybees not found in Saudi Arabia such as the Secapin-1 of *Apis cerana* and Cystathionine beta-synthase and ATP synthase of *Apis mellifera ligustica*, This finding reflected the usefulness of the honey proteomics in determining the honeybee origin of honey samples.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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