



Research article

Purification and characterization of proteins in multifloral honey from kelulut bee (stingless bee)



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ABSTRACT

The kelulut bee (*Meliponini*) is a subfamily of stingless bees that produce honey. A total of 89 species out of a total of 500 species of kelulut bees are known to originate from the Indo-Australian region. Kelulut bees do not have quality standards so they still refer to the Codex and EU Directive which basically only applied for *Apis* honey. The Codex and EU Directive are formed by several psychochemical parameters, one of it is diastase activity. Diastase activity in kelulut honey is known not to meet existing standards or even undetectable. Therefore, this study aimed to explore proteins inside kelulut honey and investigate the possibility of using a specific protein as a biomarker to differentiate honey produced by kelulut bee from other honey. This research can also be considered as an initial step to optimize the exploration of protein in kelulut honey. This research is divided into two sections which are the preliminary research and the research expansion. From preliminary section, glucose dehydrogenase enzyme (GDH) was found to be present inside *Tetragonula* spp honey. A further examination of GDH enzyme was made in four kelulut bee honeys namely *Tetragonula leaviceps*, *T. biroi*, *Heterotrigona itama*, and *Geniotrigona thoracica*. The preliminary research has five stages that are exactly the as expansion research section except it didn't include GDH activity measurement. The research includes seven main stages. First honeys were dialyzed to remove the sugar content followed by centrifugation. The samples were then purified using liquid chromatography with anion exchanger column. The molecular weight of proteins was analysed by SDS-PAGE method. The GDH activity was measured using spectrophotometer followed by qualitative analysis using LC-MS/MS. The peptide sequences resulted from LC-MS/MS were then matched with Uniprot to identify the unknown protein. The results showed that only *T. biroi* and *T. laeviceps* had GDH enzyme activity of 0,1891 U/mL and 0,1652–1,579 U/mL, respectively. Bands from both species were also qualitatively identified as GDH. With these results, it can be concluded that the GDH enzyme cannot be used as a biomarker to distinguish the kelulut honey.

1. Introduction

Kelulut bee is a subfamily of stingless bees with more than 500 species identified from 32 genera throughout the world (Michener, 2013). 89 species from 15 genus of kelulut bee are known to originate from the Indo-Australian region (Rasmussen, 2013). *Tetragonula biroi*, *Tetragonula leaviceps*, *Geniotrigona thoracica*, and *Heterotrigona itama* are the species that are known for their productivity and their honey has been commercialized.

Tetragonula sp. or Kelulut honeybee is one of the native Indonesian bee. From our previous research, this honeybee product is known having

a high degree of anti-inflammatory, antiangiogenic, antioxidant and anti-bacterial activity, anticancer, antidiabetes (Diva et al., 2019; Farida et al., 2019; Iqbal et al., 2019; Miyata et al., 2019). Bioactive compounds which is presumed gained from Kelulut honey have gained more interest recently in our previous research (Rahmawati et al., 2019; Sahlan et al., 2019; Shafira et al., 2019). The publication of kelulut bees has increased over the past 10 years from 288 to 1870 publications in 2018 (Avila et al., 2018). The growth of research in this field is based on the presence of beneficial bioactive compounds such as proteins that make honey have high antibacterial or antimicrobial and antioxidant activity (Alvarez-Suarez et al., 2018; Boorn et al., 2010; Jalil et al., 2017; Sahlan et al.,

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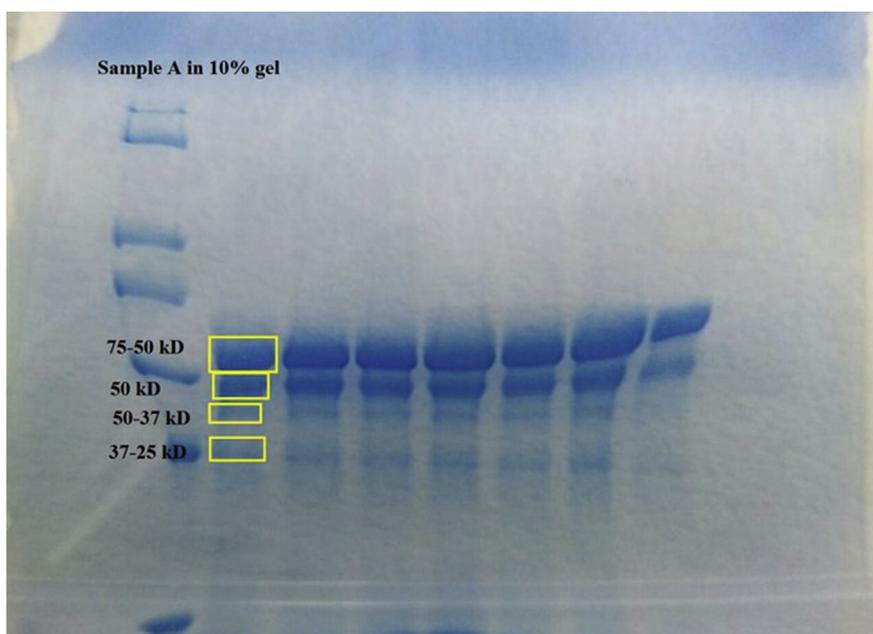


Fig. 1. *Tetragonula* spp (Sample A) show protein bands at 25–37 kDa, 37–50 kDa, and 50–75 kDa.

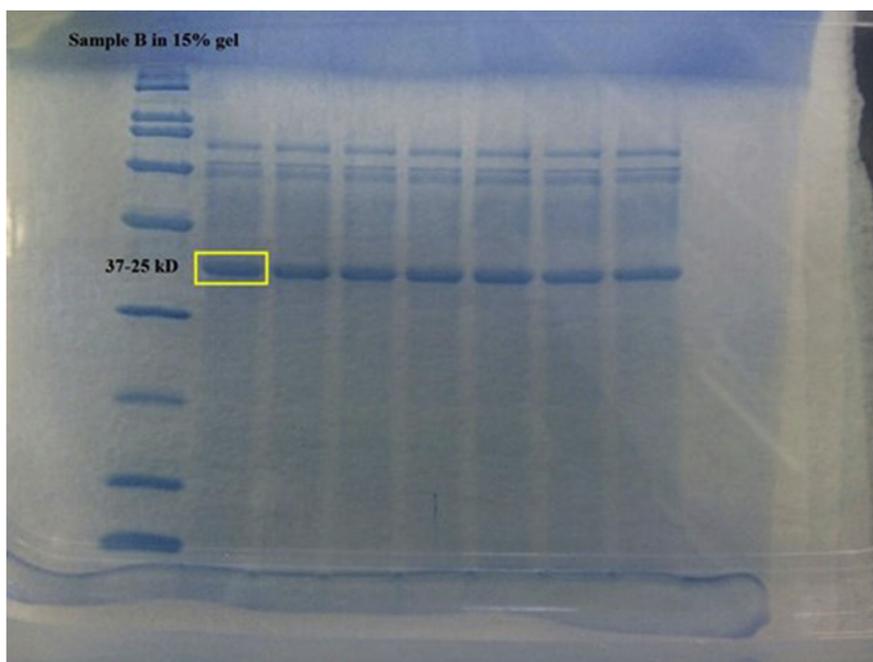


Fig. 2. *Tetragonula* spp (Sample B) show protein band at 25–37 kDa.

2018). It is known that the protein concentration in the *Tetragonula* sp. is very high, reaching 97 $\mu\text{g}/\text{mL}$ (Sahlan et al., 2018). The high economic value, the many benefits, and the absence of quality standard of kelulut bee honey makes this honey very likely to be faked.

Kelulut honey is known to be unable to meet the psychochemical parameters of existing honey standards, namely Codex and EU directive (Nordin et al., 2018). Codex is a reference for all honey at this time although it cannot be used for kelulut honey while the EU directive is only specific to *A. mellifera* which is in the *Apini* tribe. The standards are formed by several psychochemical parameters, one of which is diastase enzyme activity. Diastase enzyme activity exist in honey of *A. mellifera* and *A. dorsata*. However, it is not owned by some species of kelulut honey in Indonesia, while others are known to have diastase enzyme activity

that does not meet the standards of Codex or Eu directive.

This research was divided into two section the which were the preliminary research and the research expansion. The preliminary research was done as an exploration of proteins inside one species of kelulut honeys. Protein that present as a majority in SDS-PAGE result will be further examined through the research expansion section. The research expansion section was done to discover the existence of the identified proteins in the other kelulut honeys species. The discovery might lead to further research on the possibility of using the protein as biomarkers to differentiate kelulut honey from the others. In addition, the observed protein in kelulut honey may help to develop a quality standard for kelulut honey since it doesn't has its own quality standard yet.

In this study, we focused on the proteins contained within the

1. [KOX78881.1](#) Mass: 60070 Score: 141 Matches: 2(2) Sequences: 2(2) emPAI: 0.11
Glucose dehydrogenase [acceptor], partial [Melipona quadrifasciata]
 Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
182	664.8748	1327.7350	1327.7347	0.0003	0	71	0.017	1	U	R.EVILSAGTINSPK.L
198	832.9070	1663.7995	1663.8001	-0.0007	0	73	0.0089	1	U	R.NLGGTTLHHGMAYHR.G

2. [XP_003707372.1](#) Mass: 66621 Score: 73 Matches: 1(1) Sequences: 1(1) emPAI: 0.05
PREDICTED: glucose dehydrogenase [FAD, quinone]-like isoform X1 [Megachile rotundata]
 Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
198	832.9070	1663.7995	1663.8001	-0.0007	0	73	0.0089	1	U	K.NLGGTTIHHGMAYHR.G

Proteins matching the same set of peptides:
[XP_012149944.1](#) Mass: 54053 Score: 73 Matches: 1(1) Sequences: 1(1)
PREDICTED: glucose dehydrogenase [FAD, quinone]-like isoform X2 [Megachile rotundata]

3. [XP_011142158.1](#) Mass: 74409 Score: 71 Matches: 1(1) Sequences: 1(1) emPAI: 0.04
PREDICTED: glucose dehydrogenase [FAD, quinone] isoform X1 [Harpegnathos saltator]
 Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
182	664.8748	1327.7350	1327.7347	0.0003	0	71	0.017	1	U	K.EVILSAGTLNSPK.I

Proteins matching the same set of peptides:
[XP_011142162.1](#) Mass: 72792 Score: 71 Matches: 1(1) Sequences: 1(1)
PREDICTED: glucose dehydrogenase [FAD, quinone] isoform X2 [Harpegnathos saltator]

Fig. 3. MASCOT Search result returned possible protein identification as GDH.

multifloral honey of *Tetragonula* spp., given its high antioxidant activity and protein content, and the fact that few of these proteins have been characterized and that they could be useful biomarkers of honey quality. Proteins were characterized and separated by dialysis, ion exchange chromatography, and SDS-PAGE, which revealed bands with molecular weights divergent from those typically found for honey. The peptides at a band corresponding to 25–37 kDa were extracted and subjected to LC-MS/MS peptide mass fingerprinting analysis, with the results revealing a species of glucose dehydrogenase protein that has not been previously identified. These findings could reveal the molecular basis behind the beneficial properties of this honey and be useful for analyzing its purity.

2. Materials and methods

2.1. Samples

Preliminary research section only tested on one type of kelulut bees which was *Tetragonula* spp. The research expansion section tested on three species of kelulut bees which were *Tetragonula biroi*, *Tetragonula laeviceps*, *Geniotrigona thoracica*, and *Heterotrigona itama* multifloral honey were harvested from West Java, Lampung, and West Kalimantan Indonesia. All of the samples have a higher productivity than the other genus. After harvesting, the honeys were stored in a plastic bottle and kept at -4°C .

2.2. Preliminary research

This research contained five stages which were protein isolation, purification, recovery, characterization, and qualitative analysis. The protein isolation was done by dialysis the sample in 2 L of 50 mM Tris-HCl buffer solution (pH 8.0) to remove honey sugars. The protein purification was done using biologic duoflow chromatography system (Bio-Rad, USA) and an anion exchanger column to bind the targeted protein. The honey proteins were recovered using amicon® ultra centrifugal filters (Merck, Ireland) in order to get bands with higher intensity. The protein characterization was done using SDS-PAGE method to characterize different proteins based on their molecular weight and also to discover which bands had the highest intensity. The highest intensity bands were qualitatively identified by LC-MS/MS instrument. The more detailed process would be explained in the next research expansion section.

2.3. Research expansion

This section contained seven stages which were protein isolation, purification, characterization, concentration measurement, quantitative analysis or enzymatic activity measurement, recovery, and qualitative analysis or protein identification.

2.3.1. Honey protein isolation

20 mL of honey samples were dialyzed separately using a dialysis

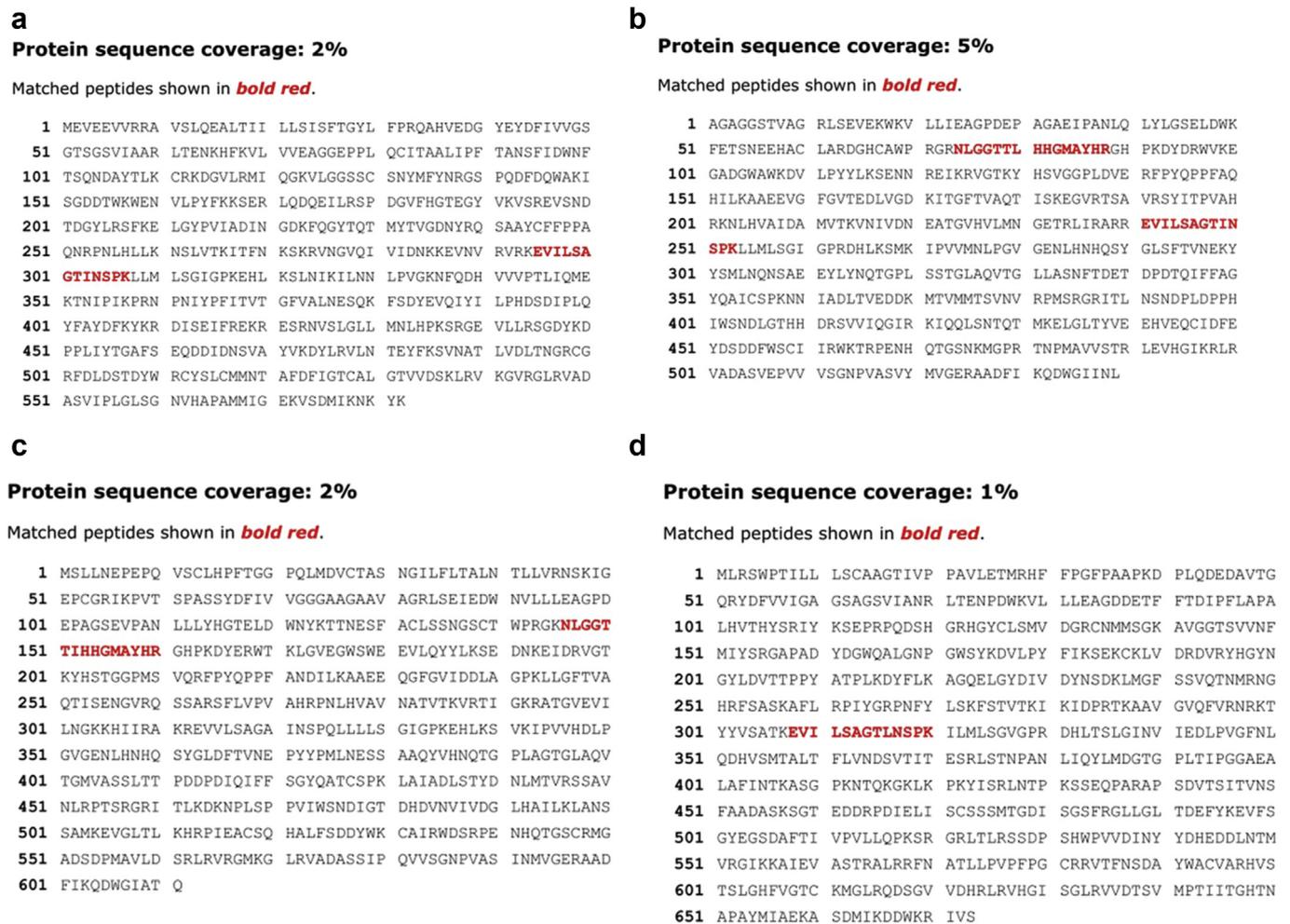


Fig. 4. Comparison to closest proteins that searched in NCBIProt. (a). CBH09301.1 with 2% protein sequence coverage. (b). KOX788881.1 with 5% protein sequence coverage. (c). XP_003707372.1 with 2% protein sequence coverage. (d). XP_011142158.1 with 1% protein sequence coverage.

Table 1
Fraction grouping and concentration measurement result.

Honey bee species	Grouped Fraction	Fraction Code	Volume (mL)	Concentration (mg/mL)
<i>T. biroi</i>	48–53	B1	12	1.0923
	54–58	B2	10	0.982
	1–6	B3	5,5	0.745
<i>T. laeviceps</i>	8–10	L1	2	1.733
	11–14	L2	2	3.427
	15–16	L3	2	1.836
	<i>G. thoracica</i>	10–11	T1	4
12		T2	2	1.3660
13		T4	2	1.1050
14		T5	2	0.9963
15		T6	2	0.8460
16–18		T7	6	0.7663
<i>H. itama</i>	8–9	I1	4	0.4123
	10–11	I2	4	0.8423
	12–14	I3	6	1.2237
	15–16	I4	4	1.2180

tubing in 2 L of 50 mM Tris-HCl buffer solution (pH 8.0). Dialysis was performed within 24 h with 3 times of buffer changing. After reaching 80–100 mL, the dialyzed samples were then centrifuged at 5000 rpm and – 4 °C for 30 min (Sahlan et al., 2018). The supernatants were collected and the pellets were disposed.

2.3.2. Honey protein purification

Biologic duoflow chromatography system (Bio-Rad, USA) was utilized as the protein purification instrument especially in wash, elution, and clean step as described by Gu et al. (2016) with slight modification (Gu et al., 2016). A peristaltic pump was also utilized to apply the sample to the column. A ResourceQ column containing quaternary ammonium cation resin was chosen as the anion exchanger. Tris-HCl buffer (buffer A) was used as the equilibration buffer solution and Tris-HCl with 0.5 M NaCl (buffer B) was used as the elution buffer in ion exchange chromatography. Before applying the sample to the column, the sample was filtered by using mixed cellulose ester having pore and diameter of 0,45 µm and 47 mm respectively (ADVANTEC, Japan) to prevent any contaminant from entering the column. The sample was applied to the column using peristaltic pump (EYELA MP-2000, Japan). After connecting the column to the purification instrument, sample was washed by isocratic flow mode of 100% buffer A. The purification process continued by setting the elution process to gradient linear mode of 100%–0% buffer A and 0%–100% buffer B. The system was kept at – 4 °C with a flow rate of 3.0 mL/min. Most peaks were detected from 12 min to 26 min of the process for all samples. Tens of fractions were collected and ready to be grouped by SDS PAGE.

2.3.3. Honey protein characterization

The characterization of purified protein was done using methods as described by Tamura et al. (2015). Purified protein samples were then subjected to SDS-PAGE. Subsequently, the membrane was washed with

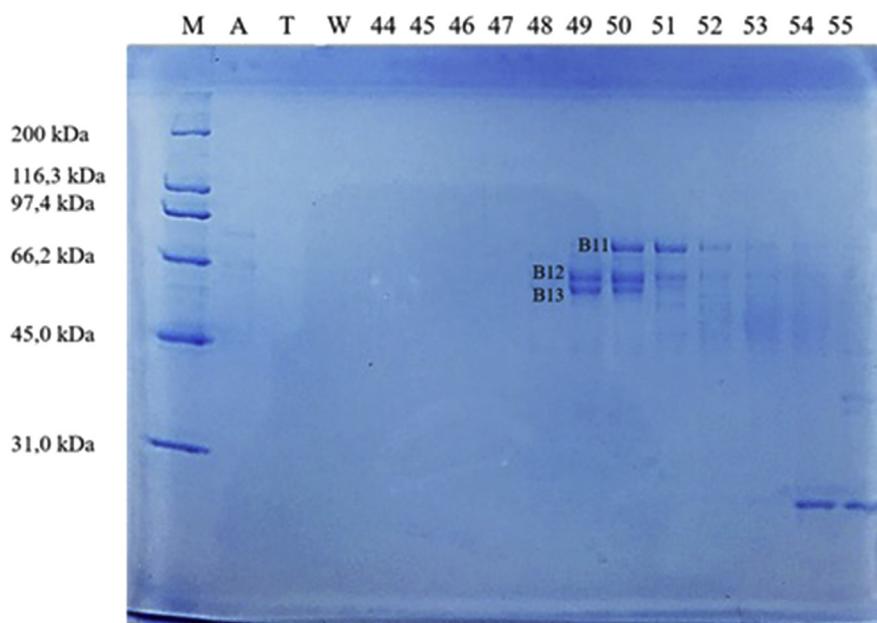


Fig. 5. Honey protein characterization of *T. biroii* resulted in 3 major bands which were B11, B12, and B13.

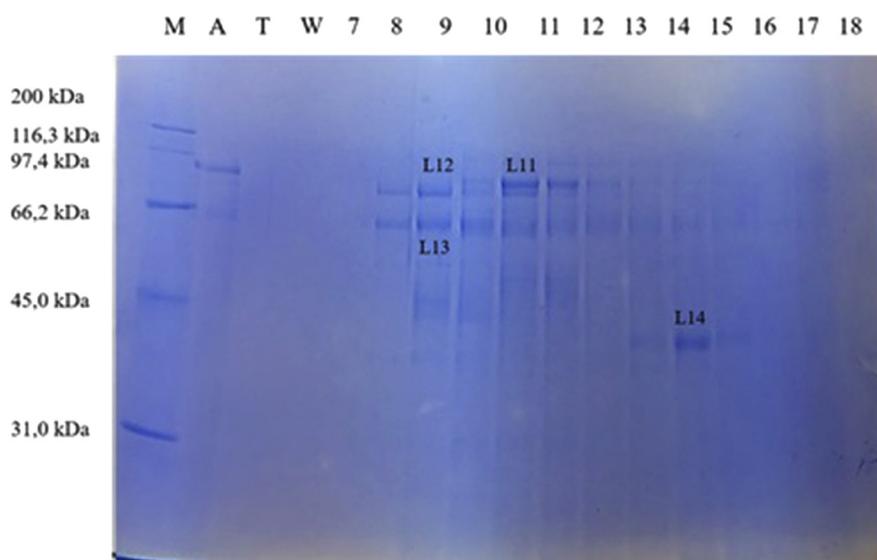


Fig. 6. Honey protein characterization of *T. laeviceps* resulted in 4 major bands which were L11, L12, L13, and L14.

water and stained using Coomassie Brilliant Blue-G (Tamura et al., 2015). Fractions with similar band were grouped into one group fractions because similar band patterns indicated the existence of similar proteins. The grouped fractions were checked for their protein concentration and enzyme activity.

2.3.4. Protein concentration measurement

Measurement of the total protein concentration of the purified samples was carried out using a nanodrop instrument. The purified samples were not plenty in volume and that was why nanodrop was used because it only required 1–2 μ L of sample on each measurement. The concentration measurement was triplicate and the results were then averaged.

2.3.5. Enzymatic activity measurement

The volume activity measurement of GDH was carried out to ascertain

the presence of the enzyme in the sample. Reagent for the measurement were consist of 1 M D-glucose solution, 0.1 M potassium phosphate pH 7.0, 0.9 mM dichlorophenolindophenol (DCIP) solution, and 30 mM PMS solution based on the Kikkoman Company protocol. 600 μ L of D-glucose and 2050 μ L of potassium phosphate buffer pH 7.0, and 150 μ L of DCIP solution were put into cuvette and mixed one at a time. The solution incubated at 37 °C for 3 min. 0.1 mL of PMS and 0,1 mL of were also added to the solution and mixed one at a time. The absorbance value was measured against time from 30 to 90 s (1 min) at 600 nm. 1 unit of enzyme activities is defined as the reduction of 1 μ mol DCIP per minute under the used assay conditions. Water was used as the blank.

2.3.6. Honey protein recovery

The sample resulted from purification was concentrated using amicon® ultra centrifugal filters (Merck, Ireland) with capacity of 4mL and

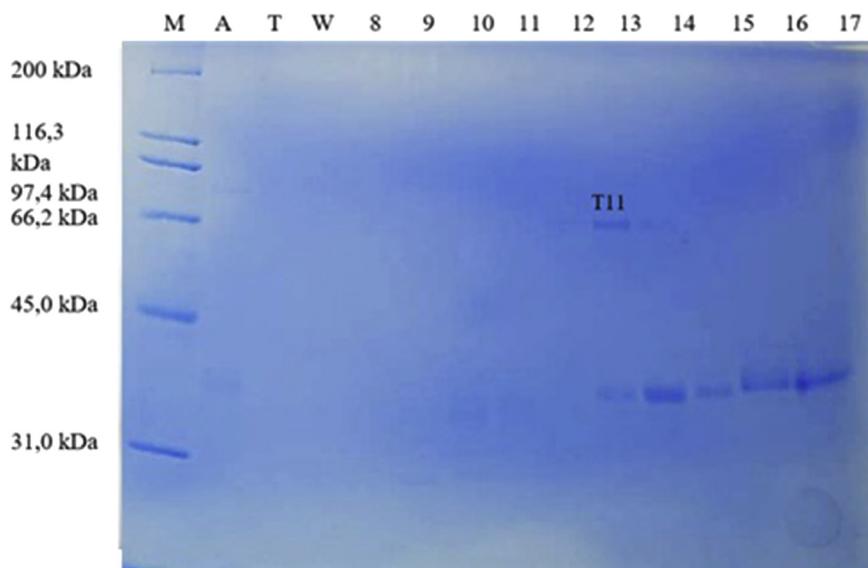


Fig. 7. Honey protein characterization of *G. thoracica* resulted in 1 major band which was T11.

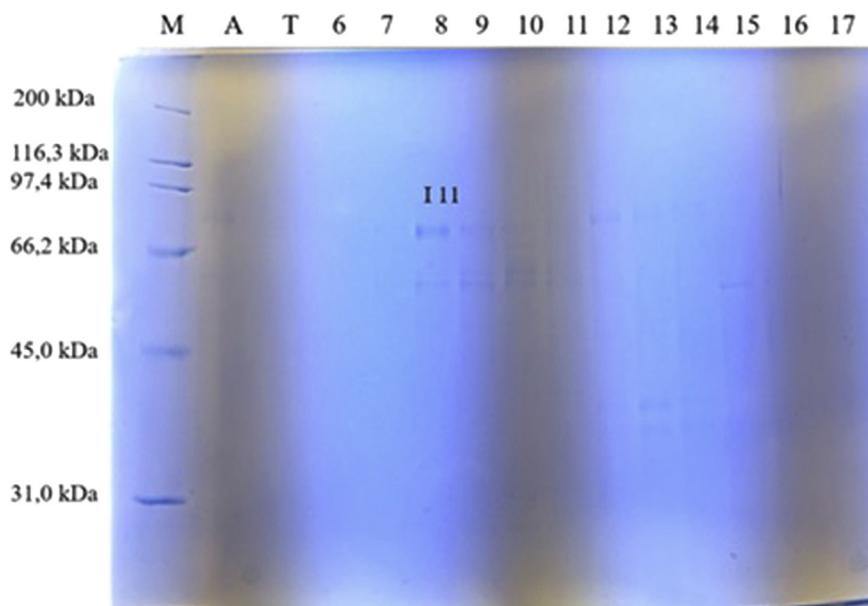


Fig. 8. Honey protein characterization of *H. itama* resulted in 1 major band which was I11.

MWCO of 10 kDa in order to get the higher bands intensity (Sahlan et al., 2018). The concentrated proteins were collected from the bottom part of the filters. The concentrated proteins were characterized through the same protocols as the previous characterization except the wells all of the wells were filled by 1 type of samples. This was done to increase the number of targeted bands to be analysed by LC-MS/MS instrument.

2.3.7. Honey protein identification

The bands corresponding to honey protein subunits were then extracted, destained, and digested in-gel using trypsin. Next, tryptic peptides were extracted and then further processed in preparation for LC-MS/MS analysis (Chua et al., 2015; Sahlan et al., 2018). The peptide sequences resulted from LC-MS/MS were then analyzed proteome discoverer 2.1. The obtained sequences were also matched with the protein databank, UniProt, so that the protein exist in the band could be identified.

3. Results and discussion

3.1. Preliminary research

3.1.1. Honey protein characterization

Major purified proteins in *Tetragonula* spp. honey were subjected to SDS-PAGE and exhibited bands at 25–37, 37–50, and 50–75 kDa for Sample A (Fig. 1) and 25–37 kDa for Sample B (Fig. 2). Typical honey protein bands were present at around 50 kDa (Won et al., 2008); thus, the existence of 25–37 and 50–75 kDa bands is interesting, potentially revealing the existence of novel honey proteins. In this research, we focus on the 25–37 kDa band because it was observed in both Samples A and B.

3.1.2. Honey protein identification with MASCOT search and PROSITE

After destaining and in-gel digestion, the 25–37 kDa band was then extracted from the gel (Rigaut et al., 1999) and was subjected to LC-MS/MS for PMF analysis, after which the NCBIProt database was

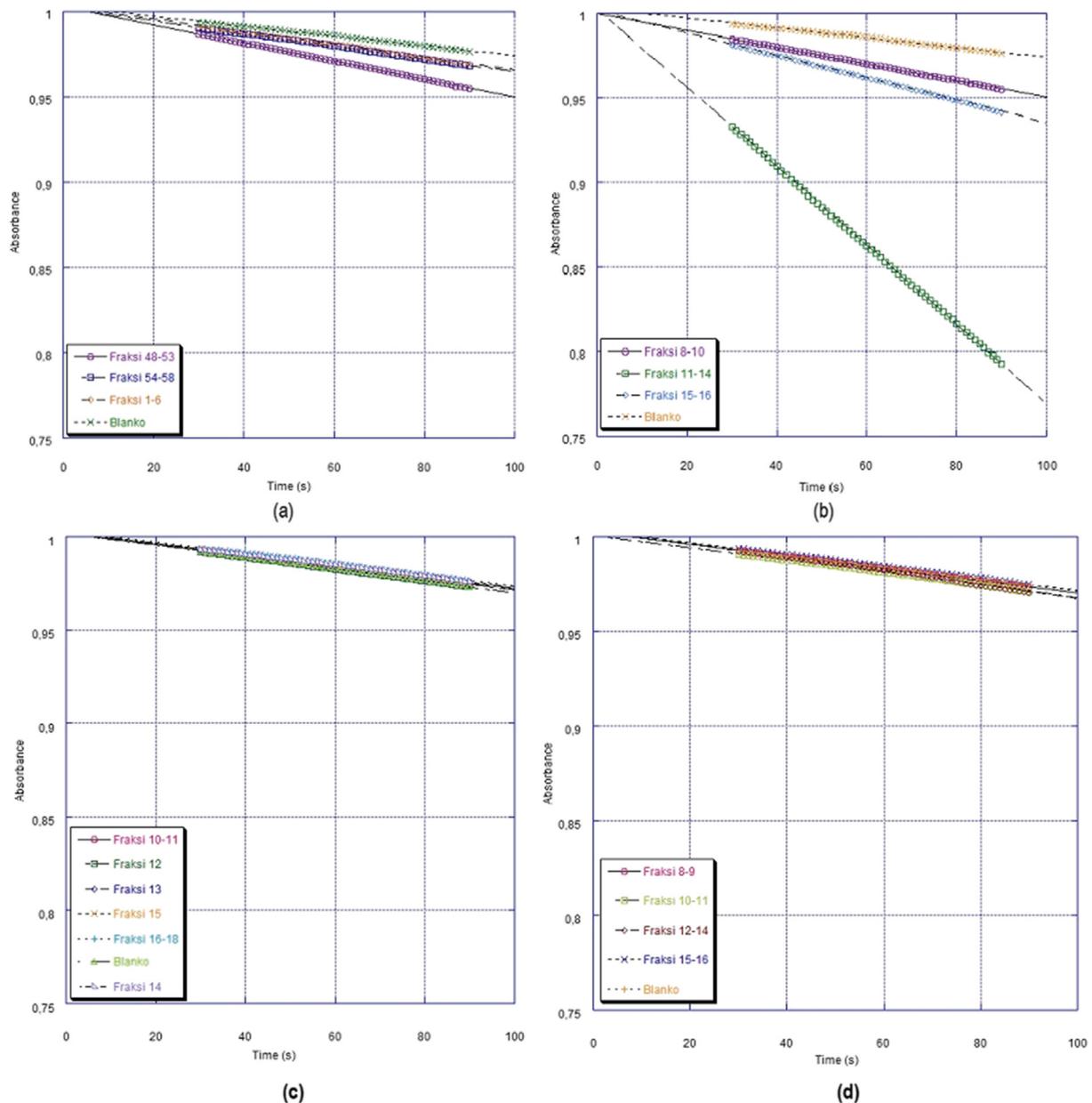


Fig. 9. Absorbance vs time graphs from spectrophotometer measurement. (a) *T. biroi* (b) *T. laeviceps* (c) *G. thoracica* (d) *H. itama*.

searched using the obtained results (Chua et al., 2015; Cottrell, 2011). The search was performed using MASCOT search.

The search result returned a possible match which is glucose dehydrogenase, for which the search revealed an ion score of 71–73. Further analysis was performed using Peptide View of MASCOT search (Fig. 3), and then the corresponding peptides were used as queries for searches using PROSITE (Fig. 4) to determine whether they matched any previously registered patterns (De Castro et al., 2006; Sigrist et al., 2002, 2012). The search results showed that some peptide sequences had the same general signature pattern sequence as glucose dehydrogenase (GMC_OXRED_2), which has the following sequence: [GS]-[PSTA]-x(2)-[ST]-[PS]-x-[LIVM](2)-x(2)-S-G-[LIVM]-G (Sigrist et al., 2012). This identified sequence strongly suggests that the protein corresponding to the band at 25–37 kDa is glucose dehydrogenase (De Castro et al., 2006). However, despite having the signature sequence of glucose dehydrogenase, the sequences had low (<5%) protein sequence coverage with any known protein sequences in the NCBIProt database,

with numerous peptides showing no results at all within the search. This indicates that the protein identified in the band corresponding to the size of 25–37 kDa is potentially a new protein species of glucose dehydrogenase that has never been found or registered in the NCBIProt database.

3.2. Research expansion

3.2.1. Fraction grouping and protein concentration measurement result

Band pattern similarity from each fraction of each sample was observed. Similar proteins have a greater chance to be found in the fractions with similar band patterns. This step also made the number of the observed fractions decrease. The grouping results are listed in Table 1. The result of SDS-PAGE also showed that bands were visible at below 31 kDa and 45–97.4 kDa for *T. biroi* sample (Fig. 5), 31–45 kDa and around 66.2–97.4 kDa for *T. laeviceps* sample (Fig. 6), 31–45 kDa and 66.2–97.4 kDa for *G. thoracica* sample (Fig. 7), and 66.2–97.4 kDa for *H. itama* sample (Fig. 8). The targeted bands for further examination were

Table 2
Volume activity measurement result.

Grouped Fraction	Fraction Code	Concentration (mg/mL)	Volume Activity (U/mL)	Total Activity (U)	Specific Activity (U/mg)
<i>T. biroi</i>					
48–53	B1	1.0923	0.189154	2.2699	0.1732
54–58	B2	0.982	0.067941	0.6794	0.0692
1–6	B3	0.745	0.064081	0.3524	0.0860
<i>T. laeviceps</i>					
8–10	L1	1.733	0.165221	0.330441	0.095351
11–14	L2	3.427	1.579632	3.159265	0.460937
15–16	L3	1.836	0.294154	0.588309	0.160186
<i>G. thoracica</i>					
10–11	T1	1.0733	-0.013897	-0.055588	-0.012948
12	T2	1.3660	0.009265	0.018529	0.006782
13	T4	1.1050	-0.000772	-0.001544	-0.000699
14	T5	0.9963	-0.016213	-0.032426	-0.016273
15	T6	0.8460	-0.000363	-0.000727	-0.000429
16–18	T7	0.7663	-0.003088	-0.018529	-0.004030
<i>H. itama</i>					
8–9	H1	0.4123	0.010037	0.040147	0.024341
10–11	H2	0.8423	0.015441	0.061765	0.018331
12–14	H3	1.2237	0.042463	0.254779	0.034702
15–16	H4	1.2180	0.002316	0.009265	0.001902

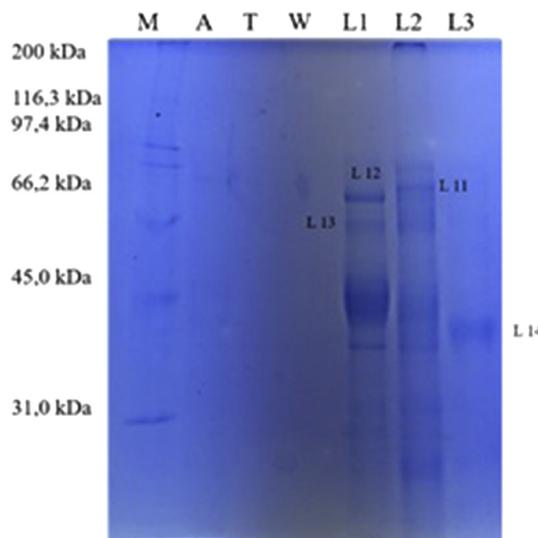


Fig. 11. Honey protein characterization after protein recovery of *T. laeviceps* resulted in 4 major bands with higher intensity.

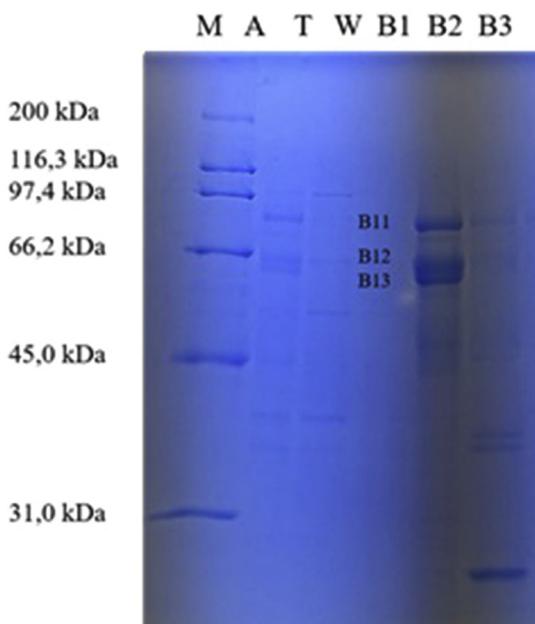


Fig. 10. Honey protein characterization after protein recovery of *T. biroi* resulted in 3 major bands with higher intensity.

predicted to be above 50 kDa.

Table 1 also provides the result of total protein measurement. It can be seen that *T. laeviceps* has the highest concentration of 1.733 mg/mL, 3.427 mg/mL, and 1.836 mg/mL for 8–10, 11–14, and 15–16 fractions respectively.

3.2.2. Enzymatic activity measurement

After purification, the GDH volume activity was measured using spectrophotometer (Fig. 9). The existence of GDH can be predicted by utilizing the time vs absorbance graph. The graph that coincide with the blank graph means that no activity present in the sample. Meanwhile, the graph with steep gradient shows high activity. *T. biroi* was predicted to have the highest activity at 48–53 because this graph was steeper among others. *T. laeviceps* was predicted to have the highest activity at 11–14. Meanwhile, no activity was predicted to be existed in *G. thoracica* and

Table 3
Volume activity result comparison.

Research	Organism	Volume Activity (U/mL)	Total Activity (U)	Specific Activity (U/mg)	Definition of 1 Unit Enzyme Activity
This research (2019)	<i>T. biroi</i>	0.1892	22.6990	0.1732	Reduction of 1 μmol DCIP per minute.
	<i>T. laeviceps</i>	1.5796	3.1593	0.4609	
(Sygmund et al., 2011)	<i>Glomerella cingulata</i>		215,000	165	Reduction of 1 μmol glucose per minute.
(Yamaoka et al., 2008)	<i>Burkholderia cepacia</i>	7			Oxidation of 1 μmol glucose per minute.

H. itama because all the graphs coincided with the blank graph. Calculation of volume activity was necessary to validate the prediction. The calculation results are listed in Table 2.

This research use Kikkoman company's protocol for determining the volume activity of GDH enzyme. The protocol also set 0.1 U/mL as that the minimum value of the volume activity. GDH only detected in *T. biroi* and *T. laeviceps*. In *T. biroi*, B1 has GDH activity of 0.189154 U/mL at a concentration of 1.092 mg/mL (Fig. 10). In *T. laeviceps*, L1, L2, and L3 has GDH activity of 0.165221 U/mL, 1.579632 U/mL, and 0.294154 U/mL respectively (Fig. 11). From here the author decided to focus only on *T. biroi* and *T. laeviceps* knowing that the enzyme activity only detected in that species.

The obtained volume activity result initially wanted to be compared but the comparison couldn't be done because the assay protocol, the definition of 1 unit enzyme activity, and the organism origin were different. It was very difficult for the authors to find the suitable one for the comparison since research on the GDH volume activity in kelulut honey had never been done before. The comparison trial can be seen in Table 3.

3.2.3. Honey protein recovery result

Honey protein had been diluted and so the protein content was lower than the original condition. Lower protein content means lower intensity of protein showed on the gels. Qualitative analysis with LC-MS/MS

Table 4
Qualitative analysis result.

Fraction	Band Code	Volume Activity (U/mL)	Origin	Coverage (%)	Unique Peptide	Score Sequest HT	Result
48–53	B11	0.189154	<i>Melipona quadrifasciata</i>	3.8961	2	4	Glucose Dehydrogenase
48–53	B12	0.189154	-	-	-	-	-
48–53	B13	0.189154	-	-	-	-	-
11–14	L11	1.579632	<i>Danaus plexippus</i>	4.2910	2	16	Glucose Dehydrogenase
8–10	L12	0.165221	-	-	-	-	-
8–10	L13	0.165221	-	-	-	-	-
15–16	L14	0.294154	-	-	-	-	-

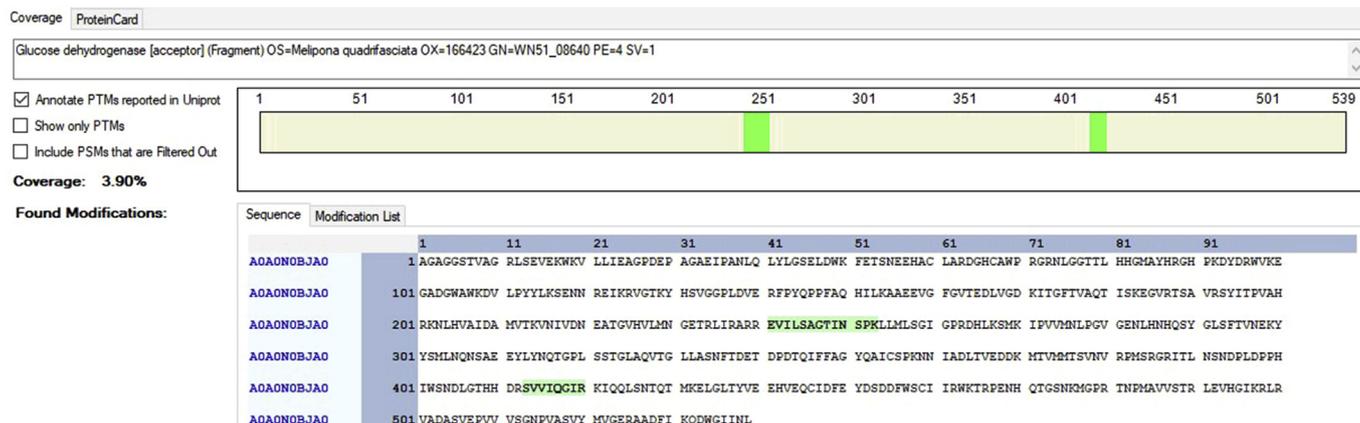


Fig. 12. Peptides sequences of B11 band codes of *T. biroi* showing 3.90% of coverage sequences.

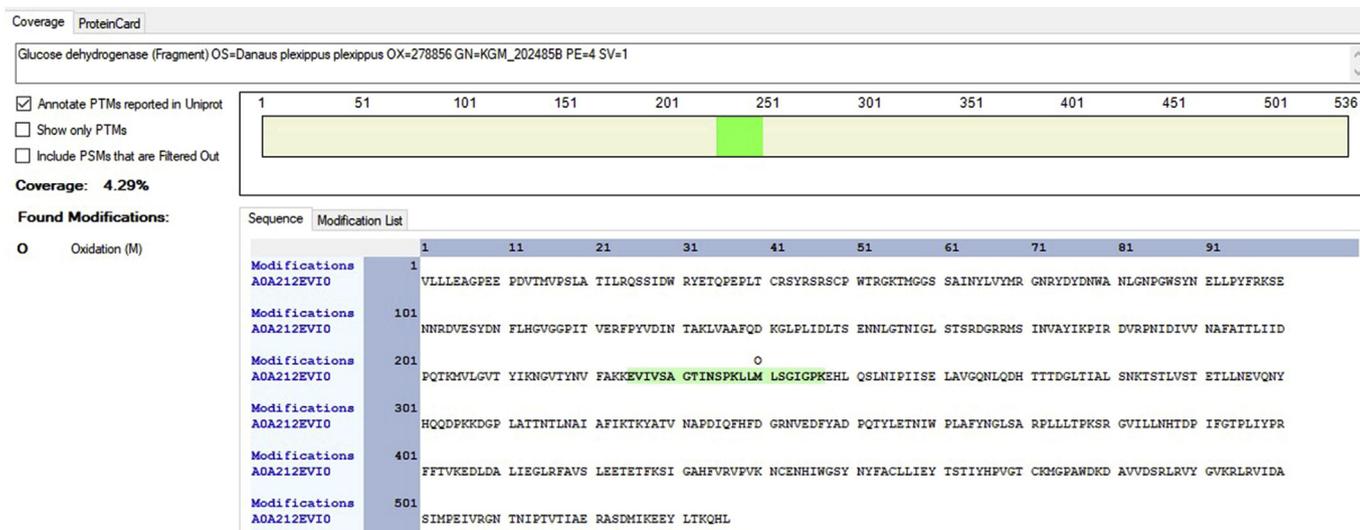


Fig. 13. Peptides sequences of L11 band code of *T. laeivipes* showing 4.29% of coverage sequences.

instruments required high intensity band so the protein needed to be concentrated through a stage called recovery. Besides concentrating protein, this stage was also intended to duplicate the fraction that had the targeted protein bands. Only two samples were concentrated which were *T. biroi* and *T. laeivipes* since they had GDH enzyme activity. There were seven high-intensity bands that would be analyzed qualitatively by LC-MS/MS instrument.

3.2.4. Honey protein identification with proteome discoverer 2.1 and sequest™ HT

After destaining and in-gel digestion, the 25–37 kDa band was then extracted from the gel (Rigaut et al., 1999) and was subjected to LC-MS/MS for PMF analysis, after which the UniProt database was

searched using the obtained results (Chua et al., 2015; Cottrell, 2011). The search was performed using Sequest™ HT search engine.

Table 4 summarized the result of protein identification. B11 was known to have as much as 3.8961% of protein sequences (Fig. 12) that was coverage while L11 have 4.2910% of protein sequences (Fig. 13) that was coverage. This value was sufficient to conclude GDH enzyme as the identified protein in this analysis because the protein coverage sequence was located on the catalytic side of the enzyme whose sequence was very specific for only one type of enzyme. In addition, the level of confidence of the results can also be seen from the number of unique peptides which were 2 for both B11 and L11 and the score sequest HT which were also above 1.4.

4. Conclusion

The preliminary research section was intended to search for a strong protein candidate that can be further examined through the research expansion section. 25–37, 37–50, and 50–75 kDa. The band at 25–37 kDa bands were found to be existed in *Tetragonula* spp. The bands were then extracted for further analysis, followed by destaining and digestion in-gel. These peptides were analyzed by PMF using LC-MS/MS. The results revealed a possible match with glucose dehydrogenase with an ion score of approximately 71–73. Further analysis confirms this match since the peptide shows a signature pattern of glucose dehydrogenase (GMC_OXRED_2). Despite showing a signature pattern, the sample exhibit a low protein coverage (<5%) to any known glucose dehydrogenase in the NCBIProt database. These findings strongly suggest the existence of a new protein species of glucose dehydrogenase in *Tetragonula* spp. multiflora honey.

The research expansion section was intended to explore the presence of GDH in some kelulut honeys species and observe GDH enzyme more deeply through the volume activity measurement. After measuring the GDH volume activity using spectrophotometer, the result showed the GDH enzyme activity was detected only in *T. biroi* and *T. laeviceps* samples and was not detected in *G. thoracica* and *H. itama* samples. This indicates the utilization of GDH enzyme as one of the biomarker candidates to distinguish kelulut honey is not visible. Qualitative analysis was done to make sure that the isolated proteins were really GDH. LC-MS/MS instrument was used to get the peptide sequences of the examined proteins. From seven analyzed peptides, only B11 and T11 were identified as GDH enzyme with the protein coverage sequence of 3.8961 % and 4.2910 %. Coverage is defined as the percentage of protein sequences covered by the identified peptide. The matching process occurred on the catalytic side of the enzyme. The catalytic side of the enzyme is very specific thus the obtained result has a high confident level. In addition, the confidence level of the results can also be seen from the number of unique peptides which were 2.0 and the score sequest HT which were also above 1.4 for both identified bands. Unique peptides represent a peptide sequence that exist only in specific protein. Meanwhile, the score sequest HT represent a value obtained from matching the experimental peptide fragment to the theoretical spectrum consisting of the order of b and y ions. A further research needs to be done to find other biomarker that can distinguish honey from kelulut since GDH only found in two out of four observed sample.

Declarations

Author contribution statement

Muhamad Sahlan: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Kaysa Faradis Mahira, Ihsan Wiratama, Alfiani Guntari Mahadewi: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Masafumi Yohda, Heri Hermansyah, Keiichi Noguchi: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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