Original Article

PROPERTIES AND ANTIMICROBIAL ACTIVITY OF *APIS DORSATA* HONEY FROM THAILAND

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ABSTRACT

Objectives: For *Apis dorsata* honey, the basic properties, namely the pH, and the total proline, protein and invert sugar contents, were determined. For proteins, the mass weight and partial amino acid sequence of the three major proteins were assayed and homologs screened for. A bioassay for antimicrobial activity was also performed.

Methodology: Proline content and the percentage of invert sugar were evaluated, whilst the total protein content was assayed and the major protein components were analyzed by reducing SDS-PAGE. From the excised bands partial amino acid sequences deduced by MALDI-TOF MS and homologs were searched for by Mascot database. Antimicrobial activity was assayed by the agar well diffusion method.

Results: The proline, invert sugar and total protein contents were 6.35µg/ml, 13.2% (w/w) and 0.16% (w/w), respectively, with three major protein bands of 50, 75 and 100 kDa. All tested (25-100% (v/v)) dilutions of honey could inhibit the growth of *S. aureus*.

Conclusion: A. dorsata honey may contain an epinecidin homolog, an antimicrobial peptide, and can inhibit the growth of some bacteria. It suggests that honey could be used as an antimicrobial agent.

KEYWORDS: *Apis dorsata*, Honey, Proline, Invert sugar, Epinecidin, Inhibition zone.

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INTRODUCTION

Honey is modified from the nectar of plants by the function of α – glucosidase by bees as a storage food source, but is widely consumed by humans (and other animals) as food. Honey is typically composed of fructose and glucose (79% (w/v)), H_2O (20% (v/v)), and minor acids such as gluconic acid (0.5% (w/v)), and minerals, such as calcium, magnesium, potassium and phosphorus, together with vitamins, such as riboflavin and niacin (0.5% (w/v)).[1] The main components of honey are the two monosaccharides, fructose and glucose; this allows it to be highly saturated and of a high osmotic potential providing at least some of the antimicrobial properties in addition to the sweet taste sought after by other animals, including bears and humans. In contrast, gluconic acid provides the sour taste. The properties of different honey batches will depend upon the nectar (and pollen) sources and, therefore, the plants foraged. For example, different honey sources can differ in terms of their taste, smell and color (from light yellow to darkish brown).[2] Not only is honey nutritional, as a rich source of monosaccharides, trace minerals and vitamins, but it also inhibits the

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growth of microbial pathogens. It was recorded that Egyptians used honey in ancient medicine to prevent localized bacterial infections by topical application in surgery and other wounds.[3] The antimicrobial activity of honey may principally be due to its low moisture content and high osmotic pressure, which can desorb water out of microbe cells (hypotonic condition) leading to either the death or the growth inhibition of bacteria, fungi and yeasts.[4-5] Nowadays, although antibiotics are widely used in the prevention of localized and systemic microbial infections, there is still the increasing problem of antibiotic resistance. Using honey for the growth inhibition of microorganisms might be an alternative way in some suitable cases for topical application and, perhaps, for some partially systemic infections following oral administration.

Not only does the property of honey depend on the plant nectar, but it also depends on the species of honeybee. This probably in the main reflects the different flower nectar and pollen collecting preferences of different bee species, as well as to some lesser extent the different biochemical and microbiological components. In Thailand, as with most of Southeast Asia, the species diversity of honeybees is high and consists of four native *Apis* species, *Apis dorsata, A. cerana*, *A. florea* and *A. andreniformis*, and one imported *Apis* species, *A. mellifera*. In this research, *A. dorsata* was selected for study since honey from this wild species is the most popular in local consumption and sales. For the purposes of the present study, the properties of honey, in terms of pH, proline and total protein and invert sugar contents, were determined. The main proteins in honey were evaluated by reducing SDS-PAGE resolution and the partial amino acid sequences of three of the main protein bands were obtained. Furthermore, antimicrobial activity from various dilutions of honey was observed on selected bacterial pathogens, yeasts and fungi.

METHODOLOGY

Sample collection: Honey of *Apis dorsata* was purchased from a bee farmer in the Samut Songkram province.

Proline content: The proline content was determined as described previously[6] with reference to a proline standard curve constructed from 100, 200, 300, 400 and 500µg/ml. Honey was diluted in water to 2% (v/v) and then 250 ml was mixed with formic acid (130 ml) and 3% (w/v) Ninhydrin solution (500 ml). The mixture was boiled for 15 min, allowed to cool, and mixed with isopropanol (2.5 ml). The absorbance was measured at an incident light of 520 nm (1 cm path length).

Total protein content: The total protein content of honey was evaluated as described previously[7] with reference to a Bovine Serum Albumin (BSA) standard curve constructed from 0, 0.05, 0.10, 0.15, 0.20, 0.25 and 0.30 mg/ml. Honey was diluted in water to 5 % (v/v) and then 20 ml of this was mixed with Bradford solution (200 ml), incubated at RT for five min, and then the absorbance was measured at 595 nm (1 cm light path).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE): The principal protein component of honey was resolved and visualized by separation on a discontinuous reducing SDS-PAGE gel, comprised of a 12% (w/v) PA separating gel and 4% (w/v) PA stacking gel. The honey sample was mixed with 5x loading dye, heated to 80°C for five min, and cooled on ice prior to loading. The electrophoresis was performed at 100 V until the dye front reached the bottom of the gel. After electrophoresis, the gel was coomassie blue-stained as summarized below.

Coomassie brilliant blue (CBB) staining and amino acid sequencing: After electrophoresis, the SDS-PA gel was incubated in 1.25% (w/v) CBB / 10% (v/v) acetic acid / 50% (v/v) methanol for 30 min, and then destained (the solution of 10% (v/v) acetic acid and 10% (v/v) methanol) until the background was clear. Major visible protein bands were cut from the gel and sent to the Bioservice Unit (BSU) of Thailand for commercial evaluation of the partial amino acid sequences by Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight Mass Spectrometry (MALDI-TOF MS). The obtained mass spectrometry was used to search for amino

acid sequences in the MSDB protein data base by Mascot searching.

Agar well diffusion method: Honey, at a dilution of 25, 50, 75 and 100 (v/v) % was made. Four microorganisms, *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillus niger*, representative of human pathogens from Gram^{+ve} and Gram^{-ve} bacteria, yeast and fungi, respectively, were selected for this study. The microbe cultures were grown in LB broth for the two bacterial species and PDB for the yeast and fungi, respectively. A culture of each of the above microorganisms was inoculated into LB or PDB media, as appropriate, and grown until the O.D. at 600 nm (1 cm light path) was 0.1. Then, 200 ml of culture was spread onto each of triplicate 11 cm diameter LB or PDB agar plates, as appropriate, per assay, and once adsorbed into the agar a hole was made in the center using a cork borer (# 6), and 200 ml of the desired honey solution (0-100 % (v/v)) was added in the hole. Three replications of each honey dilution and of each microorganism were made. All samples were then incubated at 37^{o}C for three days and the diameter of the clear inhibition zone was measured daily. Comparison of the size of the inhibition zone induced by honey at the different concentrations, for each of the various microorganisms, was analyzed by One Way ANOVA (SPSS program).

Percentage of invert sugar: The method was followed as described previously.[6] A 2% (v/v) honey solution (10 ml) was mixed with Fehling's solution (2.5 ml), boiled for 15 min, and then 0.2% (w/v) methylene blue (500 µl) was added and diluted honey was titrated into the mixture until the blue color was gone. The volume of added honey was recorded (X ml). In a new flask, 2.5 ml Fehling's solution was mixed with dd-H_2O (at the volume of 12.5–X ml), boiled as above and 0.2 % (w/v) methylene blue (500 µl) was added. Diluted honey was then titrated into the mixture until the blue color was gone and the volume of added honey was recorded (Y ml). The percentage of invert sugar was then obtained from 25,000/[(gram of honey)(Y)].

RESULTS

Since honey is a supersaturated sugar solution, the amount of invert sugar present in the honey was evaluated and found to be, in the samples analyzed here, 13.2% (w/v). Other than simple monosaccharides, honeys have been reported to be fairly complex in minor components, for example, one sample is reported to be composed of at least 181 minor components.[8] Here, the total protein concentration was quantified as was the level of proline, since this is one of amino acids that can influence the aroma of honey. These results are summarized in Table-I.

After SDS-PAGE resolution of the honey and CBB staining, five distinct bands were visible and located within the 50-150 kDa size range (Fig-1). The two largest of these distinct bands were a close doublet and rather indistinct and thus likely to be problematic for direct MALDI-TOF MS analysis and so the three other smaller and more major proteins of 50, 75 and 100 kDa were excised as bands A, B and C, respectively, as indicated in Fig-1.

Partial amino acid sequences from these three major bands (bands A-C, Figure 1) were obtained by Matrix-Assisted Laser Desorption/ Ionisation Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) and Mascot Searching using the MSDB database.

For the first of these proteins, the major band of an apparent size of 50 kDa (A in Figure 1), the MALDI-TOF deduced peptide sequences most closely matched the conserved immunoglobulin superfamily, with the highest match being the immunoglobulin heavy chain vari-

Table-I: The four estimated properties of *Apis dorsata* honey.

Honey parameter	*Quantity of measured parameter*
pH	3.8
Proline content (mg/ml)	6.35
Protein concentration (g/100 g)	0.16
Percentage of invert sugar (%)	13.2

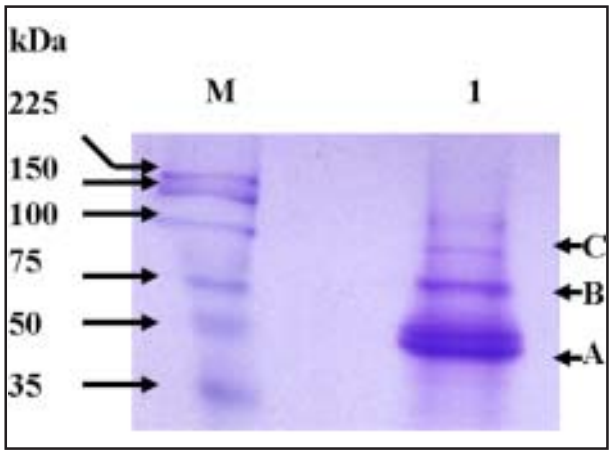

Fig-1: One dimensional reducing SDS-PAGE resolution of the total proteins in honey with coomassie brilliant blue (CBB) staining. Lane M contains protein molecular weight markers, with their sizes in kDa marked to the left. Lane 1 contain honey to total protein equivalents of 1 mg. Bands labeled A, B and C were excised from the gel, eluted and sent for commercial amino acid peptide sequence deduction via MALDI-TOF MS analysis.

able region in *Mus musculus* (Mouse), whose amino acid sequence in this region is shown below with the matching peptides from the honey protein (band A) being shown in bold.

1 CDGGSTYYPD TMERRFIISR DNTKKTLYLQ MSSLRSEDTA LYYCARRNGN
51 YVFAYWGQGT L

For the second protein band, a larger apparent MW protein of ~75 kDa (B in Fig-1), the MALDI-TOF deduced peptide sequences most closely matched the *Drosophila pseudoobscura* nsf attachment protein (snap) gene (GA 19734-PA), and its homologs from related dipteran species including *Anopheles* and *Aedes*. The fragment of the *D. pseudoobscura* snap gene that matches the derived peptides of honey protein band B is shown below with the bold text marking the matched peptides.

1 GDNEQKALQLMADAEKKLTQQKGFLGSLF GGGSNKVEDA IECYQRAGNM
51 FKMSKNWTKA GECFCEAATL HARAGSR HDA GTCYVDASNC YKKVDVENAV
101 ACLMKSIDIY TDMGRFTMAA KHHQSIAEMY EADSNTLAQS IQHYEQAADY
151 FKGEESVSSA NKCMLKVAQY AAQLEDYEKA ISIYEQVAAS SLESSLLKYS
201 AKEYFFRAAL CHLSVDLLNA QHAIQKYAEQ YPAFQDSREF KLIKILCEHL
251 EEQNIEGFTE AVKDYDSISR LDQWYTTILL RIKKAADEDP DLR

Finally, for the third protein, a band with an apparent MW of ~100 kDa (band C, Fig-1), the MALDI-TOF deduced peptide sequences most closely matched the antimicrobial 12 superfamily and in particular Epinecidin (Epinecidin-1 prepropeptide precursor) in *Epinephelus coioides* (Orange-spotted grouper). The complete sequence of this peptide is shown below, with the matching MALDI-TOF MS peptides shown in bold.

1 MRCIALFLVL SLVVLMAEPG EGFIFHIIKG LFHAGKMIHG LVTRRRHGVE
51 ELQDLDQRAF EREKAFA

Analysis of the results of the antimicrobial activity shows that neat honey is the most efficient concentration in order to inhibit the growth of all four tested microorganisms (Table-II), and indeed only honey at concentrations of >75% (v/v) were able to cause any detectable inhibition of proliferation of the tested yeast and fungi isolates. Within the two bacteria, *S. aureus* was more sensitive than *E. coli*, yielding an overall sensitivity to proliferation inhibition of *S. aureus* > *E. coli* > *C. albicans* > *A. niger* (Table-II).

DISCUSSION

Although largely composed of two monosaccharides (fructose and glucose) and some invert sugar, honey also contains a diverse array of minor components, the actual composition of which will vary from hive to hive and is likely to be principally varied by the plant species foraged and, therefore, bee species, geographical location and season, but also other factors like climate and environmental conditions will likely have an influence. Here, polyfloral honey of *A. dorsata* was assayed since the local Thai people consume and use polyfloral honey much more than unifloral honey. Four parameters were measured and reported (Table-I). Three of these parameters were expected to be involved in antimicrobial activities. The first

Table-II: The antimicrobial activity of honey, as detected by an agar well diffusion assay.

Types of *Pathogens*	Mean + S.D. of diameter of inhibition zone (cm)				
	0 %	*25 %*	*50 %*	*75 %*	*Neat*
	(Control)	*Dilution*	*Dilution*	*Dilution*	
E. coli	-	2.8 ± 0.00^{a}	3.3 ± 0.35^{b}	4.1 ± 0.23^{c}	4.3 ± 0.17^{d}
S. aureus	-	3.6 ± 0.10^{aa}	4.0 ± 0.15^{bb}	4.3 ± 0.12^{cc}	4.4 ± 0.17^{dd}
C. albicans	-	-	-	1.5 ± 0.10*	2.0 ± 0.10**
A. niger	-	-	-	1.2 ± 0.06^{A}	1.3 ± 0.10^{B}

Data are the mean + S.D. and are derived from independent repeats. – means no detectable zone of inhibition of proliferation was noted at all time points. Means within a row with different uppercase letters are significantly different at $p < 0.05$ level.

parameter was the honey pH, which is the degree of acidity. *A. dorsata* honey was found to be fairly acidic (pH 3.8), but within the reported pH range of other honey types such as pH 3.4-5.4 for *A. mellifera* honey from Switzerland.[1] The second parameter was the percentage of invert sugar and proline. Compared to the honey from *Apis mellifera* in Thailand, both the proline content and the percentage of invert sugar of *A. dorsata* honey were lower.[9] Sugar was one of the interests in this research since it provides a strong osmotic effect that is likely to play an important role in an antibacterial action.

Finally, the third component was total protein, which can contain anti-microbial. Anklam reported that honey contained about 0.2% (w/v) protein.[10] In this research, the total protein content of *A. dorsata* honey was slightly but not dramatically lower at 0.16% (w/v), and indeed was the same as that reported for heterofloral honey and close to that of chestnut honey (0.17% (w/v)).[4]

At present, the continued use, especially common misuse of antibiotics in some disease treatments may lead to an increasing resistance frequency in pathogen populations to unacceptable levels, necessitating alternative treatments. Honey, as a natural product, seems to be one possible option, and has long been used in traditional medicine for both topical applications and as an orally administered systemic prophylactic.[11-13] The microbial inhibition ability of *A. dorsata* honey revealed significant differences between the different dilutions of honey and the type of pathogens. *A. dorsata* honey was most effective against *S. aureus*, a representative of Gram^{+ve} bacteria, which agrees well with the previous results of Chanchao et al. and Marcucci et al.[9,14] Certainly, the flavonone pinocembrin, the flavonol galangin, and caffeic acid phenethyl ester have been shown to be able to inhibit the function of bacterial RNA polymerase.[15] In addition, Mirzoeva et al. reported both that there was quercetin in honey that it could increase the membrane permeability preventing bacterial ATP synthesis, membrane transport and mobility.[16] Moreover, galangin, one of the flavonoids in honey, can degrade the bacterial cytoplasm membrane.[17] Since the determination of antibacterial activity can be measured quantitatively, it can be used as an additional quality criterion for honey.

CONCLUSION

In the honey of *Apis dorsata*, three new findings relating to antimicrobial activity were reported in this study. The first one is the acidity (pH 3.8) of honey. The second is the presence of the antimicrobial peptide epinecidin (about 76 kDa) in honey as one of the three major proteins. In addition, although another of the three major protein bands (fragment of immunoglobulin heavy chain variable region) is not an antimicrobial peptide, it is still related to immune system. The last novel observation was the antimicrobial activity of this *A. dorsata* honey. *S. aureus* was the most susceptible to honey at all tested dilutions (25-100% (v/v)),

whilst *C. albicans* and *A. niger* were not susceptible to honey at 50% (v/v) and lower concentrations. It could be concluded that honey at different dilutions performs different inhibition activities and or that different microorganisms show different responses to honey. Due to the data mentioned above, *A. dorsata* honey from Thailand could be a good source for antimicrobial agents that can be used to protect health and fight against some diseases.

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