Eur Food Res Technol (2016) 242:625–629
DOI 10.1007/s00217-016-2665-5

SHORT COMMUNICATION

The MRJP1 honey glycoprotein does not contribute to the overall antibacterial activity of natural honey

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Received: 16 November 2015 / Revised: 1 February 2016 / Accepted: 13 February 2016 / Published online: 26 February 2016

Abstract A recent study has claimed that honey glycoproteins including major royal jelly protein 1 (MRJP1), the most abundant protein in honey, exhibit strong antibacterial activity at μg/ml concentrations. These were shown to be effective against a broad spectrum of multidrug-resistant clinical isolates. In this study, we investigated the antibacterial activity of the protein content of three different sterile honey samples (manuka honey, Revamil source honey and honeydew honey) used in wound care, and characterised the antibacterial activity of purified MRJP1. Following ultrafiltration, honey samples contained different amounts of proteins. The most abundant protein was MRJP1. Honey proteins with a molecular weight (MW) above 10 kDa did not inhibit the growth of the laboratory strain *Micrococcus luteus*. Similarly, purified MRJP1 did not possess any antibacterial activity against *M. luteus*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* in both an agar well diffusion assay and a broth microdilution assay. The results of this study indicate that honey proteins with a MW above 10 kDa, including MRJP1, do not possess direct antibacterial activity.

Keywords Honey · MRJP1 · Wound · Antibacterial efficacy

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Introduction

The antibacterial activity of honey has been extensively studied, and several antibacterial compounds have been identified. Two of these compounds, bee defensin-1 and glucose oxidase, which mediates hydrogen peroxide release, have been shown to be common components in honey and significantly contribute to the overall antibacterial activity of honey against both Gram-positive and Gram-negative bacteria [1, 2].

After sugars and water, the protein content is the third most important honey component. All major proteins identified in honey are of bee origin [3]. Major royal jelly protein 1 (MRJP1), a 55 kDa protein, is the most abundant protein in honey. The mean amount of MRJP1 as a proportion of the total protein content of analysed honey samples measured by ELISA was 23.4 % [4]. The MRJP1 protein is multifunctional and has a nutritional function in larval jelly [5] and a presumed function in the bee brain that is associated with learning ability [6]. It also acts as a precursor protein of short antimicrobial peptide jelleines [7], and its monomeric form in royal jelly, called royalactin, is a factor that induces the differentiation of honeybee larvae into queens [8]. Furthermore, MRJP1 has been reported to show various biological effects on animal cells [9–11].

Brudzinsky et al. [12] claimed that honey glycoproteins, including MRJP1, possess a broad spectrum of antimicrobial activity against clinical bacterial isolates, with MIC values ranging from 4.8 to 33 μg/ml (i.e. from 87 to 600 μmol/l when considering a molecular weight (MW) of 55,000, which corresponds to MRJP1). Considering the much lower molecular weight of antibiotics (e.g. ampicillin sodium salt has a MW of 371) and their almost equivalent antibacterial efficacy to honey glycoproteins, glycoproteins

are approximately ten times more effective than antibiotics. In contrast to these findings, Kwakman et al. [13] detected only one antibacterial compound, defensin-1, in the >5-kDa retentate fraction after the size-fractionation of honey by ultrafiltration. Since these results of Brudzinsky et al. are very relevant for wound care, we conducted analyses to confirm this finding. Therefore, the aim of this study was to investigate the antibacterial activity of the protein content of three different sterile honey samples (manuka honey, Revamil source honey and honeydew honey) used in wound care and to characterise the antibacterial activity of purified MRJP1.

Materials and methods

Honey samples

Two commercially available sterile honey products: Medihoney® (Derma Sciences, Inc., Canada) and Revamil® (Bfactory, The Netherlands) and one Slovak sterile honeydew honey (Medar, s.r.o, Slovakia) were used in the study.

Microorganisms

The antibacterial activity of honey retentates and purified MRJP1 was assessed against the laboratory strain *Micrococcus luteus* ATCC 272 and the isolates *Pseudomonas aeruginosa* CCM1960 and *Staphylococcus aureus* CCM4223, obtained from Department of Medical Microbiology, Slovak Medical University (Bratislava, Slovakia).

Ultrafiltration of honey samples

Each honey sample (2.5 g) was diluted in deionised water to a final volume of 5 ml until completely fluid. The liquid solution obtained was filtered through a 0.22-μm PES filter (Millipore, MA, USA) and was then concentrated and simultaneously fractionated by centrifugation at 5000×*g* at room temperature in a Vivaspin 6 concentrator tube (Sartorius, Germany), with an exclusion limit of 10 kDa, to a final volume of 500 μl.

Purification of native MRJP1

The MRJP1 protein was purified from royal jelly (RJ) according to Majtan et al. [14]. Briefly, RJ (Institute of Apiculture, Liptovsky Hradok, Slovakia) was homogenised at a concentration of 10 mg/ml in 0.05 M HEPES buffer, pH 8.3 (buffer A). The supernatant was collected from the RJ suspension following centrifugation at 10,000×*g* for 10 min at 4 °C and passed through a 0.22-μm filter. The RJ supernatant was loaded onto a HiTrap DEAE Sepharose FF column (5 ml) (GE Healthcare, UK) equilibrated with buffer A. The flow-through was collected and applied to a HiTrap Heparin HP column (5 ml) (GE Healthcare) equilibrated with buffer A. The MRJP1 containing the fractions was pooled again and concentrated using a Vivaspin 5000 MWCO concentrator (GE Healthcare). The obtained sample was applied to a Superdex 75 HR 10/300 GL column (GE Healthcare) equilibrated with 0.05 M HEPES (pH 7.2) and 0.2 M NaCl (buffer B) at a flow rate 0.5 ml/min. Finally, pooled fractions containing MRJP1 were desalted using desalting PD-10 columns (GE Healthcare), and the purity of prepared MRJP1 in distilled water was determined by 12 % SDS-PAGE, stained with Serva Blue (Serva, Germany), and the concentration of MRJP1 was measured by a Quick Start Bradford Protein Assay (Bio-Rad, Hercules, CA, USA).

To verify the presence of MRJP1 in honey retentates and the purified protein, we carried out Western blot analysis of proteins using the semi-dry blotting method. Proteins were electrophoretically transferred after SDS-PAGE to a 0.22-μm nitrocellulose membrane. The membrane was blocked for 1 h in TBST buffer (50 mM Tris–HCl, pH 7.5, 200 mM NaCl, 0.05 % Tween 20) containing 10 % powdered non-fat milk (TBST-M buffer) and was then incubated overnight with a purified rabbit polyclonal antibody against recombinant MRJP1 diluted 1:4000 in TBST-B. After washing with TBST, the membrane was incubated for 2 h in TBST-M buffer containing goat anti-rabbit HRP-linked antibodies (Promega, WI, USA) diluted 1:2500. Immunoreactive bands were detected in solution containing dissolved SigmaFast 3,3-diaminobenzidine tablets (Sigma-Aldrich, UK).

Determination of antibacterial activity

The antibacterial efficacy of honey retentates, purified MRJP1 and ampicillin (as a positive control) was evaluated by the agar well diffusion assay using *M. luteus*, a model Gram-positive bacterium frequently used for testing antibacterial properties. Briefly, an overnight culture of *M. luteus* was suspended in 2 ml PBS by vortexing for 60 s, and the turbidity of the suspension was adjusted to 10^8 colony forming units (CFU)/ml. A 100-μl aliquot of the suspension was inoculated into 10 ml 0.7 % (w/v) agar in MHB broth (at 48 °C) and poured into 90-mm Petri dishes. After MHB agar solidification, 2-mm-diameter wells were punched into the agar and a 5-μl sample was added to each well. The antibacterial activity of the samples was compared on the basis of the radius of a clear inhibition zone around the wells after 18 h of incubation at 37 °C. The results are shown as mean values from triplicate measurements.

The antibacterial activity of MRJP1 was also evaluated by the broth microdilution assay in a 96-well microtitre

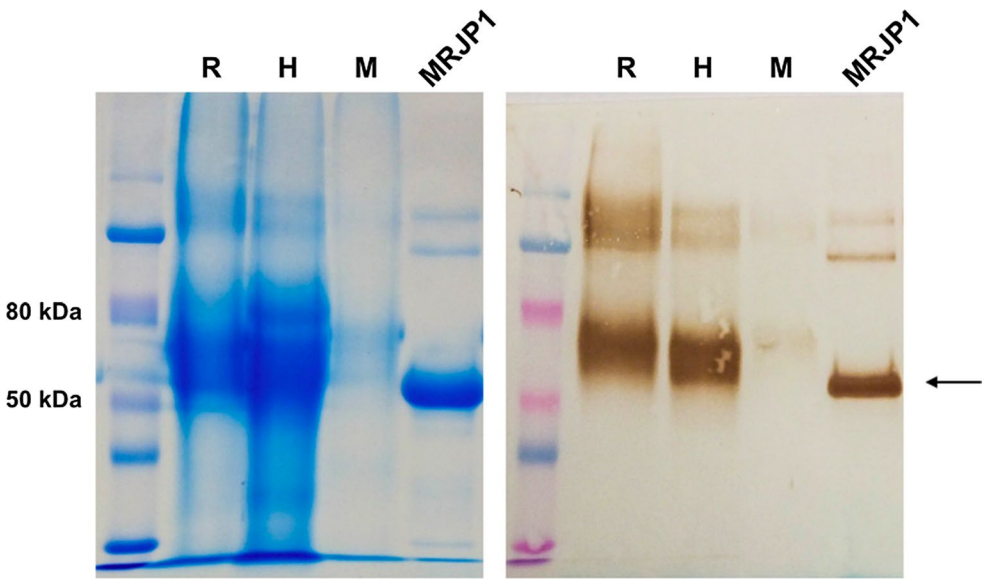

Fig. 1 Protein content of medical-grade honeys and purification of MRJP1. Honey retentates (15 µl) containing proteins with a molecular weight above 10 kDa and purified MRJP1 from royal jelly were electrophoresed on a 12 % SDS-PAGE gel and **a** stained with Coomassie Blue R or **b** immunoblotted with a purified rabbit polyclonal antibody against recombinant MRJP1. An *arrow* indicates the position of the MRJP1 protein. *R* Revamil source honey; *H* honeydew honey; *M* manuka honey

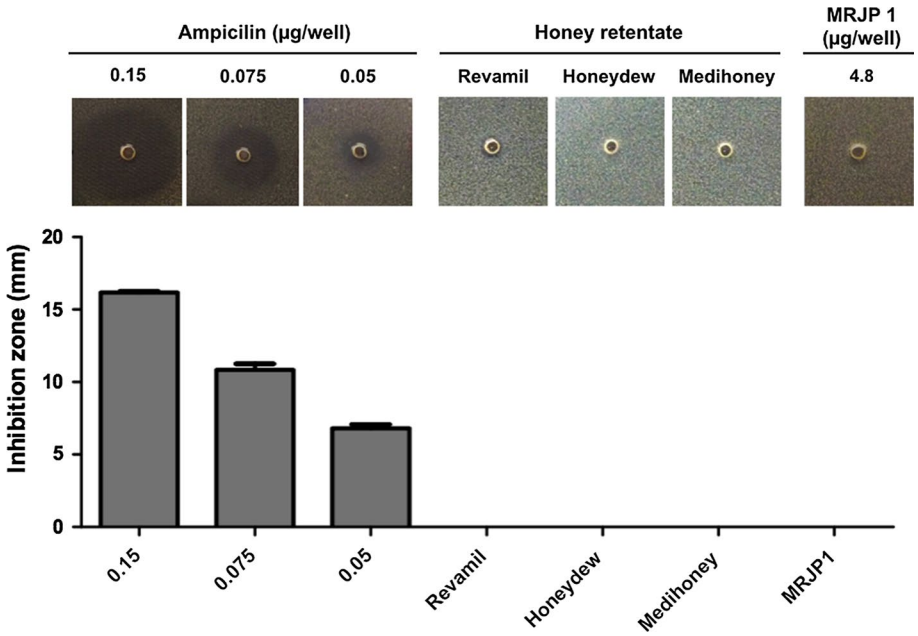

Fig. 2 Antibacterial activity of honey samples, purified MRJP1 and ampicillin against *M. luteus*. The antibacterial activity of ampicillin, MRJP1 and retentates of aqueous solutions of honey samples after their concentration and fractionation in an ultrafiltration column (10 kDa MWCO) was determined by a radial diffusion assay using *M. luteus* as a model bacterium. The zone of inhibition was measured using a digital electronic calliper

plate using *M. luteus* and bacterial wound isolates *P. aeruginosa* and *S. aureus*. Each well contained 10 µl bacterial suspension at a concentration of 10^8 CFU/ml and 90 µl of twofold serial dilutions of MRJP1 (starting from 48.5 µg/well). Bacterial growth was measured at an optical density at 490 nm after 18 h incubation at 37 °C. All tests were performed in duplicate and were repeated three times.

Results and discussion

Bee-derived proteinous components are the major group of proteins/peptides identified in honey. Recently published mass spectrometry data showed that honey contained MRJPs such as MRJP1, MRJP2, MRJP5 and MRJP7, as well as several other proteins from *Apis mellifera* [15]. Some of these components, such as bee defensin-1 and the enzyme glucose oxidase contribute to the overall antibacterial action of honey. In addition to well-characterised honey antibacterial factors (low pH, high osmolarity, defensin-1, methylglyoxal and H_2O_2), honey is thought to contain other unknown proteinous compound(s) with bactericidal/bacteriostatic activity. According to recent studies [12, 16], MRJP1-containing glycoproteins in honey have been described as novel antibacterial drugs that exhibit antibacterial activity at mg/ml concentrations.

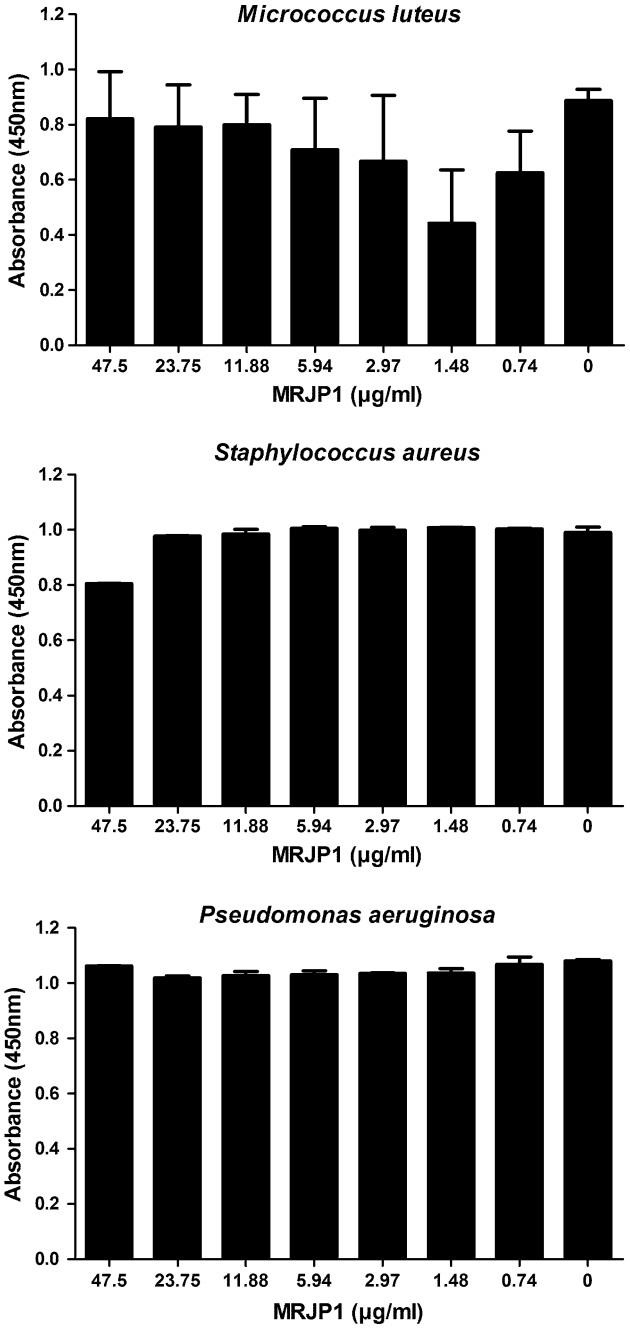

Fig. 3 Concentration-dependent growth inhibition of the laboratory strain *M. luteus* and two wound isolates *S. aureus* and *P. aeruginosa* by a purified MRJP1 using a broth microdilution assay. The concentration of MRJP1 is expressed as the amount of purified MRJP1 in the wells in a total volume of 100 µl

In this study, the protein concentration in honey retentates of Revamil, manuka and honeydew honey was 96, 16 and 223 µg/ml, respectively. As expected, the most abundant protein in all honey retentates was MRJP1, with a MW of 55 kDa (Fig. 1). Notably, the protein concentration in manuka honey retentate was very low, probably due to a methylglyoxal-induced structural modification of all proteins in manuka honey [17]. These modified proteins form high molecular weight adducts that cannot be resolved by SDS-PAGE or become stuck at the membrane of the ultrafiltration device.

To investigate the antibacterial activity of honeybee MRJP1, we purified a large quantity of the protein from RJ. During the purification, several columns were employed to obtain protein of high purity. The MRJP1 protein was purified from RJ instead of honey, because honey contains only minute quantities of proteins and its high sugar content negatively affects the purification procedure.

The in vitro evaluation of the sensitivity of *M. luteus* to honey retentates showed that the >10 kDa protein content of manuka, Revamil and honeydew honey at a final concentration of 0.081, 0.48 and 1.11 µg/well, respectively, exhibited no antibacterial activity (Fig. 2). Furthermore, purified MRJP1 at a concentration of 4.8 µg/well did not inhibit the growth of *M. luteus*. Similarly, no inhibition zone was detected when the wound isolates *Staphylococcus aureus* and *Pseudomonas aeruginosa* were used (data not shown). In contrast to our findings, honey glycoproteins containing MRJP1 isolated from buckwheat honey using Concavalin A spin columns showed a concentration-dependent inhibition of the growth of multidrug-resistant clinical isolates with susceptibility lower than 0.1 µg/well [12].

Honey glycoproteins are molecules with a high MW, and most exist in a multimeric form under native conditions, including MRJP1, which forms a 420-kDa oligomeric subunit [18]. Due to its high MW, the rate of diffusion of MRJP1 through the agar matrix is significantly reduced and no visible inhibition zone can be detected. Therefore, quantitative liquid bactericidal activity against *M. luteus* was performed. In the broth microdilution assay, MRJP1 showed no antibacterial activity against *M. luteus* in the concentration range from 0.74 to 47.50 µg/well (Fig. 3). Similarly, no growth inhibition was detected when the wound isolates *S. aureus* and *P. aeruginosa* were used (Fig. 3). On the other hand, MRJP1 at particular concentration of 1.48 µg/well exhibited some antibacterial action against *M. lutes*, but no activity was detected against wound isolates.

These results indicate that honey proteins with a MW above 10 kDa, including MRJP1, do not possess direct antibacterial activity. Jelleines, a very short antimicrobial peptide derived from MRJP1 and found in royal jelly, have been proposed to be responsible for MRJP1-1-mediated antibacterial activity. However, the contribution of these peptides to the antibacterial activity is questionable, since their concentrations in royal jelly are far too low to account for all the observed activity. In addition, jelleines have not been identified in honey to date.

In conclusion, honey proteins with a MW above 10 kDa, including MRJP1, do not possess direct antibacterial activity and are not responsible for the antibacterial activity of honey.

Acknowledgments We thank Dr. Jaroslav Klaudiny (Institute of Chemistry, Slovak Academy of Sciences) for providing a polyclonal antibody against MRJP1. This study was supported by the Scientific Grant Agency of the Ministry of Education of the Slovak Republic and the Slovak Academy of Sciences VEGA 2/0007/14.

Compliance with ethical standards

Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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