Journal of proteome research

Article

pubs.acs.org/jpr

In-Depth N-Glycosylation Reveals Species-Specific Modifications and Functions of the Royal Jelly Protein from Western (*Apis mellifera*) and Eastern Honeybees (*Apis cerana*)

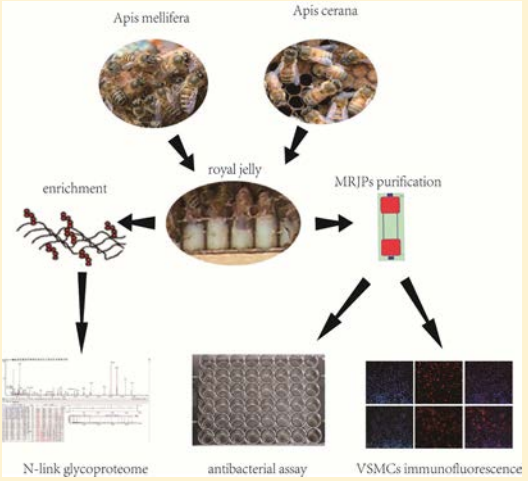
Mao Feng,[†,§] Yu Fang,[†,§] Bin Han,[†] Xiang Xu,[†] Pei Fan,[†,‡] Yue Hao,[†] Yuping Qi,[†] Han Hu,[†] Xinmei Huo,[†] Lifeng Meng,[†] Bin Wu,[†] and Jianke Li*[,†]

[†]Institute of Apicultural Research/Key Laboratory of Pollinating Insect Biology, Ministry of Agriculture, Chinese Academy of Agricultural Sciences, Beijing 100093, China

[‡]College of Bioengineering, Henan University of Technology, Zhengzhou 450001, China

Supporting Information

ABSTRACT: Royal jelly (RJ), secreted by honeybee workers, plays diverse roles as nutrients and defense agents for honeybee biology and human health. Despite being reported to be glycoproteins, the glycosylation characterization and functionality of RJ proteins in different honeybee species are largely unknown. An in-depth N-glycoproteome analysis and functional assay of RJ produced by *Apis mellifera lingustica* (Aml) and *Apis cerana cerana* (Acc) were conducted. RJ produced by Aml yielded 80 nonredundant N-glycoproteins carrying 190 glycosites, of which 23 novel proteins harboring 35 glycosites were identified. For Acc, all 43 proteins glycosylated at 138 glycosites were reported for the first time. Proteins with distinct N-glycoproteomic characteristics in terms of glycoprotein species, number of N-glycosylated sites, glycosylation motif, abundance level of glycoproteins, and N-glycosites were observed in this two RJ samples. The fact that the low inhibitory efficiency of N-glycosylated major royal jelly protein 2 (MRJP2) against *Paenibacillus larvae* (*P. larvae*) and the absence of antibacterial related glycosylated apidaecin, hymenoptaecin, and peritrophic matrix in the Aml RJ compared to Acc reveal the mechanism for why the Aml larvae are susceptible to *P. larvae*, the causative agent of a fatal brood disease (American foulbrood, AFB). The observed antihypertension activity of N-glycosylated MRJP1 in two RJ samples and a stronger activity found in Acc than in Aml reveal that specific RJ protein and modification are potentially useful for the treatment of hypertensive disease for humans. Our data gain novel understanding that the western and eastern bees have evolved species-specific strategies of glycosylation to fine-tune protein activity for optimizing molecular function as nutrients and immune agents for the good of honeybee and influence on the health promoting activity for human as well. This serves as a valuable resource for the targeted probing of the biological functions of RJ proteins for honeybee and medical communities.



KEYWORDS: *N-glycoproteome, royal jelly, Apis mellifera ligustica, Apis cerana cerana, antihypertension, Paenibacillus larvae*

1. INTRODUCTION

Royal jelly (RJ) is the principal food of honeybee larvae within the first 3 days and the exclusive food for the honeybee queen during her lifetime.[1] It is a cocktail solution derived from the hypopharyngeal, mandibular, and thoracic glands of worker honeybees aged from 5−15 days old.[2] Apart from its pivotal role in honeybee nutrition and determination the fate of young larvae development as worker or queen bees,[3] RJ is also widely consumed with its broad spectrum functional properties of antihypertensive, antitumor, anti-inflammatory, antifatigue, and antioxidative activities.[4−7] In addition, important components in RJ, 9-oxo- and 10-hydroxy-2(E)-decenoic acids or queen substance, can be artificailly synthezed and used as pheromone in regulating the honeybee worker behavior; thus, this is important for social organization in the honeybee community.[8,9]

Apis mellifera lingustica (Aml), one of the western honeybee species, is widely kept in China (>8 million colonies) since it was introduced at the beginning of the 1900s and now has become a major bee species. Having benefited from a breeding program launched in the 1980s, China has successfully selected a strain of high RJ producing bees (RJB) from Aml that can produce >10 kg of RJ a colony per year, which makes China the biggest RJ producer and accounts for 90% of world total RJ output. In contrast, *Apis cerana cerana* (Acc), an indigenous Chinese honeybee, is an eastern bee species whose RJ

Received: September 5, 2015
Published: October 26, 2015

DOI: 10.1021/acs.jproteome.5b00829
J. Proteome Res. 2015, 14, 5327−5340

production is at least 10-times lower than that of RJB.[10] Despite its low RJ producing capacity, the Acc has evolved several biological merits in regards to resisting the mite, *Varroa destructor*, adapting to extreme weather conditions, and collecting nectar from scattered floral resources.[11] Therefore, the Acc has become the second biggest bee species in China (about 2 million colonies) as a honey producer.

To understand the biological differences of RJ from the western and eastern bees, the proteome and the function of RJ from the two bee species have been compared, and profound differences were found. Although the same protein species are observed, the abundance level of major RJ proteins (MRJPs) in Aml is significant higher than that in Acc,[10] whereas the abundance level of phosphorylated proteins displays a reverse trend, and the phosphorylated peptides show stronger activity in against bacteria and fungi in RJ of Acc.[12] These observations reveal the fact that the western bees use the high abundance of MRJPs as nutrients and defense agents to support their large body sizes, while the eastern bees use higher level of phosphorylated peptides as a countermeasure of low levels of MRJPs to ensure the larval development.[12]

Nutritional crossbreeding of the larvae of Aml and Acc has significant influence on the morphology and the gene expression pattern. The Aml larvae fed with RJ produced by the Acc exhibit morphological characteristic of Acc such as proboscis, forewings, abdomen, and body weight of newly emerged adult bees.[13] Alternative splicing, an important RNA-mediated post-transcriptional process can be influenced if Aml larvae are fed with RJ produced by Acc.[14] Strikingly, the resistivity to *varroa* mites, a major parasitic threaten to Aml, is improved if the Aml larvae are fed with Acc RJ. Moreover, the Acc larvae enhance the capacity to fight against Chinese sacbrood virus, a fatal viral disease of Acc, when fed with RJ produced by Aml.[13] These results reveal a notion that there are remarkable functional differences between the RJ produced by Aml and Acc.

Glycosylation is one of the ubiquitous modifications of the eukaryotic proteins.[15] Over 50% of the proteins found in nature are glycosylated, of which more than 70% are N-glycosylated.[16] N-linked glycans are almost always covalently attached to the asparagine residues (N) side chain that are present as a part of Asn-X-Ser/Thr (N-X-S/T, X ≠ proline/p) consensus sequences and can be generally classified as high mannose, complex, and hybrid types depending on their monosaccharide composition and their branching.[17] Protein glycosylation is important in regulating many biological processes including cell differentiation, cell growth, and immunity.[18]

Proteins are the main components of RJ, accounting for ~50% of RJ dry mass,[19] while nine MRJP families represent 80−90% of the total proteins in RJ.[20] Despite being reported as glycoproteins,[21] only 12 different N-glycans have been identified in RJ with the high-mannose as main type.[22] A 350 kDa RJ protein, which stimulates cell growth,[23] has been documented to contain the high-mannose type N-glycans with four different structures analyzed by nuclear magnetic resonance (NMR) spectroscopy,[24] while a 55 kDa RJ protein, which maintains the high viability of rat liver primary cultured cell, possesses only one kind N-linked sugar chain.[25] It is also reported that the glycosylation of a minority homologue of MRJP2 can inhibit the growth of *P. larvae*, the etiological agent of the American foulbrood (AFB).[26]

Mass spectrometry (MS) is currently the most powerful tool for global glycoproteomic studies. To better characterize the glycoproteome, it is important to enrich the low abundant glycosylated proteins/peptides to reduce the sample complexity before submitting to MS analysis.[27] Lectin affinity and hydrazide resin are widely used as complementary protocols in enrichment of glycopeptides and glycoproteins.[28] These technological advantages have made great advances in the study of RJ glycoproteome; hence, a total of 25 N-linked glycoproteins carrying 53 N-glycosites have been mapped into Aml RJ proteins.[29] It is reported that extending the elution gradient time is an efficient way to increase the chromatographic resolution, the detected isotope patterns, and peptide amounts, thus improving the protein identification on liquid chromatography−mass spectrometry (LC−MS) system.[30] This promotes us adopting this strategy to optimize the parameter setting of the LC−MS system for the in-depth profiling of glycoproteomics of Aml RJ proteins. Given that knowledge on N-glycosylation in the Acc RJ proteins is still lacking, it is necessary to characterize the glycoproteomes of RJ produced by the western and eastern bees and reveal the functional differences of glycosylated proteins of RJ from these two bee species to better understand how glycosylated RJ proteins benefit the honeybee physiology and human health.

2. MATERIALS AND METHODS

2.1. Chemical Reagents

Unless otherwise specified, all chemicals were bought from Sigma-Aldrich (St. Louis, MO, USA). PNGase F was purchased from NEB (Ipswich, MA, UK).

2.2. RJ Sample Preparation

Two-hundred-fifty queen cell cups of RJ were collected from five colonies each of Aml and Acc around 70 h after the bee larvae (>24 h) had been transferred into the queen cell at the apiary of the Institute of Apicultural Research, Chinese Academy of Agricultural Science, Beijing.

RJ proteins were extracted, and the N-linked glycoproteins were prepared according to the previous protocol.[29] In brief, 1 mg of RJ protein pellet precipitated by acetone was resolved in 250 μL of coupling buffer [100 mM sodium acetate, 150 mM NaCl, pH 5.5] and then oxidized with sodium perodate and captured by the well equilibrated hydrazide resin. Then the attached glycoproteins were desalted, reduced, and alkylated by dithiothreitol (DTT) and iodoacetamide (IAA), followed by digesting with trypsin (sequencing grade, modified, Roche) overnight. Glycans attached to the glycopeptide backbone were removed, and the N residues were labeled with ^{18}O simultaneously during enzymatic deglycosylation using PNGase F prepared with H_2 ^{18}O. The ^{18}O labeled N-linked glycopeptides were eluted by 80% ACN (100% ACN diluted with H_2 ^{18}O) and concentrated with Speed-Vac system for subsequent LC−MS analysis.

The enrichment of N-linked glycopeptides with lectin was carried out according to a filter aided sample preparation (FASP) based method.[28,29] Briefly, 1 mg of RJ protein pellet was suspended in UA buffer (8 M urea, 100 mM of Tris-HCl, pH 8.5) and then transferred into an Ultracel YM-10 10 000 MWCO centrifugal filter unit (Millipore). The glycoproteins were reduced by DTT, alkylated by IAA, and digested by trypsin overnight. Then the peptides were mixed with lectin solution (mixture of concanavalin A, wheat germ agglutinin, and *Ricinus communis* agglutinin (RCA) 120). The unbound peptides were removed by centrifugation, and the captured ones were washed after deglycosylation with PNGase F

DOI: 10.1021/acs.jproteome.5b00829
J. Proteome Res. 2015, 14, 5327−5340

prepared with H_2 ^{18}O. Finally, the labeled N-linked glycopeptides were eluted with 40 mM NH_4HCO_3 in H_2 ^{18}O.

2.3. LC–MS Analysis

The enriched N-linked glycopeptides were analyzed using a Q-Exactive system (Thermo Fisher Scientific) coupled to an Easy-nLC 1000 (Thermo Fisher Scientific) via a nanoelectrospray ion source. Samples were loaded onto a fused silica trap column containing 5.0 μm Aqua C18 beads (100 μm × 20 mm, Thermo Fisher Scientific) for 2 min in buffer A (0.1% formic acid) at a flow rate of 5 μL/min. Peptides were separated with a fused silica analytical column filled with 2 μm Aqua C18 beads (100 Å, 75 μm × 500 mm, Thermo Fisher Scientific) at a flow rate of 350 nL/min. The elution gradient was as follows: from 3 to 8% buffer B (0.1% formic acid in acetonitrile) for 10 min, from 8 to 23% buffer B for 130 min, from 23 to 30% buffer B for 20 min, from 30 to 90% buffer B for 8 min, and 90% buffer B for 12 min. A data dependent acquisition mode was applied for full MS scans ranging from m/z 300–1800 with a resolution of 70 000; the 15 most abundant precursor ions were fragmented by higher energy collisional dissociation (HCD) mode with a resolution of 17 500 and started from m/z 100, isolation window 2 m/z, with normalized collision energy of 27. The dynamical exclusion setting was as follows: charge exclusion, unassigned 1, > 8; peptide match, preferred; exclude isotopes, on; dynamic exclusion, 10 s. The MS/MS data were acquired in raw files using the Xcalibur software (version 2.2, Thermo Fisher Scientific).

2.4. Data Analysis and Location of N-glycosylation Sites

The raw MS data were searched against the constructed database containing protein sequences of *Apis* (downloaded from NCBI on April, 2013) and a common repository of adventitious proteins (cRAP, from The Global Proteome Machine Organization, downloaded April, 2013), totaling 32 515 entries, by using the in-house PEAKS software (version 7.0, Bioinformatics Solutions Inc., Waterloo, Canada). The searching parameters were: precursor mass tolerances, 20 ppm; fragment mass tolerances, 0.05 Da; enzyme, trypsin; allowing a nonspecific cleavage, maximum missed cleavages per peptide, 2; maximum allowed variable PTM per peptide, 3; fixed modification, carbamidomethyl (C, + 57.02); variable modifications, oxidation (M, + 15.99), deamidation (N, 0.98), and deamidation ^{18}O (N, + 2.9890). The fusion-decoy database searching strategy was applied to control the false discovery rate (FDR) by ⩽1.0% both at protein and peptide levels.[31] Protein identifications were accepted only as those with at least one unique peptide that had at least one spectrum.

The glycosites were assigned using Scaffold PTM (version 1.1.3, Proteome Software) on the basis of the Ascore algorithm.[32] Only a site with a confidence level no less than 95%, and the manual check of spectrum with continuous y or b ions, was considered to be a localized glycosite in RJ proteins.

2.5. MRJP1–3 Deglycosylation and Glycan Purification

MRJP1–3 from both RJ samples were purified by following the methods described previously.[33,34] N-glycan was released by incubating 100 μL of the target protein solution with 2 μL of PNGase F overnight at 37 °C under the guide of the manufacturer's instruction.

The released N-glycans were separated, desalted, and enriched by using solid phase extraction and a C8 cartridge (Waters) as described.[35] Then the N-glycans were eluted with 0.05% trifluoroacetic acid (TFA) in 40% of ACN solution and dried in the Speed-vac system.

2.6. Protein Quantification by Spectral Counting Analysis

A spectral counting based label-free strategy was used to quantify the glycoprotein expression profiles in RJ as it is a simple but reliable index for relative protein quantification.[36] Given that proteins with larger molecular weight could be expected to produce more peptides and therefore more spectral counts than smaller ones, a normalized spectral abundance factor (NSAF) was applied to eliminate the bias generated from protein length.[37] Then the ratio of NSAF normalized spectral counts (Acc/Aml) was used for one-sample t test analysis (SPSS version 16.0, SPSS Inc., IBM). Proteins with ratios above 1.5 and $P < 0.05$ were considered to have significant higher abundance level in Acc, while proteins with ratios below 0.67 and $P < 0.05$ were considered to have significant higher abundance level in Aml. Spectral counting assigned to each glycosite was conducted to evaluate the abundance level at each glycosite.

2.7. N-Glycosylated Motif Analysis

N-glycosylation consensus sequences of RJ from Aml and Acc were extracted using Motif-X algorithm (http://motif-x.med.harvard.edu/motif-x.html),[38] and the uploaded honeybee proteome (randomly reduced >10M) was chosen as the background.[10] Only those N-glycosites localized with confidence level ⩾95% by Scaffold PTM were used for motif extraction. The prealigned mode was used, and all candidate sequences were centered on the glycosylated residue and extended six residues on each side. The minimal reported number of occurrences for a given motif was set to 10, and the threshold for significance was set to $p < 10^{-5}$.

2.8. Antibacterial Assay

Antibacterial assays of glycosylated RJ proteins on *Staphylococcus aureus* (ATCC 6538), *Bacilus subtilis* (ATCC 6051), and *P. larvae* (ATCC 13537) were performed according to a previous method with minor modification.[12] Bacteria were incubated at 37 °C in Luria–Bertani medium at a concentration of 10^5 colony-forming units (cfu)/mL, and an inoculum corresponding to 2×10^3 cfu was placed in each well of a 96-well plate. Purified MRJP1–3, the deglycosylated MRJP1–3, and their corresponding glycans were serially diluted and then added to each well containing the bacterial inoculum. Following incubation at 37 °C for 24 h with shaking at 220 rpm (rpm), the antibacterial activity was visualized as bacterial growth inhibition by measuring the absorbance at 595 nm of each well using a microplate reader (Model 3550, Bio-Rad). The minimal inhibitory concentration (MIC) was determined as the lowest concentration causing 50% bacterial growth inhibition.[39]

2.9. Scratch Wound Assay and Immunofluorescence

An in vitro scratch wound healing assay is usually used to investigate the effects of chemical exposure or gene knockdown on mammalian cells. To assess the migration and differentiation ability of mice aortic vascular smooth muscle cells (VSMCs) in the presence of glycosylated RJ proteins, VSMCs were obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37 °C under a humidified 95% air/5% CO_2 mixture (v/v). VSMCs (2×10^4) were seeded to a six-well dish and cultured overnight. Then the media were removed and incubated in serum-free DMEM for 24 h. A

DOI: 10.1021/acs.jproteome.5b00829
J. Proteome Res. 2015, 14, 5327–5340

transverse scratch wound on each monolayer of VSMC was made by using a sterilized 200 μL pipet tip. The scratch wounded VSMC monolayers were rinsed twice with phosphate buffer saline (PBS) to remove the detached cells and then stimulated with Angiotensin II (Ang II) (final concentration 1×10^{-6} M) and glycosylated MRJP1 (P+), deglycosylated MRJP1 (P), and glycans (+) at a concentration of 0.1 mg/mL for an additional 16 h; the intersection of the previously marked line and the wound edge were re-examined with an inverted microscope (IX 71, Olympus, Japan) and analyzed using image J software (1.48v, NIH).

Cells were seeded upon coverslips at the bottom of culture dishes until subconfluence and then were fixed with 4% paraformaldehyde for 20 min, followed by a wash with PBS for three times. To block nonspecific reactions, the sections were treated with blocking buffer (5% normal goat serum, 3% bovine serum albumin, and 0.1% Triton X-100 in PBS) for 30 min at room temperature. Then anti-α-smooth muscle actin (α-SMA) polyclonal antibody (1:200 dilution in blocking buffer) was incubated overnight at 4 °C. After three washes with phosphate buffered saline with Tween 20 (PBST), the sections were incubated with Dylight 594 goat antimouse IgG (Abbkine, 1:400 dilution in blocking buffer) for 50 min away from light. Then 4′,6-diamidino-2-phenylindole (DAPI) (1:200 in PBS, Roche, Mannheim, Germany) was added as a stain for total nuclei after washing. Imaging was performed by inverted microscope system.

2.10. Western Blot

To investigate the expression level of α-SMA, a VSMC-specific marker, after being treated with MRJP1, Western blot analysis was also performed following the standard procedure. The total amount of protein was extracted from cells, and the concentration was determined by Bradford Kit (Sangon Biotech, Shanghai, China). Then equal amounts of proteins were separated by stacking (4%) and separating (12%) SDS-PAGE, followed by transfer to polyvinylidene difluoride (PVDF) membranes (Invitrogen). After being blocked with 5% nonfat milk in Tris buffered saline with Tween 20 (TBST) at 37 °C for 1 h, the membrane was washed and incubated with primary mice antibodies for α-SMA at a dilution of 1:500. Then the membrane was washed with TBST three times and incubated with secondary antibodies (goat antimouse, Invitrogen) at room temperature for 1 h. The blots were visualized using the Chemic Doc XRS imaging system (Bio-Rad) under the presence of ECL Western blotting substrate (Pierce). The bands were quantified using Quantity One software (v4.6.2, Bio-Rad) and normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was used as a loading control.

3. RESULTS

In an effort to in-depth characterize the N-glycoproteome in RJ proteins of the western and eastern honeybees, a high-mass-accuracy and high-resolution MS and long elution time were used. To remove the potential contamination of the proteins exfoliated from the secretory glands, the newly identified proteins in RJ were predicted by using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/, D-cutoff for signal-no TM networks was 0.45), and only those with the N-termial signal peptids were reported as RJ proteins. Moreover, the functional assay of the glycosylated MRJPs was also conducted to assess their influence on the proliferation and migration of VSMCs and their antibacterial activity.

3.1. An Expanded RJ N-Glycoproteome of Aml

Although the RJ N-glycoproteome of Aml has been investigated by our group,[29] the present results yielded significantly extending the N-glycoproteome by using longer elution time (180 min vs 90 min), which covered 92% of the N-glycoproteins and 60% of the N-glycosites that were previously reported. For those not identified in this study, it may attribute to the fact that a rigorous parameter comparing to the previous investigation was applied for the candidate glycopeptides evaluation this time. For instance, glycosites with localization probability less than 95% were not reported, but this criterion was not applied last time. Finally, several novel glycoproteins and glycosites were found here. In total, 80 nonredundant glycoproteins carrying 190 glycosites were identified in the RJ of Aml, of which 57 N-glycoproteins and 78 N-glycosylation sites were known before, and 23 novel proteins harboring 35 glycosites were identified. Briefly, 70 proteins glycosylated on 174 sites were identified by lectin enrichment, while 40 proteins with 97 glycosites were identified by hydrazide resin enrichment. Overall, 30 proteins and 81 glycosites were identified by both of the enrichment strategies. Forty-one proteins glycosylated on 93 glycosites, and 10 proteins glycosylated on 18 sites, were uniquely identified by lectin and hydrazide resin, respectively (Supplementary Table S1). The integration of the two enrichment strategies, together with the expansion of the elution time of the Easy-nLC 1000 system and state of the art MS technology, significantly increased the coverage of the RJ glycoproteome in Aml.

The novel proteins identified in the Aml RJ include the following: cysteine proteinase CG12163-like isoform X1, X2, membrane metallo-endopeptidase-like 1-like isoform X1, X2, X3, alpha-N-acetylgalactosaminidase-like, glucosylceramidase-like isoform X1, X2, X3, carboxypeptidase Q-like isoform X2, X3, ferritin-4_chloroplastic, beta-glucuronidase-like isoform X2, apolipophorin-III-like protein, defensin precursor, defensin 1 precursor, icarapin variant 1 precursor, yellow-e3 isoform X1, and five other functionally unknown proteins. All the identified proteins were functionally categorized into five groups: metabolic process (26 proteins), immune response (24 proteins), MRJPs (16 proteins), developmental regulation (six proteins), and eight other functionally unknown proteins (Figure 1).

3.2. Profile of the RJ N-Glycoproteome of Acc

At present, the N-glycosylation profile of RJ produced by Acc is still lacking. Here, in total, 138 new glycosites residing on 43 nonredundant glycoproteins were identified for the first time. Among these, 117 glycosites in 40 glycoproteins were identified by lectin enrichment, and 61 glycosites from 20 glycoproteins were identified by hydrazide resin enrichment. Moreover, 23 proteins were glycosylated on 77 sites, three glycoproteins modified on 21 glycosites, were uniquely identified by these two methods, respectively (Supplementary Table S2).

Among the 43 identified glycoproteins, despite 21 being previously reported but without any lycosylation information,[10,12] 22 proteins carrying signal peptides in the N-terminal were found in the RJ of Acc in this study, indicating they are RJ proteins (Supplementary Table S2). These newly identified 22 glycoproteins were: opioid-binding protein/cell adhesion molecule, hemolymph protein, apidaecin precursor, apolipophorins, laccase, ferritin heavy chain, antithrombin-III, venom protein 2, glucose dehydrogenase, lysosomal aspartic protease, hypothetical protein ACCB08249, putative cysteine proteinase

DOI: 10.1021/acs.jproteome.5b00829
J. Proteome Res. 2015, 14, 5327−5340

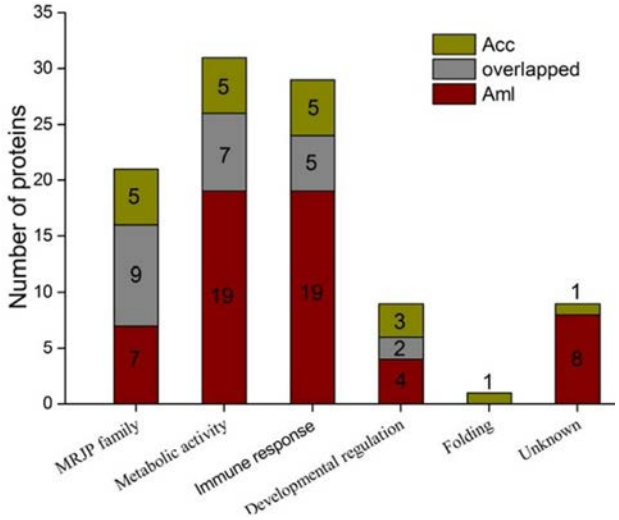


Figure 1. Functional category of the identified N-glycosylated RJ proteins produced by Aml and Acc. The color codes represent the number of protein identified in RJ produced by the two honeybee species.

CG12163, alpha-amylase 4N, leucine-rich repeat-containing protein 15, hypoxia up-regulated protein 1, MRJP6, MRJP9, Chitinase-like protein Idgf4, glucosylceramidase, regucalcin, protein takeout, and proactivator polypeptide (Supplementary Table S2). The 43 glycoproteins were functionally assigned into six groups, of which MRJPs were the most abundant (14 proteins). The second biggest group was proteins related to metabolic processes (12), followed by proteins involved in immune response (10), proteins associated with developmental processes (five), protein with folding activity (one), and one functionally unknown protein (Figure 1).

3.3. Novel Glycosylation Motifs Found in RJ Proteins

Only those peptides with site confidence levels no less than 95% assessed by Scaffold PTM (Supplementary Tables S3−6) and spectra with good quality after manually checked (Supplementary Figures S1−2) were submitted for N-linked motif extraction. N-X-S/T sequences were dominated in this study, among which N-X-S sequences represented 29.5% in Aml and 27.5% in Acc, respectively, while N-X-T sequences accounted for 57.4% in Aml and 43.5% in Acc, respectively (Figure 2A). Moreover, two new sequence motifs, N-G-X and N-X-V, were found in the RJ produced by the two bee species: N-G-X represented 8.9% in Aml and 17.4% in Acc, while N-X-V represented 4.2% in Aml and 11.6% in Acc (Figure 2A). As expected, the canonical N-X-S/T motif was the major form of the N-glycosylated peptides in the RJ proteins of Aml and Acc. Moreover, one of the new sequence motifs, N-G-X, was also

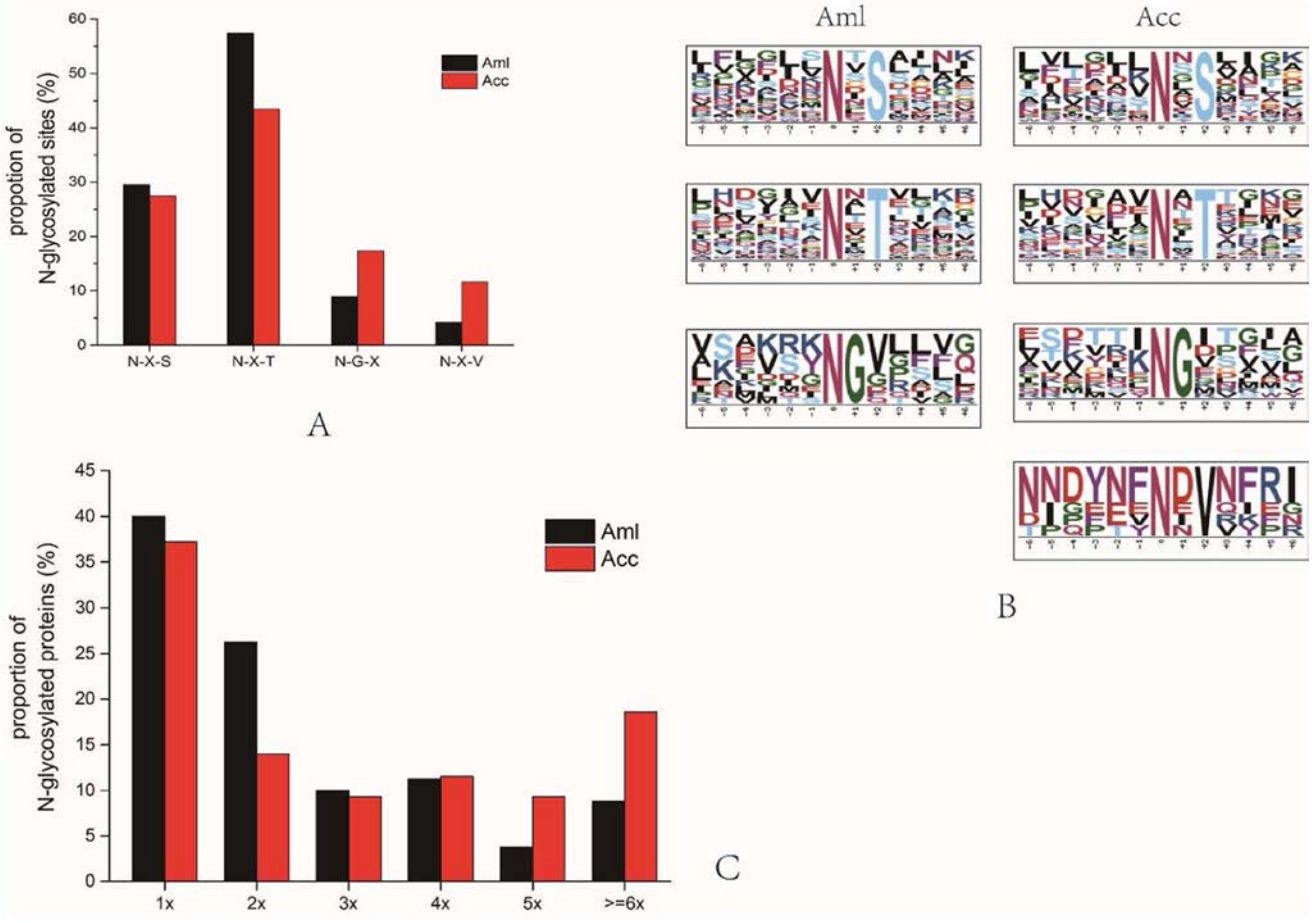


Figure 2. Common characteristics of the identified N-glycoproteomes of RJ proteins produced by Aml and Acc. (A) The proportion of recognized sequence motifs from the N-glycopeptides, for N-X-S/T (X ≠ P/proline); for N-G-X and N-X-V, X can be any amino acid; (B) the extracted motifs using Motif-X algorithm. Only glycosites with a localization probability greater than 95% are considered; (C) the distribution of N-glycosylated RJ proteins harboring single, double, and multiple glycosites.

DOI: 10.1021/acs.jproteome.5b00829
J. Proteome Res. 2015, 14, 5327−5340

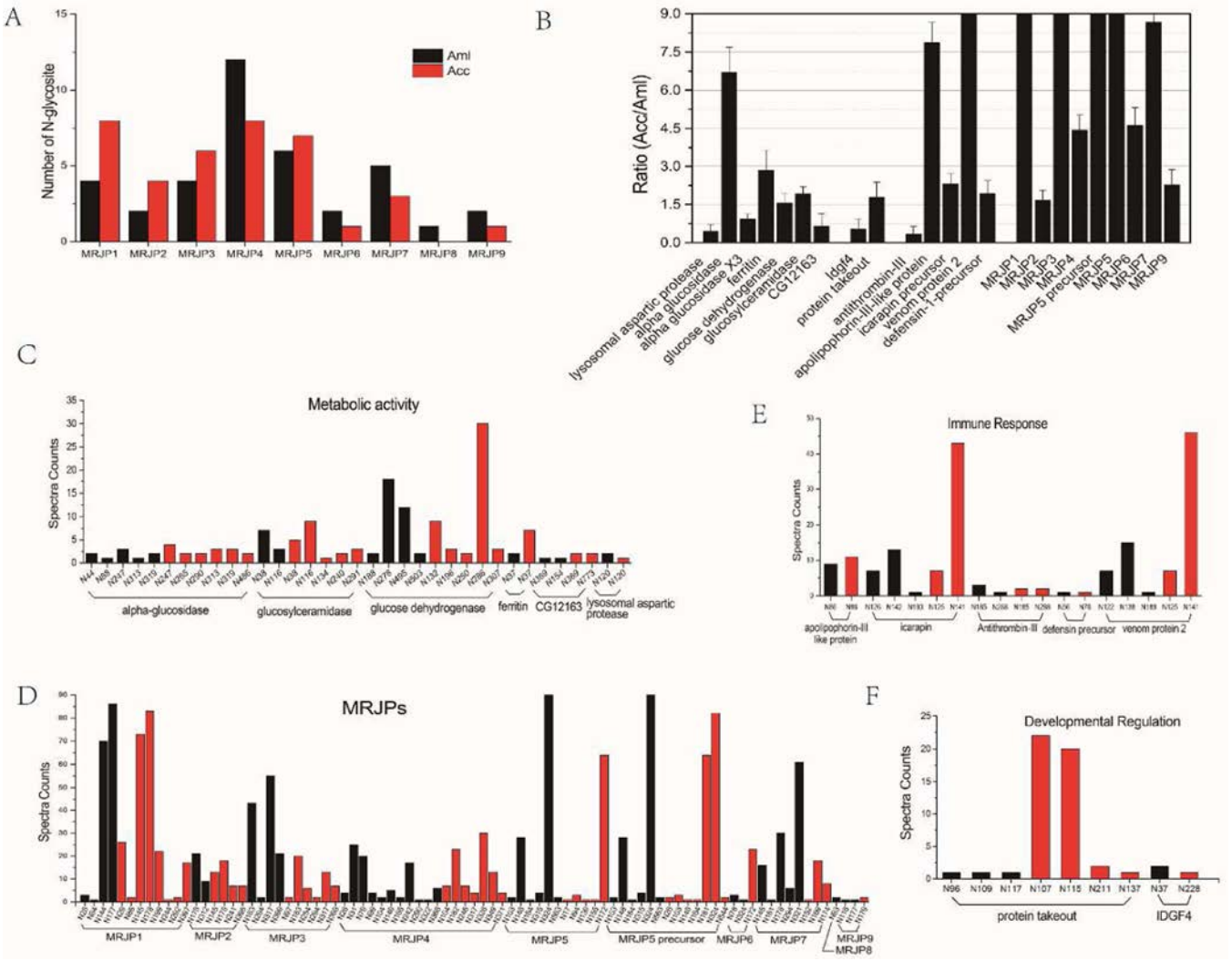


Figure 3. Qualitative and quantitative analysis of the N-glycosylated royal jelly proteins produced by Aml and Acc. (A) The N-glycosites distribution in MRJP1−9; (B) the quantitative comparison of abundance level of the N-glycoproteins shared by Aml and Acc; the ratio of the protein abundance is shown as Acc to Aml, and the ratios above 1.5 or below 0.66 represent proteins showed significant higher abundance level in Acc and Aml, respectively ($P < 0.05$), and error bars represent the standard deviation; (C−F) the spectra counts detected in each N-glycosite of the individual glycoprotein across each protein functional group.

identified in the glycopeptides of both Acc and Aml. N-X-V was exclusively extracted from glycopeptides of Acc for the first time ($p < 10^{-5}$) (Figure 2B). Although these two motifs have been found in the mouse glycoproteome,[28] they were reported in the RJ for the first time, indicating that protein N-glycosylation in RJ is governed in a more complicated way than previously expected N-glycosylation machinery.[40]

The site occupancy analysis showed that most of the identified glycoproteins carried only a single N-linked glycosylated site, with 40.0% in Aml and 37.2% in Acc. Proteins with two glycosylated sites represented 26.3% in Aml and 14.0% in Acc. Proteins with three sites were 10.0% in Aml and 9.3% in Acc, and the ones with four sites were 11.3% in Aml and 11.6% in Acc. Moreover, proteins glycosylated on five sites were 3.8% in Aml and 9.3% in Acc, and the rest carried six or more sites (Figure 2C).

3.4. Different N-Glycosylation Sites Observed in MRJPs of Two RJ Samples

Given that the MRJPs are the most abundant proteins in RJ, special attention was paid with their N-glycosylation modification. Characterization at each glycosite of the proteins is essential for understanding their glycosylation status and functionality.[41] Although all the nine members of MRJPs were identified as being glycosylated proteins, the site numbers in the MRJPs were different within the two bee species. MRJP1−3 and 5 carried more N-glycosites in Acc than that of Aml. For instance, MRJP1 produced by Acc was glycosylated at six sites (N^{247}, N^{265}, N^{290}, N^{313}, N^{316}, and N^{486}) and MRJP2 at two sites (N^{38} and N^{116}), while Aml derived MRJP1 was glycosylated at five sites (N^{44}, N^{88}, N^{247}, N^{313}, and N^{319}) and MRJP2 at five sites (N^{38}, N^{116}, N^{134}, N^{240}, and N^{291}) (Figure 3C). However, MRJP4 and MRJP6−9 harbored more N-glycosites in Aml than that of Acc. For instance, MRJP4 in Aml was heavily glycosylated with 11 N-glycosites, while there were only seven in Acc (Figure 3A).

3.5. Quantitative Comparisons of the Glycoproteins and Glycopeptides between Two RJ Samples

To evaluate the abundance levels of the glycosylated RJ proteins, a spectral counting-based quantitative strategy was used. Here we only compared the RJ glycoproteins identified in

DOI: 10.1021/acs.jproteome.5b00829
J. Proteome Res. 2015, 14, 5327−5340

both of the bee species. Among the nine shared MRJP proteins, the abundance levels of MRJP1−7, MRJP5 precursor, and MRJP 9 were all significantly higher in Acc than in Aml ($p < 0.05$). The seven shared proteins, which are related to metabolic process, alpha-glucosidase, ferritin, glucose dehydrogenase, and glucosylceramidase showed higher levels in Acc than in Aml; lysosomal aspartic protease and cysteine proteinase CG12163-like isoform X1 are higher in Aml than in Acc ($p < 0.05$). The abundance of alpha-glucosidase X3 was not significantly different ($p > 0.05$). Of the two proteins related to development, Idgf4 had a higher abundance level in Aml, while protein takeout was significantly higher in Acc ($p < 0.05$). Among the five immune response related proteins, four of them showed high abundance level in Acc: apolipophorin-III-like, defensing-1-precursor, icarapin precursor, and venom venom protein 2. However, only antithrombin-III was higher in Aml ($p < 0.05$) (Figure 3B, Supplementary Table S7).

The protein glycosylation is commonly reflected in the changes of protein expression and site-specific glycosylation. To differentiate these scenarios, the spectral counting based relative abundance of each glycosylation site is needed to compare with that of its parent protein to verify the differential glycosylation. For the proteins with a single N-glycosite in the two RJ samples, ferritin and apolipophorin-III-like protein exhibited higher abundance levels in Acc both in protein and glycosite, while Idgf4 was higher in Aml both at protein and glycosite levels. For the proteins with two or more glycosites, varied abundance patterns were found at the glycosite level across all the protein groups between two RJ samples. For instance, glucosylceramidase was higher in Acc at protein level (Figure 3B), but one of its N-glycosites, N^{38}, showed higher abundance levels in Aml, and the other site, N^{116}, showed higher abundance levels in Acc (Figure 3C−F).

3.6. Glycosylated MRJP2 Inhibits the Growth of *P. larvae*

To test the influence of protein glycosylation on the antibacterial activity of MRJPs, the MRJP1−3 from both Aml and Acc were purified and deglycosylated. Figure 4 shows that the MRJP1−3 from both of the bee species were well purified and deglycosylated.

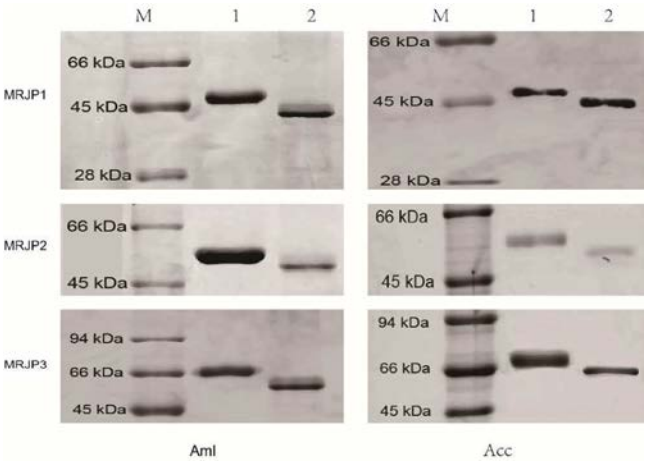


Figure 4. SDS-PAGE analysis of the purified glycosylated MRJP1, 2, and 3 and their deglycosylated froms. Aml and Acc represent the RJ proteins produced by Aml and Acc, respectively. M represents the molecular weight marker; lane 1 is the glycosylated MRJP1−3; lane 2 is for the deglycosylated MRJP1−3 after treated with PNGase F.

The glycosylated MRJP1−3 (P+) as well as their deglycosylated forms (P), and the glycans (L) released from MRJP1−3, were prepared and exposed to the medium containing *Staphylococcus aureus*, *Bacilus subtilis*, and *P. larvae*. MRJP1 (P+, P, and L) and MRJP3 (P+, P, and L) from the Aml and Acc did not exhibit any antibacterial activity against *Staphylococcus aureus*, *Bacilus subtilis*, and *P. larvae*. Interestingly, MRJP2-P+ from produced by both bees was found to effectively inhibit the growth of *P. larvae*, and the resistance of *P. larvae* by Acc was significantly higher than that of Aml ($P < 0.05$), whereas the MRJP2-P and MRJP2-L were not (Table 1).

3.7. Glycosylated MRJP1 Hinders VSMCs Differentiation and Migration

VSMCs are primary components of the blood vessel wall and are essential regulators of vascular function. Increased proliferation and migration of VSMCs are critical phenotypes of several primary cardiovascular diseases such as atherosclerosis and restenosis.[42] AngII, one of the multifunctional peptides involved in vascular alterations in hypertension, can remodel and stimulate the growth of VSMCs and is widely used as an inducer for the preparation of the hypertensive model.[43] Ang II-induced expression of α-SMA, one of the differentiation marker genes of VSMCs, significantly decreased in the presence of glycosylated MRJP1-P+ in both Aml and Acc compared to the control and MRJP1-P and MRJP1-L ($p < 0.05$) (Figure 5A). Moreover, the glycosylated MRJP1 produced by Acc was more efficient for the decreasing of α-SMA (Figure 5B). The immunofluorescence analysis of α-SMA treated with glycosylated MRJP1 and that of its deglycosylated form showed similar trends (Figure 5C).

The glycosylated MRJP1 from both Aml and Acc significantly inhibited the migration of VSMCs, while its deglycosylated form and glycans did not. Furthermore, the glycosylated MRJP1 from Acc exhibited stronger inhibition effects than that of Aml (Figure 5D,E).

4. DISCUSSION

Profiling the N-glycoproteome of the RJ proteins produced by the western and eastern bees is of great importance in understanding the functionality of N-glycosylated RJ proteins. Our improved protocol by optimizing the elution gradients through prolonging the elution time resulted in identification of 80 nonredundant glycoproteins carrying 190 glycosites in the RJ of Aml, which cover almost all of the N-glycosites previously reported,[29] and 23 proteins were identified as novel RJ proteins harboring 35 glycosites, which expands the N-glycoproteome coverage of RJ of Aml to an unprecedented depth. All the 138 glycosites from 43 nonredundant glycoproteins in the RJ of Acc were newly reported. The N-glycosylated wide spectrum of RJ proteins produced by Aml and Acc display species-specific glycosylation features and functionalities. The N-glycosylated MRJP1 from both bee species reveals its significant role in fighting against hypertension disease, one of the major culprit threats to human health, but an enhanced activity was found in MRJP1 of Acc. Moreover, the N-glycosylated MRJP2 from Acc demonstrates a stronger inhibitory efficiency on *P. larvae* than that of Aml, a destructive disease of the western honeybees.

4.1. N-Glycosylation May Influence on Enzyme Activity in RJ

Glycosylation has been reported in regulating the enzymatic processing by attaching glycans to peptide backbones and then influencing the enzymatic ctivities.[44] Apart from previously

DOI: 10.1021/acs.jproteome.5b00829
J. Proteome Res. 2015, 14, 5327−5340

Table 1. Antimicrobical Assay of N-Glycosylated MRJP1−3[a]

	MIC50 (uM)								
	MRJP1 Aml/Acc			MRJP2 Aml/Acc			MRJP3 Aml/Acc		
microorganism	P+	P	L	P+	P	L	P+	P	L
Staphylococcus aureus	N/N	N/N	N/N	N/N	N/N	N/N	N/N	N/N	N/N
Bacilus subtilis	N/N	N/N	N/N	N/N	N/N	N/N	N/N	N/N	N/N
P. larvae	N/N	N/N	N/N	25 ± 1.36/13 ± 1.18	N/N	N/N	N/N	N/N	N/N

[a]Note: N-glycosylated MRJP1−3 produced by Aml and Acc were purified and deglycosylated by PNGase F, respectively. Then the N-glycosylated MRJP1−3 (P+), deglycosylated MRJP1−3 (P), and removed glycans (L) were prepared for antimicrobical assay. The results were indicated by the values of minimal inhibitory concentration (MIC). "N" means not detected. MIC50 is showed as "means ± SD".

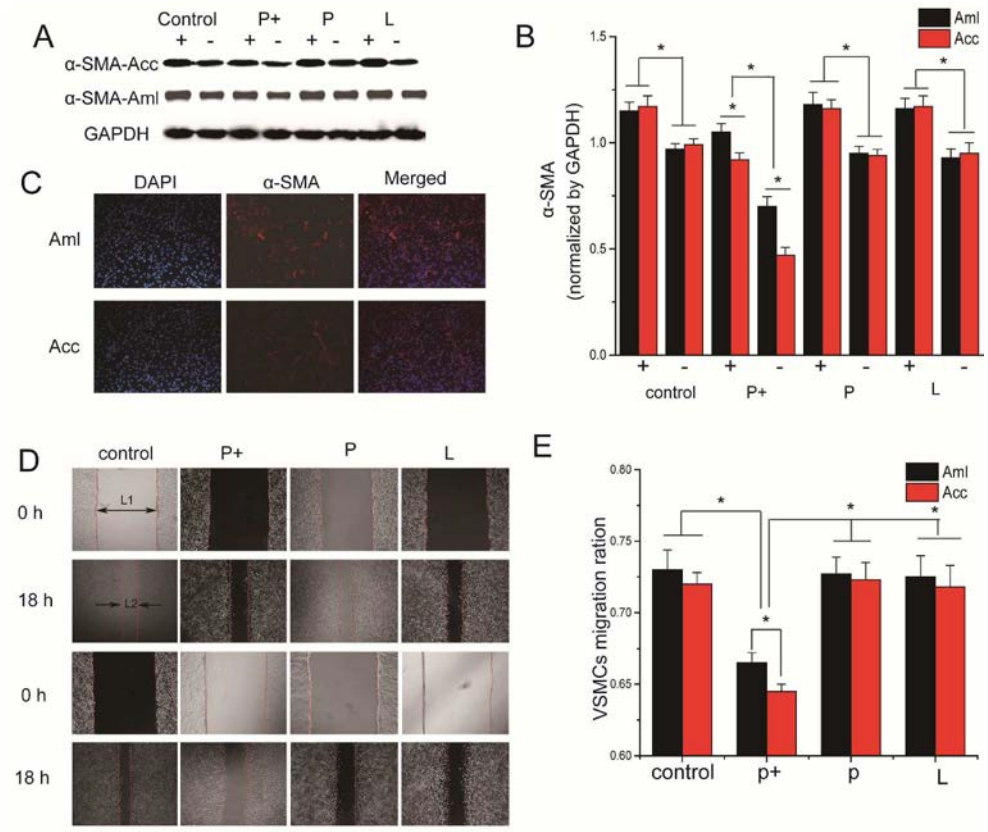


Figure 5. Western blot, fluorescent immunostaining, and scratch wound assay of N-glycosylated MRJP1 produced by Aml and Acc on mice aortic VSMCs. (A) Western blot analysis. VSMCs (2×10^4) were prepared and starved for 12 h. After that, the glycosylated MRJP1 (P+), deglycosylated MRJP1 (P), and the glycans (L) are added to the media at the concentration of 1 mg/mL, the Angiotensin II (Ang II) (final concentration 1×10^{-6} M) was added to stimulate the proliferation of the VSMCs as control ("+" indicates in the presence of Ang II, "−" is not). After 24 h incubation, total proteins are extracted from cells, and the concentration is determined, and equal amount of proteins are separated by stacking (4%) and separating (12%) SDS-PAGE, followed by Western blot analysis. The primary mice antibodies for α smooth muscle actin (α-SMA) are added at a dilution of 1:500. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is used as reference control. (B) Histograph of Western blot. The protein bands in panel A are scaled with ImageJ software, and the relative expression of α-SMA is calculated and showed in histograph. "+" indicates in the presence of Ang II, "−" is not. (C) Fluorescent immunostaining of α-SMA. The left and the middle panel are the immunofluorescence with DAPI (blue) and Dylight 594 (red), the right panel shows the merged immunofluorescence staining. (D) Scratch wound assay of VSMCs. The panels from left to right represent untreated VSMCs (control), VSMCs treated with glycosylated MRJP1(P+), deglycosylated MRJP1(P), and glycans (L) at 0 and 16 h, respectively. (E) The VSMCs migration ratio is calculated according to (L1-L2)/L1. The error bars represent the standard deviation; "∗" indicates the statistically significant differences ($p < 0.05$).

reported enzymes as glycoproteins in RJ,[29] several new enzyme isoforms and N-glycosites were identified in this study. For instance, cysteine proteinase CG12163 is involved in protein metabolism,[45] N-acetylgalactosaminidase metabolizes the glycolipid in *Bactrocera dorsalis*,[46] and beta-glucuronidase is linked to the carbohydrate metabolic process.[47] They work togrther to provide enough energetic fuel for the achievable developing speed of the larvae.[48]

N-glycans can help glycoprotein folding by creating a series of checkpoints that determine the life of the nascent proteins prior to exit from the endoplasmic reticulum (ER). To achieve this goal, the N-glycans are recognized by two lectin-like chaperones in the ER, calnexin (CNX), and calreticulin (CRT), and then the nascent proteins are retained in the ER until proper folding occurs. α-Glucosidases and mannosidases are central to this process. α-Glucosidases I and II sequentially trim

DOI: 10.1021/acs.jproteome.5b00829
J. Proteome Res. 2015, 14, 5327−5340

glucose residues from the $Glc_3Man_9GlcNAc_2$ glycan, the precursor sugar chain initially transfers to the protein, and then release the glycoprotein from CNX/CRT. However, ER α-mannosidase and Golgi α1−2 mannosidases are important in removing the terminal mannose from $Man_9GlcNAc_2$ to yield $Man_5GlcNAc_2$, a key intermediate step to form the hybrid and N-glycans.[49,50] Therefore, the α-glucosidase and α-mannosidase in RJ may be important for both glycoprotein quality control and in N-glycan chain formation for the RJ proteins.

For the honeybees, the greater number of metabolism-related glycoproteins found in the RJ of Aml (26 proteins) compared to Acc (12 proteins) may respond to the high energy demand by the larger body weight of Aml compared to Acc[51] to consolidate the larval growth via the modulation of enzymatic efficiency[44] and also to respond to the quality control and N-glycan chain formation for the high number of glycoproteins and large RJ production in Aml.[52]

The higher abundance level of six proteins in RJ of Acc and the varied the abundance level at the glycosite indicate the species-specific N-glycosylation modification of the metabolism associated glycoproteins and also reveal that protein glycosylation may be achieved its role for energy production to underpin the different larval growth of Aml and Acc at both protein and site level.

4.2. N-Glycosylation May Modulate Biological Functions of MRJPs

Glycosylated MRJPs in RJ play key roles in attaining the biological roles of RJ and ultimately in the determination of the RJ's nutritional quality. MRJPs are a well-balanced nutritional source for the rapidly developing larvae and the fecundity of the honeybee queen owing to their high level of essential amino acids and concentration of nitrogen-rich amino acids.[20,53] The glycosylation on MRJPs is likely to enhance their solubility[54] to improve the assimilation efficiency of honeybee larvae.[29]

Noticeably, albeit the protein abundance level of all the MRJPs in RJ is significantly higher in Aml than in Acc at the global proteome level,[10] a reverse trend was found at the glycoprotein level; all the nine shared glycosylated MRJPs showed a significantly higher abundance level in Acc than in Aml (Figure 3B). This is similar to the findings of phosphorylated MRJPs.[12] The multiple glycosylation sites occurred in the MRJPs from Aml and Acc could reflect that many of the MRJPs' cellular activities and functions might be independently regulated via glycosylation at distinct sites.[55] The heterogeneous distribution of the N-glycosites and the inconsistency of abundance levels of glycoproteins and glycosites of MRJPs from western and eastern honeybees indicate that the two bee species have evolved species-specific glycosylation strategies to modify their MRJPs to satisfy their own biological demands as nutrition and defense agents.

4.3. N-Glycosylated RJ Proteins May Enhance Immune Acitivity

In addition to acting as important nutrients, RJ also provides honeybee larvae and humans with a number of physiological advantages such as enhanced immunity and health promoting activities.[56] In a living organism, innate and adaptive immune responses are mobilized by the immune system to fight against invading pathogens. As for honeybee larvae, the first line of defense against pathogens before the innate and adaptive immune system generate a specific response is the cell-free immune repertoire, which mainly consists of four antibiotic polypeptides, defensin, abaecin, apidaecin, and hymenoptaecin.[57] Defensins are actively resistant against some Gram-positive bacteria and fungi, including *P. larvae*.[58] Apidaecin has a wide spectrum of antimicrobial activity against Gram-negative bacteria.[59] Hymenoptaecin can inhibit the viability of Gram-negative and Gram-positive bacteria.[60] The latter two proteins also play a crucial role in defending against *P. larvae*.[61]

In addition, several other identified proteins were also found to be involved in the improvement of honeybee immunity. Apolipophorin III is constitutively expressed in the fat body, epidermis, and venom gland of *Apis cerana* and functions in boosting innate immunity.[39] Venom serine protease 34 can fight against intruding microorganisms and parasites in insects.[62] Icarapin/venom protein 2 is involved in promoting the innate immunity in honeybees.[12,29] Venom protease is also a strong antithrombotic agent.[63] Antithrombin-III is a serine protease inhibitor that plays important roles not only in controlling innate immunity and development when ingested by the larvae,[64] but also in its effect on antiseptic properties of RJ.[2] Toll-like receptors (TLRs) are linked to an innate and adaptive immunity in *Drosophila*.[59] TLR-13 like, a glycoprotein in RJ, is deduced to be related to both development and innate immunity of honeybee larvae.[29] Here the identified glycoproteins significantly extend the RJ glycoproteome coverage since most of the previously reported proteins lacked glycosylation annotation.[29]

Apart from the roles in immune response to honeybee, the above-mentioned proteins also play key roles in human health promotion. For instance, hymenoptaecin can inhibit several human pathogens such as *Bordetella bronchiseptica*, *Enterobacter cloacae*, and *Haemophilus influenza*.[60] Regucalcin acts as a suppressor protein in signal transduction to suppress the proliferation of various types of tumor tissue in animal and human.[65] Venom dipeptidyl peptidase could enhance the immune response of Mammalia by stimulating the T-cells or inhibiting malignant phenotypes of prostate cancer cells by blocking basic fibroblast growth.[66,67]

The biological significance of glycomodification on those immune response related polypeptides and proteins in RJ may (1) act as a molecular switch to modulate a peptide/protein's activity on or off.[68] This is supported by the fact that the glycosylated MRJP2 could inhibit the growth of *P. larvae*, while its deglycosylated form could not, which is in concordance with the notion that glycosylation is implicated as a key player in host/pathogen interaction;[69] it could (2) protect the bioactive proteins and peptides from digestion in the gastrointestinal system and allow them to remain their intact form to perform their bioactive activities.[17] The protection from digestion is achieved by blocking the access of the endopeptidases to the peptide backbone by steric hindrance of the attached glycans.[70] Then the polypeptides and proteins can be kept intact and active in the honeybee midgut, the main digestive organ, and the natural pathogen habitat, and work together for immune response.

Interestingly, despite a higher number of immune response related glycoproteins in the RJ of Aml (24) than Acc (10), only five proteins were shared, and four of them showed a higher level of abundance in Acc. The varied pattern of abundance levels in protein and N-glycosites of those proteins with immunity activities indicates that the two honeybee species have devised different defense strategies by using those species-dependent exogenous N-glycoproteins and glycopeptides to combat the different pathogens for the good of the sepcies.[71]

DOI: 10.1021/acs.jproteome.5b00829
J. Proteome Res. 2015, 14, 5327−5340

4.4. N-Glycosylation May Regulate Development

As in other living organisms, developmental regulation factors are required to modulate the honeybee larvae growth. Three N-glycosyalted developmental regulators, protein takeout, Chitinase-like protein Idgf4, and laccase, were newly identified in the Acc RJ. Although four proteins are known as glycoproreins in the Aml, novel N-glycosites, Chitinase-like protein Idgf4-like isoform X1(N^{137}), X2 (N^{410}), protein takeout-like isoform X2 (N^{96}), and protein CREG1 (N^{254}) were observed here. Idgf4, a cell growth factor secreted by the honeybee salivary system, affects the physiology status and growth of the other individuals by binding to the insulin-like peptides and then activating intracellular-signaling.[72] Protein takeout is linked between circadian rhythms and feeding behavior in *Drosophila*.[73] In the honeybee genome, eight takeout related proteins play key roles in modulating the circadian rhythms.[74] The takeout proteins in RJ are also potentially important for caste differentiation of larvae and division of labor of the adults by modulating the JH function.[2] Laccase participates in the pigmentation and sclerotization of newly synthesized cuticle by catalyzing the oxidation of phenolic compounds to their corresponding quinones in insects.[75] During the larval stage, the honeybee molts five times in each instar,[19] and the laccase in RJ may perform a role in helping the honeybee brood ecdysis as it does in *Drosophila* puparium.[76] The glycosylated CREG1 in RJ may act as a ligand to enhance the honeybee larvae cell growth and differentiation under the regulation of the mitogen-activated protein kinase pathway.[77] Besides its biological roles in honeybee larvae, the CREG1 in RJ may be useful for cardiometabolic disease treatment as exogenous CREG1 can protect the heart tissue from Ang II-induced fibrosis by activating autophagy in mice.[78]

Glycosylation modification can act as a regulatory switch to modulate the function of developmental regulators. For instance, only the glycosylated epidermal growth factor can bind to its receptor and then activate the downstream pathways to trigger cell proliferation, but its deglycosylated form could not. The glycosylation on the developmental regulators in the RJ may serve as the same purpose to promote the cell growth and differentiation of honeybee larvae.

4.5. N-Glycosylated MRJP2 Has Different Antibiotic Activity on *P. larvae* in Two RJ Samples

It is reported that the phosphorylated Jelleines, products of the tryptic digestion of MRJP1,[79] in RJ produced by Aml and Acc have antibiotic activity on *Bacilus subtilis* and *P. larvae*.[12] In this investigation, however, no inhibitory effect was observed on *Staphylococcus aureus* or *Bacilus subtilis* in the presence of glycosylated MRJP1−3 (P+), the deglycosylated MRJP1−3 (P), and glycans (L) from two bee species, but the glycosylated MRJP2 from both bees exhibit resistance to *P. larvae*, which is similar to the fact that the glycosylated minority homology of MRJP2 can prohibit the growth of *P. larvae*.[26] However, the observed stronger inhibitory activity of Acc glycosylated MRJP2 on *P. larvae* than Aml suggests that the different bee species have employed a distinct strategy of glycosylation to prime the functional activity of RJ proteins for the good of their respective physiology. This is reflected in our data that a quite distinct glycosylation pattern occurred on MRJP2 in two RJ samples in terms of number of glycosites, protein, and site abundance. Importantly, only the glycosylated MRJP2 can fight against *P. larvae* rather than deglycosylated MRJP2 and glycans. This indicates that the attached glycans to the MRJP2 protein backbone are functionally important for MRJP2 to achieve antibiotic activity by acting as a decoy receptor to inhibit *P. larvae* interaction with the epithelial wall.[80]

Furthermore, two polypeptides, apidaecin and hymenoptaecin, which play a crucial role in fighting against *P. larvae*,[61] were identified in Acc RJ only. Also, a leucine-rich repeat-containing protein 15 was found in the RJ of Acc but not in Aml. Leucine-rich repeat glycoprotein is a kind of protein family associated with the formation of the insect peritrophic matrix (PM).[81] PM is the first barrier in protecting the epithelial cell layer from the attacks of *P. larvae* in the honeybee larval midgut.[82] The degradation of the larval PM is a key step in invading AFB disease pathogenesis.[83] The higher resistance of Acc larvae to AFB disease compared to Aml larvae may be attributed to the existence of leucine-rich repeat-containing protein in Acc larval food, which might help maintaining the integrity of the Acc larval PM so as to block the contact between the epithelial cell and *P. larvae*. Together with the enhanced acitivity in inhibiting the proliferation of *P. larvae* of N-glycosylated MRJP2 in Acc, they may work as a integrated mechanism to ensure the Acc larvae against the invasion of AFB disease. Our findings reveal the mechanism how different bee species fight against AFB and provide sound clues for the potential avenue to contain this disease.[84]

4.6. N-Glycosylated MRJP1 Has Antihypertensive Activity

Hypertension has become a major risk factor that can result in cerebral stroke, heart failure, and acute myocardial infarction in humans.[85] RJ has an antihypertension effect since the gastrointestinal enzyme hydrolysate of RJ can decrease blood pressure in spontaneously hypertensive rats[7] and in humans.[86] Although there are plenty of descriptions that MRJPs have a potential function in resisting hypertension,[87] the specific functional constituent is still not yet clear.

The VSMC is an ideal cell model for the research of vascular diseases in vitro.[88] The abnormal contraction and migration of VSMCs are key driving force of various vascular diseases including hypertension, atherosclerosis, and vascular stenosis after vascular remodeling.[89,90] Ang II is known to be an important stimulation for the contraction and migration of VSMCs,[91] and scratch wound assay is a useful way to measure the cell migration ability of VSMCs.[92] Here, the significantly decreased expression level of α-SMA and the effectively inhibited cell migration of Ang II-induced VSMCs after introduction of glycosylated MRJP1 from Aml and Acc suggest that only the glycosylated MRJP1, other than its deglycosylated form and glycans, is a key player in RJ for blood pressure regulation. Moreover, the glycosylated MRJP1 derived from Acc has a stronger activity on hypertension regulation. Although the mechanism of this regulation still remains to be uncovered, this will hold a new hope for the potential pharmacotherapeutics to fight against hypertensive disease using glycosylated MRJP1.

4.7. Species-Specific N-Glycoproteome of RJ

One of the most striking characteristics of our data is that the two RJ samples display a species-specific glyco-modification pattern. This is reflected by the significant differences in the two RJ samples in terms of N-glycoprotein species (Figure 1), number of N-glycosylated sites and glycopeptide motifs (Figure 2A,B), and abundance levels of glycoproteins and the N-glycosites (Figure 3). This distinct N-glycoproteome characteristic is a further manifestation of the functional differences in the glycosylated MRJP1 and MRJP2 produced by Aml and Acc

DOI: 10.1021/acs.jproteome.5b00829
J. Proteome Res. 2015, 14, 5327−5340

on blood pressure regulation and against *P. larvae*, respectively (Figure 5, Table 1). These observations indicate that different honeybees have evolved species-specific glycosylation modification strategies to tune protein activity for optimizing cellular behavior for the good of each species.

Given the regular functionality of MRJPs to work in glycosylated form, the identified information on glycoproteins and glycosites in Aml and Acc RJ may potentially be important for further functional investigation and utilization of targeted RJ proteins such as expression and biosynthesis of N-linked MRJP1 and 2 with glycoengineering by applying engineering bacteria, *E. coli*,[93] for the treatment of hypertension in humans and in AFB disease in the honeybee.

5. CONCLUSIONS

The in-depth and large-scale analysis on RJ of Aml and Acc manifests the species-specific N-linked glycoproteome characteristics in the western and eastern honeybees. The reported 23 novel proteins harboring 35 glycosites of the 80 nonredundant glycoproteins with 190 glycosites in Aml, and 43 nonredundant glycoproteins carrying 138 glycosites in Acc, extend the RJ glycoproteome coverage of Aml RJ to an unprecedented depth and characterizes the Acc RJ glycoproteome for the first time.

The wide range of N-glycosylation modified proteins in RJ is associated with MRJPs, developmental regulation, metabolism processes, and immunity activities. The glycosylation of RJ is functionally important to help RJ achieve its biological functions benefiting for the honeybee and human health. The species-specific glycosylation of RJ from Aml and Acc endows RJ with similar but different functional properties to perform different biological activities in fighting against *P. larvae* for honeybee and blood regulation for humans. The reported N-glycoproteome of RJ from the western and eastern honeybee and functional assay on MRJPs significantly gain new understanding of regulatory mechanisms underlying the honeybee biology and promoting the human health. This will act as a valuable resource for functional probing of targeted molecules via glycoengineering technology for honeybee and medical communities.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.5b00829.

Annotated tandem mass spectra of the N-linked glycopeptides of royal jelly proteins derived from Aml enriched by lectin chemistry and hydrazide resin; annotated tandem mass spectra of the N-linked glycopeptides of RJ proteins derived from Acc enriched by lectin chemistry and hydrazide resin (PDF)

Identification of N-glycosylated RJ proteins produced by Aml; identification of N-glycosylated RJ proteins produced by Acc; identification of N-linked glycopeptides of RJ proteins derived from Aml enriched with lectin chemistry; identification of N-linked glycopeptides of RJ proteins derived from Aml enriched with hydrazide resin; identification of N-linked glycopeptides of RJ proteins derived from Acc enrichment by lectin chemistry; identification of N-linked glycopeptides of RJ proteins derived from Acc enriched by hydrazide chemistry; quantitative analysis of N-linked N-linked glycoproteins in RJ proteins derived from Aml and Acc (XLSX)

Detailed protocols for N-glycosylated MRJP 1−3 purification, hydrazide and lectin enrichment, and glycan purification (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: apislijk@126.com. Phone: +86 10 6259 1449.

Author Contributions

§These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work is supported by the Agricultural Science and Technology Innovation Program (CAAS-ASTIP-2015-IAR), 948 project (2014-S3) of Ministry of Agriculture of the People Republic of China, and the earmarked fund for Modern Agro-Industry Technology Research System (CARS-45) in China. We thank Katrina Klett from the United States for her help with the manuscript.

■ REFERENCES

(1) Winston, M. L. *The Biology of the Honey Bee*; Harvard University Press: Cambridge, MA, 1987; pp 316−318.

(2) Fujita, T.; Kozuka-Hata, H.; Ao-Kondo, H.; Kunieda, T.; Oyama, M.; Kubo, T. Proteomic analysis of the royal jelly and characterization of the functions of its derivation glands in the honeybee. *J. Proteome Res.* **2013**, *12*, 404−411.

(3) Kamakura, M. Royalactin induces queen differentiation in honeybees. *Nature* **2011**, *473*, 478−483.

(4) Kamakura, M.; Suenobu, N.; Fukushima, M. Fifty-seven-kDa protein in royal jelly enhances proliferation of primary cultured rat hepatocytes and increases albumin production in the absence of serum. *Biochem. Biophys. Res. Commun.* **2001**, *282*, 865−874.

(5) Šver, L.; Oršolić, N.; Tadić, Z.; Njari, B.; Valpotic, I.; Bašic, I. A royal jelly as a new potential immunomodulator in rats and mice. *Comp. Immunol. Microbiol. Infect Dis.* **1996**, *19*, 31−38.

(6) Kamakura, M.; Sakaki, T. A hypopharyngeal gland protein of the worker honeybee Apis mellifera L. enhances proliferation of primary-cultured rat hepatocytes and suppresses apoptosis in the absence of serum. *Protein Expression Purif.* **2006**, *45*, 307−314.

(7) Matsui, T.; Yukiyoshi, A.; Doi, S.; Sugimoto, H.; Yamada, H.; Matsumoto, K. Gastrointestinal enzyme production of bioactive peptides from royal jelly protein and their antihypertensive ability in SHR. *J. Nutr. Biochem.* **2002**, *13*, 80−86.

(8) Olszewski, T. K.; Bomont, C.; Coutrot, P.; Grison, C. Lithiated anions derived from (alkenyl) pentamethyl phosphoric triamides: Useful synthons for the stereoselective synthesis of 9-oxo-and 10-hydroxy-2 (E)-decenoic acids, important components of queen substance and royal jelly of honeybee Apis mellifera. *J. Organomet. Chem.* **2010**, *695*, 2354−2358.

(9) Olszewski Claude Grison, T. K.; Bomont, C.; Coutrot, P. In *Synthesis of insect pheromones: Improved method for the preparation of queen substance and royal jelly of honeybees Apis mellifera. Ann. Soc. Entomol. Fr.* **2011**, *47*, 45−54.

(10) Yu, F.; Mao, F.; Jianke, L. Royal jelly proteome comparison between A. mellifera ligustica and A. cerana cerana. *J. Proteome Res.* **2010**, *9*, 2207−2215.

(11) Park, D.; Jung, J. W.; Choi, B. S.; Jayakodi, M.; Lee, J.; Lim, J.; Yu, Y.; Choi, Y. S.; Lee, M. L.; Park, Y.; Choi, I. Y.; Yang, T. J.; Edwards, O. R.; Nah, G.; Kwon, H. W. Uncovering the novel characteristics of Asian honey bee, Apis cerana, by whole genome sequencing. *BMC Genomics* **2015**, *16*, 1.

DOI: 10.1021/acs.jproteome.5b00829
J. Proteome Res. 2015, 14, 5327−5340

(12) Han, B.; Fang, Y.; Feng, M.; Lu, X.; Huo, X.; Meng, L.; Wu, B.; Li, J. In-depth phosphoproteomic analysis of royal jelly derived from western and eastern honeybee species. *J. Proteome Res.* **2014**, *13*, 5928−5943.
(13) Zhang, Y.; Zhang, G.; Huang, X.; Han, R. Proteomic Analysis of Apis cerana and Apis mellifera Larvae Fed with Heterospecific Royal Jelly and by CSBV Challenge. *PLoS One* **2014**, *9*, e102663.
(14) Shi, Y. Y.; Huang, Z. Y.; Wu, X. B.; Wang, Z. L.; Yan, W. Y.; Zeng, Z. J. Changes in Alternative Splicing in Apis Mellifera Bees Fed Apis Cerana Royal Jelly. *J. Apic. Sci.* **2014**, *58*, 25−31.
(15) Spiro, R. G. Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. *Glycobiology* **2002**, *12*, 43R−56R.
(16) Apweiler, R.; Hermjakob, H.; Sharon, N. On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochim. Biophys. Acta, Gen. Subj.* **1999**, *1473*, 4−8.
(17) Lis, H.; Sharon, N. Protein glycosylation. Structural and functional aspects. *Eur. J. Biochem.* **1993**, *218*, 1−27.
(18) Rudd, P. M.; Elliott, T.; Cresswell, P.; Wilson, I. A.; Dwek, R. A. Glycosylation and the immune system. *Science* **2001**, *291*, 2370−2376.
(19) Graham, J. M. *The Hive and the Honey Bee*; Dadant & Sons: Hamiltin, IL, 1992.
(20) Schmitzova, J.; Klaudiny, J.; Albert; Schröder, W.; Schreckengost, W.; Hanes, J.; Judova, J.; Simuth, J. A family of major royal jelly proteins of the honeybee Apis mellifera L. *Cell. Mol. Life Sci.* **1998**, *54*, 1020−1030.
(21) Furusawa, T.; Rakwal, R.; Nam, H. W.; Shibato, J.; Agrawal, G. K.; Kim, Y. S.; Ogawa, Y.; Yoshida, Y.; Kouzuma, Y.; Masuo, Y.; Yonekura, M. Comprehensive royal jelly (RJ) proteomics using one- and two-dimensional proteomics platforms reveals novel RJ proteins and potential phospho/glycoproteins. *J. Proteome Res.* **2008**, *7*, 3194−3229.
(22) Kimura, Y.; Miyagi, C.; Kimura, M.; Nitoda, T.; Kawai, N.; Sugimoto, H. Structural Features of N-Glycans Linked to Royal Jelly Glycoproteins: Structures of high-mannose type, hybrid type, and biantennary type glycans. *Biosci., Biotechnol., Biochem.* **2000**, *64*, 2109−2120.
(23) Kimura, M.; Kimura, Y.; Tsumura, K.; Okihara, K.; Sugimoto, H.; Yamada, H.; Yonekura, M. 350-kDa Royal Jelly Glycoprotein (Apisin), Which Stimulates Proliferation of Human Monocytes, Bears the. BETA. 1−3Galactosylated N-Glycan: Analysis of the N-Glycosylation Site. *Biosci., Biotechnol., Biochem.* **2003**, *67*, 2055−2058.
(24) Kimura, Y.; Washino, N.; Yonekura, M. N-linked sugar chains of 350-kDa royal jelly glycoprotein. *Biosci., Biotechnol., Biochem.* **1995**, *59*, 507−509.
(25) Kimura, Y.; Kajiyama, S.-i.; Kanaeda, J.; Izukawa, T.; Yonekura, M. N-linked sugar chain of 55-kDa royal jelly glycoprotein. *Biosci., Biotechnol., Biochem.* **1996**, *60*, 2099−2102.
(26) Bíliková, K.; Mirgorodskaya, E.; Bukovská, G.; Gobom, J.; Lehrach, H.; Simúth, J. Towards functional proteomics of minority component of honeybee royal jelly: The effect of post-translational modifications on the antimicrobial activity of apalbumin2. *Proteomics* **2009**, *9*, 2131−2138.
(27) Novotny, M. V.; Mechref, Y. New hyphenated methodologies in high-sensitivity glycoprotein analysis. *J. Sep. Sci.* **2005**, *28*, 1956−1968.
(28) Zielinska, D. F.; Gnad, F.; Wiśniewski, J. R.; Mann, M. Precision mapping of an in vivo N-glycoproteome reveals rigid topological and sequence constraints. *Cell* **2010**, *141*, 897−907.
(29) Zhang, L.; Han, B.; Li, R.; Lu, X.; Nie, A.; Guo, L.; Fang, Y.; Feng, M.; Li, J. Comprehensive identification of novel proteins and N-glycosylation sites in royal jelly. *BMC Genomics* **2014**, *15*, 135.
(30) Thakur, S. S.; Geiger, T.; Chatterjee, B.; Bandilla, P.; Frohlich, F.; Cox, J.; Mann, M. Deep and Highly Sensitive Proteome Coverage by LC-MS/MS Without Prefractionation. *Mol. Cell. Proteomics* **2011**, *10*, M110.003699.
(31) Elias, J. E.; Gygi, S. P. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nat. Methods* **2007**, *4*, 207−214.
(32) Beausoleil, S. A.; Villen, J.; Gerber, S. A.; Rush, J.; Gygi, S. P. A probability-based approach for high-throughput protein phosphorylation analysis and site localization. *Nat. Biotechnol.* **2006**, *24*, 1285−1292.
(33) Nozaki, R.; Tamura, S.; Ito, A.; Moriyama, T.; Yamaguchi, K.; Kono, T. A rapid method to isolate soluble royal jelly proteins. *Food Chem.* **2012**, *134*, 2332−2337.
(34) Tamura, S.; Amano, S.; Kono, T.; Kondoh, J.; Yamaguchi, K.; Kobayashi, S.; Ayabe, T.; Moriyama, T. Molecular characteristics and physiological functions of major royal jelly protein 1 oligomer. *Proteomics* **2009**, *9*, 5534−5543.
(35) Nwosu, C. C.; Aldredge, D. L.; Lee, H.; Lerno, L. A.; Zivkovic, A. M.; German, J. B.; Lebrilla, C. B. Comparison of the human and bovine milk N-glycome via high-performance microfluidic chip liquid chromatography and tandem mass spectrometry. *J. Proteome Res.* **2012**, *11*, 2912−2924.
(36) Liu, H.; Sadygov, R. G.; Yates, J. R., 3rd A model for random sampling and estimation of relative protein abundance in shotgun proteomics. *Anal. Chem.* **2004**, *76*, 4193−201.
(37) Zybailov, B.; Mosley, A. L.; Sardiu, M. E.; Coleman, M. K.; Florens, L.; Washburn, M. P. Statistical analysis of membrane proteome expression changes in Saccharomyces cerevisiae. *J. Proteome Res.* **2006**, *5*, 2339−2347.
(38) Schwartz, D.; Gygi, S. P. An iterative statistical approach to the identification of protein phosphorylation motifs from large-scale data sets. *Nat. Biotechnol.* **2005**, *23*, 1391−1398.
(39) Kim, B. Y.; Jin, B. R. Apolipophorin III from honeybees (Apis cerana) exhibits antibacterial activity. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* **2015**, *182*, 6−13.
(40) Valliere-Douglass, J. F.; Eakin, C. M.; Wallace, A.; Ketchem, R. R.; Wang, W.; Treuheit, M. J.; Balland, A. Glutamine-linked and non-consensus asparagine-linked oligosaccharides present in human recombinant antibodies define novel protein glycosylation motifs. *J. Biol. Chem.* **2010**, *285*, 16012−16022.
(41) Hounsell, E. F. Characterization of the glycosylation status of proteins. *Mol. Biotechnol.* **1994**, *2*, 45−60.
(42) Schwartz, S. M.; Heimark, R. L.; Majesky, M. W. Developmental mechanisms underlying pathology of arteries. *Physiol. Rev.* **1990**, *70*, 1177−1209.
(43) Gao, P.; Xu, T. T.; Lu, J.; Li, L.; Xu, J.; Hao, D. L.; Chen, H. Z.; Liu, D. P. Overexpression of SIRT1 in vascular smooth muscle cells attenuates angiotensin II-induced vascular remodeling and hypertension in mice. *J. Mol. Med. (Heidelberg, Ger.)* **2014**, *92*, 347−357.
(44) Nyberg, G. B.; Balcarcel, R. R.; Follstad, B. D.; Stephanopoulos, G.; Wang, D. I. Metabolic effects on recombinant interferon-γ glycosylation in continuous culture of Chinese hamster ovary cells. *Biotechnol. Bioeng.* **1999**, *62*, 336−347.
(45) Yamamoto, Y.; Takimoto, K.; Izumi, S.; Toriyama-Sakurai, M.; Kageyama, T.; Takahashi, S. Y. Molecular cloning and sequencing of cDNA that encodes cysteine proteinase in the eggs of the silkmoth, Bombyx mori. *J. Biochem.* **1994**, *116*, 1330−1335.
(46) Geib, S. M.; Calla, B.; Hall, B.; Hou, S.; Manoukis, N. C. Characterizing the developmental transcriptome of the oriental fruit fly, Bactrocera dorsalis (Diptera: Tephritidae) through comparative genomic analysis with Drosophila melanogaster utilizing modENCODE datasets. *BMC Genomics* **2014**, *15*, 942.
(47) D'Amore, M. A.; Gallagher, P. M.; Korfhagen, T. R.; Ganschow, R. E. Complete sequence and organization of the murine beta-glucuronidase gene. *Biochemistry* **1988**, *27*, 7131−7140.
(48) Chan, Q. W.; Foster, L. J. Changes in protein expression during honey bee larval development. *Genome Biol.* **2008**, *9*, R156.
(49) Fiedler, K.; Simons, K. The role of N-glycans in the secretory pathway. *Cell* **1995**, *81*, 309−312.
(50) Freeze, H. H.; Esko, J. D.; Parodi, A. J. Glycans in glycoprotein quality control. In *Essentials of Glycobiology*, 2nd ed.; Cold Spring Harbor Laboratory Press: NewYork, 2009; Chapter 36.
(51) Feng, M.; Ramadan, H.; Han, B.; Fang, Y.; Li, J. Hemolymph proteome changes during worker brood development match the

DOI: 10.1021/acs.jproteome.5b00829
J. Proteome Res. 2015, 14, 5327−5340

biological divergences between western honey bees (Apis mellifera) and eastern honey bees (Apis cerana). *BMC Genomics* **2014**, *15*, 563.
(52) Yoshiyama, M.; Kimura, K. Bacteria in the gut of Japanese honeybee, Apis cerana japonica, and their antagonistic effect against Paenibacillus larvae, the causal agent of American foulbrood. *J. Invertebr. Pathol.* **2009**, *102*, 91−96.
(53) Albertova, V.; Su, S.; Brockmann, A.; Gadau, J.; Albert. Organization and potential function of the mrjp3 locus in four honeybee species. *J. Agric. Food Chem.* **2005**, *53*, 8075−8081.
(54) Tams, J. W.; Vind, J.; Welinder, K. G. Adapting protein solubility by glycosylation. N-glycosylation mutants of Coprinus cinereus peroxidase in salt and organic solutions. *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.* **1999**, *1432*, 214−221.
(55) Huttlin, E. L.; Jedrychowski, M. P.; Elias, J. E.; Goswami, T.; Rad, R.; Beausoleil, S. A.; Villen, J.; Haas, W.; Sowa, M. E.; Gygi, S. P. A tissue-specific atlas of mouse protein phosphorylation and expression. *Cell* **2010**, *143*, 1174−1189.
(56) Ramadan, M. F.; Al-Ghamdi, A. Bioactive compounds and health-promoting properties of royal jelly: A review. *J. Funct. Foods* **2012**, *4*, 39−52.
(57) Bulet, P.; Stocklin, R. Insect antimicrobial peptides: structures, properties and gene regulation. *Protein Pept. Lett.* **2005**, *12*, 3−11.
(58) Fujiwara, S.; Imai, J.; Fujiwara, M.; Yaeshima, T.; Kawashima, T.; Kobayashi, K. A potent antibacterial protein in royal jelly. Purification and determination of the primary structure of royalisin. *J. Biol. Chem.* **1990**, *265*, 11333−11337.
(59) Casteels, P.; Ampe, C.; Jacobs, F.; Vaeck, M.; Tempst, P. Apidaecins: antibacterial peptides from honeybees. *EMBO J.* **1989**, *8*, 2387−2391.
(60) Casteels, P.; Ampe, C.; Jacobs, F.; Tempst, P. Functional and chemical characterization of Hymenoptaecin, an antibacterial polypeptide that is infection-inducible in the honeybee (Apis mellifera). *J. Biol. Chem.* **1993**, *268*, 7044−7054.
(61) Chan, Q.; Melathopoulos, A.; Pernal, S.; Foster, L. The innate immune and systemic response in honey bees to a bacterial pathogen, Paenibacillus larvae. *BMC Genomics* **2009**, *10*, 387.
(62) Asgari, S.; Zhang, G.; Zareie, R.; Schmidt, O. A serine proteinase homolog venom protein from an endoparasitoid wasp inhibits melanization of the host hemolymph. *Insect Biochem. Mol. Biol.* **2003**, *33*, 1017−1024.
(63) Hung, C. C.; Huang, K. F.; Chiou, S. H. Characterization of one novel venom protease with beta-fibrinogenase activity from the Taiwan habu (Trimeresurus mucrosquamatus): purification and cDNA sequence analysis. *Biochem. Biophys. Res. Commun.* **1994**, *205*, 1707−1715.
(64) Reichhart, J. M. Tip of another iceberg: Drosophila serpins. *Trends Cell Biol.* **2005**, *15*, 659−665.
(65) Yamaguchi, M.; Murata, T. Suppressive effects of exogenous regucalcin on the proliferation of human pancreatic cancer MIA PaCa-2 cells in vitro. *Int. J. Mol. Med.* **2015**, *35*, 1773−1778.
(66) Kikkawa, F.; Kajiyama, H.; Shibata, K.; Ino, K.; Nomura, S.; Mizutani, S. Dipeptidyl peptidase IV in tumor progression. *Biochim. Biophys. Acta, Proteins Proteomics* **2005**, *1751*, 45−51.
(67) Wesley, U. V.; McGroarty, M.; Homoyouni, A. Dipeptidyl peptidase inhibits malignant phenotype of prostate cancer cells by blocking basic fibroblast growth factor signaling pathway. *Cancer Res.* **2005**, *65*, 1325−1334.
(68) Dallas, D. C.; Sela, D.; Underwood, M. A.; German, J. B.; Lebrilla, C. Protein-Linked Glycan Degradation in Infants Fed Human Milk. *J. Glycomics Lipidomics* **2012**, No. Suppl. 1, 002.
(69) Venkatakrishnan, V.; Packer, N. H.; Thaysen-Andersen, M. Host mucin glycosylation plays a role in bacterial adhesion in lungs of individuals with cystic fibrosis. *Expert Rev. Respir. Med.* **2013**, *7*, 553−576.
(70) Boutrou, R.; Jardin, J.; Blais, A.; Tomé, D.; Léonil, J. l. Glycosylations of κ-casein-derived caseinomacropeptide reduce its accessibility to endo-but not exointestinal brush border membrane peptidases. *J. Agric. Food Chem.* **2008**, *56*, 8166−8173.
(71) Evans, J.; Aronstein, K.; Chen, Y.; Hetru, C.; Imler, J. L.; Jiang, H.; Kanost, M.; Thompson, G.; Zou, Z.; Hultmark, D. Immune pathways and defence mechanisms in honey bees Apis mellifera. *Insect Mol. Biol.* **2006**, *15*, 645−656.
(72) Fujita, T.; Kozuka-Hata, H.; Uno, Y.; Nishikori, K.; Morioka, M.; Oyama, M.; Kubo, T. Functional analysis of the honeybee (Apis mellifera L.) salivary system using proteomics. *Biochem. Biophys. Res. Commun.* **2010**, *397*, 740−744.
(73) Sarov-Blat, L.; So, W. V.; Liu, L.; Rosbash, M. The Drosophila takeout gene is a novel molecular link between circadian rhythms and feeding behavior. *Cell* **2000**, *101*, 647−656.
(74) Hagai, T.; Cohen, M.; Bloch, G. Genes encoding putative Takeout/juvenile hormone binding proteins in the honeybee (Apis mellifera) and modulation by age and juvenile hormone of the takeout-like gene GB19811. *Insect Biochem. Mol. Biol.* **2007**, *37*, 689−701.
(75) Dittmer, N. T.; Kanost, M. R. Insect multicopper oxidases: diversity, properties, and physiological roles. *Insect Biochem. Mol. Biol.* **2010**, *40*, 179−188.
(76) Sugumaran, M.; Giglio, L.; Kundzicz, H.; Saul, S.; Semensi, V. Studies on the enzymes involved in puparial cuticle sclerotization in Drosophila melanogaster. *Arch. Insect Biochem. Physiol.* **1992**, *19*, 271−283.
(77) Sacher, M.; Di Bacco, A.; Lunin, V. V.; Ye, Z.; Wagner, J.; Gill, G.; Cygler, M. The crystal structure of CREG, a secreted glycoprotein involved in cellular growth and differentiation. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 18326−18331.
(78) Yan, C. H.; Li, Y.; Tian, X. X.; Zhu, N.; Song, H. X.; Zhang, J.; Sun, M. Y.; Han, Y. L. CREG1 ameliorates myocardial fibrosis associated with autophagy activation and Rab7 expression. *Biochim. Biophys. Acta, Mol. Basis Dis.* **2015**, *1852*, 353−364.
(79) Fontana, R.; Mendes, M. A.; Souza, B. M. d.; Konno, K.; César, L. M. M.; Malaspina, O.; Palma, M. S. Jelleines: a family of antimicrobial peptides from the Royal Jelly of honeybees (Apis mellifera). *Peptides* **2004**, *25*, 919−928.
(80) Yue, D.; Nordhoff, M.; Wieler, L. H.; Genersch, E. Fluorescence in situ hybridization (FISH) analysis of the interactions between honeybee larvae and Paenibacillus larvae, the causative agent of American foulbrood of honeybees (Apis mellifera). *Environ. Microbiol.* **2008**, *10*, 1612−1620.
(81) Hocking, A. M.; Shinomura, T.; McQuillan, D. J. Leucine-rich repeat glycoproteins of the extracellular matrix. *Matrix Biol.* **1998**, *17*, 1−19.
(82) Müller, S.; Garcia-Gonzalez, E.; Genersch, E.; Süssmuth, R. D. Involvement of secondary metabolites in the pathogenesis of the American foulbrood of honey bees caused by Paenibacillus larvae. *Nat. Prod. Rep.* **2015**, *32*, 765−778.
(83) Garcia-Gonzalez, E.; Genersch, E. Honey bee larval peritrophic matrix degradation during infection with Paenibacillus larvae, the aetiological agent of American foulbrood of honey bees, is a key step in pathogenesis. *Environ. Microbiol.* **2013**, *15*, 2894−2901.
(84) Gregorc, A. Histopathological and histochemical changes in honeybee larvae (Apis mellifera L.) after infection with Bacillus larvae, the causative agent of American foulbrood disease. *Cell Biol. Int.* **1998**, *22*, 137−144.
(85) Takaki-Doi, S.; Hashimoto, K.; Yamamura, M.; Kamei, C. Antihypertensive activities of royal jelly protein hydrolysate and its fractions in spontaneously hypertensive rats. *Acta Med. Okayama.* **2009**, *63*, 57−64.
(86) Kajimoto, O.; Maruyama, H.; Yoshida, C.; Araki, Y.; Mishima, S.; Sakamoto, A.; Kajimoto, Y. Antihypertensive effects and safety of drink containing protease-treated royal jelly in subjects with high-normal or mild hypertension. *Health Sciences* **2005**, *21*, 229.
(87) Tokunaga, K.; Yoshida, C.; Suzuki, K.; Maruyama, H.; Futamura, Y.; Araki, Y.; Mishima, S. Antihypertensive effect of peptides from royal jelly in spontaneously hypertensive rats. *Biol. Pharm. Bull.* **2004**, *27*, 189−192.
(88) Devlin, A. M.; Clark, J. S.; Reid, J. L.; Dominiczak, A. F. DNA synthesis and apoptosis in smooth muscle cells from a model of genetic hypertension. *Hypertension* **2000**, *36*, 110−115.

DOI: 10.1021/acs.jproteome.5b00829
J. Proteome Res. 2015, 14, 5327−5340

(89) Chu, X.; Filali, M.; Stanic, B.; Takapoo, M.; Sheehan, A.; Bhalla, R.; Lamb, F. S.; Miller, F. J., Jr. A critical role for chloride channel-3 (CIC-3) in smooth muscle cell activation and neointima formation. *Arterioscler., Thromb., Vasc. Biol.* **2011**, *31*, 345−351.
(90) Kim, J.; Zhang, L.; Peppel, K.; Wu, J. H.; Zidar, D. A.; Brian, L.; DeWire, S. M.; Exum, S. T.; Lefkowitz, R. J.; Freedman, N. J. Beta-arrestins regulate atherosclerosis and neointimal hyperplasia by controlling smooth muscle cell proliferation and migration. *Circ. Res.* **2008**, *103*, 70−79.
(91) Daemen, M. J.; Lombardi, D. M.; Bosman, F. T.; Schwartz, S. M. Angiotensin II induces smooth muscle cell proliferation in the normal and injured rat arterial wall. *Circ. Res.* **1991**, *68*, 450−456.
(92) Kotha, J.; Zhang, C.; Longhurst, C. M.; Lu, Y.; Jacobs, J.; Cheng, Y.; Jennings, L. K. Functional relevance of tetraspanin CD9 in vascular smooth muscle cell injury phenotypes: a novel target for the prevention of neointimal hyperplasia. *Atherosclerosis* **2009**, *203*, 377−386.
(93) Merritt, J. H.; Ollis, A. A.; Fisher, A. C.; DeLisa, M. P. Glycans-by-design: engineering bacteria for the biosynthesis of complex glycans and glycoconjugates. *Biotechnol. Bioeng.* **2013**, *110*, 1550−1564.

DOI: 10.1021/acs.jproteome.5b00829
J. Proteome Res. 2015, 14, 5327−5340