Characterization of Immunomodulatory Activities of Honey Glycoproteins and Glycopeptides

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ABSTRACT: Recent evidence suggests an important role for natural honey in modulating immune response. To identify active components responsible, this study investigated the immunomodulatory properties of glycoproteins and glycopeptides fractionated from Ziziphus honey. Honey proteins/peptides were fractionated by size exclusion chromatography into five peaks with molecular masses in the range of 2–450 kDa. The fractionated proteins exhibited potent, concentration-dependent inhibition of reactive oxygen species production in zymosan-activated human neutrophils (IC50 = 6–14 ng/mL) and murine macrophages (IC50 = 2–9 ng/mL). Honey proteins significantly suppressed the nitric oxide production by LPS-activated murine macrophages (IC50 = 96–450 ng/mL). Moreover, honey proteins inhibited the phagocytosis latex bead macrophages. The production of pro-inflammatory cytokines IL-1β and TNF-α by human monocytic cell line in the presence of honey proteins was analyzed. Honey proteins did not affect the production of IL-1β; however, TNF-α production was significantly suppressed. These findings indicated that honey glycoproteins and glycopeptides significantly interfere with molecules of the innate immune system.

KEYWORDS: honey, glycoproteins, immunomodulatory, phagocytosis, oxidative burst

INTRODUCTION

Natural honey is a well-known nutraceutical that is mainly composed of fructose, glucose, sucrose, and maltose. Small amounts of oligosaccharides, proteins, amino acids, minerals, trace elements, vitamins, aroma compounds, and polyphenols are also present (reviewed in ref 1). Honey exerts a number of pharmacological effects including antimicrobial, immunomodulatory, prebiotic, antinematodal, anti-inflammatory, and anti-nociceptive activities.1–9 It showed both bacteriostatic and bactericidal potentials against pathogenic bacteria including drug-resistant strains.2 Honey was found to exert anti-inflammatory effects via inhibiting (a) thrombin-induced reactive oxygen species (ROS) production6 and (b) myeloperoxidase-dependent oxidative burst by professional phagocytes.7 It inhibited nitric oxide (NO) production in a carrageenin-induced inflammatory rat model.10 In addition, manuka, pasture, and jellybush honeys were found to reduce ROS production11 and stimulate monocytes (MonoMac-6 cell line) to produce tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6, which are known to play vital roles in tissue repair.12 The effect of honey on cytokine production was found to be associated with a 5.8 kDa component isolated from manuka honey, which interacted with TLR-4.13 Natural honey exhibited antitumor activity by inhibiting the growth of Lewis lung carcinoma/2 cells.14 An immunostimulatory effect of honey on antibody production against thymus-dependent (sheep red blood cells) and thymus-independent (Escherichia coli) antigens in mice has also been observed.15 Likewise, honey also inhibited mitogen-activated T cell proliferation in vitro. Moreover, honey regulates homeostasis by inhibiting human platelet aggregation and blood coagulation proteins.16

Natural honeys have a low protein content, which varies from type to type. Characterization of honey proteins from various sources was found to be valuable in determining the floral and geographical origin.18 A number of studies pointed out the immunomodulatory potential of honey proteins. Four immunologically characterized IgE-binding glycoproteins (belonging to the major royal jelly protein-1 (MRJP-1) family) were identified in honey, and N-linked glycosylation in these proteins played an important role in binding of IgE.19,21 Ultracentrifugation of royal jelly (RJ) revealed that the MRJP1 was the most abundant protein of RJ present in monomeric (55 kDa) as well as oligomeric (ca. 450 kDa) forms.20 Honey contains MRJP1 protein as well, which exerts its immunostimulatory effect by stimulating TNF-α production in murine peritoneal macrophages, and the protein moiety of this glycoprotein was found be responsible for this property.22 Recently, highly glycosylated arabinogalactan proteins (AGPs) with molecular masses of ∼110 kDa have been characterized from New Zealand kanuka honey.23 AGPs are proteoglycans that contain type II arabinogalactans and 2–10% covalently attached protein that is typically rich in hydroxyproline and serine. AGPs are compact molecules consisting of a protein core to which highly branched polysaccharide chains are attached.24 AGPs are found in the female sexual tissues of flowers and thus could be a potential source of AGPs in honey. AGPs and type II arabinogalactans from different sources have been shown to exert immunomodulatory properties.25 As a
continuation of our research, we have elaborated the role(s) of honey glycoproteins and glycopeptides in the modulation of innate immune systems.

### MATERIALS AND METHODS

**Fractionation and Characterization of Honey Proteins.**

**Honey Samples.** Monofloral *Ziziais* species honey samples produced by *Apis mellifera* in bee farms located in Himalayan and Pothohar plateau regions in northern Pakistan were used during this study.

**Ammonium Sulfate Precipitation.** Honey samples were diluted in buffer A (20 mM Tris-HCl, 150 mM NaCl, and 0.05% sodium azide, pH 7.4) and centrifuged at 2500g for 20 min at 4 °C to remove particulates, particularly pollen. The supernatants were collected and processed for ammonium sulfate precipitation at 4 °C with 80% saturation. The solution was kept on a stirrer for 1–2 h followed by centrifugation at 8300g at 4 °C. The pellets were dissolved in buffer and stored at −20 °C until use.

**Gel Filtration Chromatography (GFC).** GFC of crude honey proteins was carried out by using a HiLoad 16/60 Superdex 200 Prep grade column (GE Healthcare, Uppsala, Sweden) with buffer A as mobile phase. Chromatography was done using an AKTA FPLC system (GE Healthcare) at the flow rate of 1 mL/min, and fractions of 2 mL were collected. The resultant five chromatographic peaks were termed GFC-P1–GFC-P5. Fractions containing a high molecular mass peak, GFC-P1, were pooled and treated with 8 M urea (Sigma-Aldrich, Rockford, IL, USA). The molecular masses of proteins were estimated by using a Gel Filtration Calibration Kit HMW (GE Healthcare BioSciences AB, Uppsala, Sweden), which contains five proteins and Blue dextran 2000 (ovalbumin M, 43,000; conalbumin M, 75,000; aldolase M, 158,000; ferritin M, 440,000; thyroglobulin M, 669,000; and Blue dextran 2000 M, 2,000,000).

**Reverse Phase HPLC of GFC Peaks.** The gel chromatography peaks GFC-P1 and GFC-P5 were subjected to reverse phase HPLC using a C-18 column (Purospher STAR, RP-18 end-capped (5 μm, 150-4.6, Merck, Darmstadt, Germany). The column was equilibrated with 0.1% TFA, and 100 μL of filtered protein sample was applied with a flow rate of 1.0 mL/min. Gradient elutions were made with acetonitrile as 5 min/0%, 40 min/70%, and 45 min/0%. Peaks were collected, concentrated, and stored at −20 °C until next use.

**Mass Spectrometry.** MALDI-TOF analysis of peaks obtained by RPLC was carried out as follows. Half a microliter of peptide suspension was mixed with 0.5 μL of matrix solution (sinapinic acid in 1:1 0.1% TFA/acetonitrile). The matrix/analyte solution was applied to the sample plate. Mass spectra were recorded on a Bruker Ultraflex III TOF/TOF spectrometer. Two hundred shots of light from a smart beam laser were irradiated to record mass spectra. Bioinformatics analysis of mass spectrometric data was done by using Glycosuite Database and GlycoMod tool of ExPaSy server (www.expasy.org).

**Immunomodulatory Activity Assays.**

**Isolation of Phagocytic Cells.** Polymorphonuclear leukocytes (PMNLs) were collected from human blood by using Ficoll paque gradient centrifugation. For this purpose, blood was drawn from healthy human volunteers (36–45 years old) who had not received any medication for 1 week. Blood was mixed with equal volumes of lymphocyte separation medium (MP Biomedical, Inc., Kaysersberg, France) and Hank’s balanced salt solution (HBSS) without calcium and magnesium (Sigma-Aldrich, Steinheim, Germany). The diluted blood was kept undisturbed at room temperature for 30–45 min. When red blood cells (RBCs) settled, supernatant was layered onto 5 mL of LSM in a separate centrifuge tube and centrifuged at 400g for 20 min at 4 °C. Supernatant was discarded, and pellet was resuspended in HBSS free from calcium and magnesium and washed at 300g for 15 min at 4 °C.

In the presence of RBCs contamination, RBCs were lysed with sterile distilled water; finally, pellet was resuspended in HBSS containing calcium and magnesium. Cell viability was determined using trypan blue dye, and cell count was adjusted to 1,000,000 cells per milliliter.

In addition, murine peritoneal macrophages were collected after the injection of 10 mL of sterile phosphate buffer saline (PBS) into mouse peritoneum (immunized with 1 mL of sterile fetal bovine serum 72 h earlier). Cells were spun at 300g for 15 min at 4 °C. Pellet was resuspended in 1 mL of sterile PBS, viability was checked with trypan blue exclusion method (cells were >95% viable), and count was adjusted as 1,000,000 cells per milliliter.

**Chemiluminescence Assay for Oxidative Burst.** The effect of honey protein fractions on ROS production was measured on PMNLs and mouse peritoneal macrophages. In brief, cells were plated in flat-bottomed 96-well plates and activated by serum opsonized zymosan-A (Sigma-Aldrich) at 250 μg/mL final concentration. Protein/peptide/glycopeptide samples added at various concentrations (0.125–2.0 μg/mL) and plates were incubated for 20 min at room temperature. Luminol (3-aminophthalhydrazide) (Alfa Aesar, Karlsruhe, Germany) was added at 1.5 μg/mL final concentration. Plates were immediately kept in the thermostated chamber of a Luminoskan, and chemiluminescence kinetics of ROS production was monitored as relative light units (RLUs) for 50 min in the Luminoskan (Labsystem, Helsinki, Finland) with repeated scans.

**Nitric Oxide Assay.** The murine macrophage cells (J774.2 cell line), were purchased from the European Collection of Cell Cultures (Salisbury, UK). Cells were cultured in complete DMEM (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 μ/mL penicillin, and 100 μg/mL streptomycin. The effect of honey proteins on NO production was monitored according to the method of Inoue et al. Briefly, cells were seeded in a 96-well plate as 1.8 × 10^5 cells/well and activated by 30 μg/mL of LPS (Escherichia coli serotype 0111:B4, DIFCO Laboratories, Detroit, MI, USA) for the production of nitric oxide. Protein samples were added at various concentrations, and the plate was incubated at 37 °C for 48 h. Supernatants (50 μL) from each well were mixed with an equal amount of Griess reagent (0.1% naphthy11orylendiamine dihydrochloride in distilled water and 1% sulfanilic acid in 5% H3PO4, in 1:1 ratio) (Fisher Scientific, Leicester, UK), and absorbance was recorded at 550 nm using a Spectra Max plus 340 (Molecular Devices, Sunnyvale, CA, USA).

**Phagocytosis Assay.** Phagocytosis assay was performed according to the method of Foulas et al. with some modifications. The murine macrophage cells (J774.2 cell line) were cultured in complete DMEM and seeded into a 96-well tissue culture-treated plate with 5 × 10^4 cells/well. Culture reaction plate was incubated in CO2 environment at 37 °C with 5% CO2 for 24 h. The next day, latex beads (fluorescent coated carbosylated modified polystyrene beads, 1 μm, Sigma-Aldrich) were added as 100 beads/cell. Protein samples were added as 800 ng/mL final concentration, and the plate was further incubated for 24 h at 37 °C in a CO2 environment. Afterward, supernatant was removed and wells were washed twice by gently inverting the plate with 1× PBS. Trypan blue (1×, 50 μL) was added to each well, and the plate was kept at room temperature for 1 min. The trypan blue was removed, and fluorescence was recorded at 575–610 nm using a fluorescence reader (Molecular Devices).

**Cytokine (TNF-α and IL-1β) Production Assay.** THP-1 cell line (human mononcytic leukemia cells) was purchased from the European Collection of Cell Cultures. Cells were cultured in a 75 mm2 tissue culture flask with complete RPMI-1640 medium (MP Biomedical, Solon, OH, USA) supplemented with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, 2 mM l-glutamine, 5.5 mM 1-glucose, 50 μM l-mercaptoethanol, and 1% penicillin and streptomycin. Cells were seeded into a 24-well polystyrene culture plate with 20 μg/mL PMA (SERVA, Heidelberg, Germany), which causes differentiation of monocytes to macrophages. The culture plate was incubated at 37 °C for 24 h. The next day, cells were activated with 50 ng/mL LPS, protein samples were added in various concentrations, and the plate was incubated at 37 °C with 5% CO2. Supernatants were collected after 6 h, and levels of TNF-α and IL-1β were estimated using an
ELISA kit (R&D System, Minneapolis, MN, USA). Absorbance was recorded at 450 nm using a microplate reader (DIA Reader, Gmbh, Wr. Neudorf, Austria).30

**Statistical Analysis.** All experiments were run in triplicates, and data are presented as the statistical mean ± standard deviation (STD). A one-way analysis of variance (ANOVA) was applied to measure the significant difference between the results of test proteins and controls. Significance level was set as $P < 0.05$, $P < 0.01$, and $P < 0.005$.

**RESULTS AND DISCUSSION**

Separation and Characterization of Honey Glycoproteins and Glycopeptides. Previously, we have reported immunomodulatory activity of honey.6,8 In the present study, we characterized immunomodulatory properties of proteoglycans, glycoproteins, and glycopeptides from *Ziziphus* honey. We and others have already reported that honey proteins can be separated into five peaks (namely, GFC-P1–GFC-P5) (Figure 1A). In agreement with previous results,19,31 we found an array of proteins with molecular masses in the range from 10 to ∼450 kDa in peaks GFC-P1–GFC-P4 as revealed by nondenaturing (i.e., GFC) as well as reducing/denaturing (SDS-PAGE) conditions (Figure 1B), whereas peak GFC-P5 contained low molecular mass peptides/glycopeptides (2–5 kDa).

The high molecular mass protein peak GFC-P1 was treated with 8 M urea followed by rechromatography with the same column (Hiload Superdex 200 prep grade). Untreated and 8 M urea treated peak GFC-P1 eluted at the same time (Supporting Information, supplementary Figure 1), whereas SDS-PAGE of this peak (Figure 1B) showed a band at 55 kDa, which indicated that high molecular mass proteins in the peak were dissociated into smaller subunits. Reverse phase liquid chromatography (RPLC) of GFC-P1 showed two distinct peaks (Supporting Information, supplementary Figure 2). These peaks were eluted during the start of the gradient, which indicated their hydrophilic nature. Typically, proteins show $\lambda_{\text{max}}$ at 280 nm, whereas the UV spectra of RPLC peaks determined by diode array detector showed $\lambda_{\text{max}}$ at 266 nm (Supporting Information, supplementary Figure 2). These observations pointed out covalently attached glycans, polyphenols, and other natural products to the protein core. Manuka, kanuka, and clover honeys have been reported to contain type II arabinogalactan proteins with molecular masses of >100 kDa.23−25 Hence, high molecular mass protein peak (GFC-P1) presumably contained MRJP1 proteins. Molecular masses of proteins in peaks GFC-P2–GFC-P4 were found to be in the range of 10–200 kDa.19 SDS-PAGE of GFC-P2 showed multiple bands with an intense band of 50–55 kDa, whereas GFC-P3 contained proteins of 45–70 kDa masses.

Peptides and glycopeptides in peak GFC-P5 were further separated by RPLC, which resulted in 13 fractions (Figure 2). Peaks 8, 12, and 13 of RPLC were found to possess potent ROS production inhibitory activity (see below). MALDI mass spectrometric analysis of these peaks was carried out (Figure 3 and Supporting Information, supplementary Figure 3). Mass spectra were analyzed using the GlycoMod tool (www.expasy.org), which showed that peak 8 contained highly glycosylated peptide(s) with a molecular mass of 1157 Da (Figure 3A). A number of possible structures of O-linked glycopeptide(s) were found. This glycopeptide was characterized as O-linked glycopeptide with a glycan structure of (Hex)3 (HexNAc)1 (Deoxyhexose)2 (Sulph)1 (HexA)1 (Figure 3A). Peak 12 was found to be a hybrid type N-linked glycoconjugate with the core region consisting of two N-acetylgalactosamine residues and three mannose residues (Figure 3B). The peptide mass was calculated as 1066 Da, whereas the mass of the glycosylated peptide was 3086 (Figure 3B). The MALDI MS spectrum of relatively nonpolar peak 13 showed a single mass peak corresponding to peptide mass 1939 Da (Supporting Information, supplementary Figure 3). [M + H]$^+$, [M + Na]$^+$, and [M + NH$_4$]$^+$ adducts were expected to form during mass spectrometry; therefore, possible structures of these adducts were considered using GlycoMod. We used proteins and peptides fractionated by gel filtration chromatography for immunomodulatory assays.

**Effect of Honey Glycoproteins/Glycopeptides on ROS Production.** Activation of immune cells by any antigen induces the respiratory burst that results in ROS release. The
ROS are known to play a crucial role in the pathogenesis of various inflammatory diseases including cardiovascular diseases, rheumatoid arthritis, and other inflammatory diseases. The manuka and pasture honeys were reported to have potential to reduce the production of ROS in MM-6 cells.\(^1\) We previously reported the ability of natural honey to inhibit thrombin-induced ROS production\(^6\) as well as myeloperoxidase-dependent oxidative burst of professional phagocytes.\(^7\) The current experiments revealed that ROS production by human polymorph nuclear cells and mouse peritoneal macrophages was significantly suppressed by honey proteins/peptides of different molecular masses, that is, peaks GFC-P1–GFC-P5 (Figure 4; Table 1). Production and level of ROS were detected by luminol probe, which captures intracellular ROS (mainly HOCl besides the H$_2$O$_2$ and OH$^-$ species) and converts them into a luminescent product, which was detected and recorded by luminoskan in the form of RLUs. All protein peaks showed potent inhibition of ROS production by neutrophils ($IC_{50} = 6–14$ ng/mL) and macrophages ($IC_{50} = 2–9$ ng/mL) stimulated with serum opsonized zymosan (SOZ). Zymosan is a fungal cell wall component, when coated with serum; it binds to cell surface phagocytosis receptors and serves as a potent activator of NADPH oxidase. The activation of NADPH oxidase results in the production of superoxide (O$_2^-$), which is a major player of host defense against bacteria and fungi. However, superoxide and other ROS must be controlled, and their continuous and excessive production can lead to inflammation.\(^3\) The SOZ elicits phagocytosis through the Fc$\gamma$ receptors on phagocytic cells.\(^3\) Therefore, inhibition of ROS by honey proteins can be linked to the interference with or down-regulation of Fc$\gamma$ receptors.

Among different fractions of peak GFC-P5 that were separated by RPLC, peaks RP-8, RP-12, and RP-13 suppressed ROS generation by neutrophils. These peptides showed significant inhibition of ROS production with $IC_{50}$ values of 120 nM (RP-8), 50 nM (RP-12), and 150 nM (RP-13) (Figure 5). These results revealed that the proteins and peptides of honey play vital roles in ROS inhibition.

**Effect of Honey Glycoproteins and Glycopeptides on Phagocytosis.** Phagocytosis is the foremost mechanism of
immune system to fight invading microorganisms and a key feature of innate immune response. Macrophages are the most proficient phagocytes. The binding of bacterial molecules to phagocyte surface elicits them to engulf and wipe out the invaders through the professional killing mechanism that includes respiratory burst process. In the current study, we investigated the effect of honey proteins on receptor-mediated phagocytosis of red fluorescent coated polystyrene modified unopsonized latex beads by J774.2 mouse macrophage cells. Honey proteins/peptides exhibited significant inhibitory effect at 800 ng/mL (Figure 6A; Table 1). Peaks GFC-P3 and GFC-P4 presented the highest degree of inhibition on phagocytosis (70%), whereas the other honey protein peaks showed moderate inhibitory effects. Phagocytosis of unopsonized particles involves a number of cell surface receptors including scavenger receptors (MARCO, SR-A, SR-B, SR-C, LOX-1, etc.) and nonscavenger receptors (β-integrin, mannose, CD-48, β-glucan, asialoglycoproteins receptors, etc.). Among these macrophage receptors, MARCO has been extensively studied for its role in latex beads and other inert and unopsonized environmental particles phagocytosis. In the present study, honey proteins might inhibit phagocytosis through interfering with one or more of these receptors. Hence, honey proteins exerted their anti-inflammatory potential by interfering with phagocytotic process.
Effect of Honey Glycoproteins and Glycopeptides on Nitric Oxide Production. Effect of honey proteins on NO production by macrophages was also evaluated. Nitric oxide, the product of inducible nitric oxide synthase, is a key modulator of immune response. The increase in NO production results in inflammation and progression of various chronic inflammatory diseases including rheumatoid arthritis, septic shock, inflammatory bowel disease, cardiovascular diseases, and several autoimmune disorders. Natural honey was reported to inhibit NO production in carrageenan-induced inflammatory rat model. In the current study, the inhibitory effect of honey proteins on NO production was investigated using murine macrophage cells J774.2 that generate the inducible form of nitric oxide (Figure 6B; Table 1). All protein peaks showed a significant inhibition of NO production (IC$_{50}$ = 130–450 ng/mL) by murine macrophage cells J774.2, which were stimulated with bacterial LPS. As a component of bacterial outer cell membrane, LPS serves as a potent activator of macrophage immune response. It binds to the macrophages through the CD14 and Toll-like receptors, which in turn activate NF-$\kappa$B transcription factor. The NF-$\kappa$B regulates a large number of genes involved in inflammation, cell proliferation, cell survival, and apoptosis.

Effect of Honey Proteins and Peptides on Pro-inflamatory Cytokine Production. We further investigated the effect of honey proteins on pro-inflammatory cytokine production using human monocytes. Honey has been shown to stimulate the production of TNF-$\alpha$, IL-1$\beta$, TGF-$\beta$, and matrix metalloproteinases by human monocytes and keratinocytes. Moreover, the protein content of the honey glycoproteins was found to be responsible for stimulation of pro-inflammatory cytokine production. In the current study, honey proteins were tested at different concentrations for their effects on the production of TNF-$\alpha$ and IL-1$\beta$ by human monocyte cell line (THP-1 cells). For cytokine production, these cells were first differentiated into macrophages by PMA and then activated by LPS for the
production of cytokines. In our experiments, honey proteins did not affect the production of IL-1β at the concentrations tested (Figure 7B) but exerted significant effects on TNF-α production (Figure 7A). We found that the production of pro-inflammatory cytokine TNF-α production was 10–20% down-regulated by peak GFC-P2. Peaks GFC-P3–GFC-P5 showed suppression of TNF-α production at higher protein concentration (2000 ng/mL) and stimulation at lower concentration (80 ng/mL). These results pointed out the involvement of TNF-α in the immunomodulatory activity of honey proteins and peptides. The high molecular mass glycoconjugates/ proteoglycans of honey might exert their effects by binding with cell surface receptors followed by activation of signaling pathways(s), whereas (glyco)peptides in peak GFC-P5 might be effective by interaction with intracellular receptor(s) after an internalization mechanism.

**ASSOCIATED CONTENT**

Supplementary Figures 1–3. This material is available free of charge via the Internet at http://pubs.acs.org.

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