Structure and immunomodulatory activity of glycogen derived from honeybee larvae (*Apis mellifera*)

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ABBSTRACT

Honeybee larvae have been recognized as nutrient-rich food in many countries. Although glycogen, a storage form of glucose in animals, is synthesized in honeybee larvae, there is no information on the structure of glycan and its biological activity. In this study, we successfully extracted glycogen from honeybee larvae using hot water extraction and investigated the structure and biological activity of glycan. It was found that the molecular weight of glycogen from honeybee larvae is higher than that of glycogen from bovine liver and oysters. In addition, treatment of RAW264.7 cells with glycogen from honeybee larvae resulted in a much higher production of TNF-α and IL-6 than treatment with glycogen from either bovine liver or oysters. These results suggest that the high molecular weight glycogen from honeybee larvae is a functional food ingredient with immunomodulatory activity.

**Key words:** honeybee larvae, glycogen, immunomodulatory activity
INTRODUCTION

Glycogen is a highly branched α (1-6)-and α (1-4)-linked d-glucan with a molecular weight of $10^6 - 10^9$. Although glycogen is generally synthesized as a storage form of glucose in animals, there are several reports related to the biological activities of glycogen from natural products or that synthesized enzymatically. For example, glycogen extracted from scallops shows antitumor activity through the modulation of cytokine production. The immunomodulatory activities of enzymatically synthesized glycogen (ESG) were also reported in RAW264.7 cells activated by interferon (IFN)-γ, an in vitro co-culture model comprising Caco-2/RAW264.7 cells and RBL-2H3 cells. It was reported that molecular weight with a defined range may affect the immunomodulatory activity of ESG because the effect of ESG with $M_w \sim 5000K$ on the production of tumor necrosis factor (TNF)-α and interleukin (IL)-6 was stronger than that of ESG at 10,000K. Other studies suggest that the association of ESG with toll-like receptor 2 (TLR2) rather than TLR4 is important for cytokine production. However, it should be noted that comb-like branched α-d-glucan with α (1-6) and α (1-4) linkages activate the TLR4-dependent immunomodulatory activity of RAW264.7 cells. In addition, an anti-inflammatory response by ESG without a TLR2 response was also observed. Thus, identification of novel glycan structures from natural products is important to better understand the immunomodulatory activity of functional glycans, including glycogen.

Honeybee larvae have been recognized as a nutrient-rich food containing vitamins, amino acids, fatty acids, and carbohydrates, especially glycogen. Interestingly, oral administration of lyophilized powder prepared from enzymalyzed honeybee larvae relieves depression associated with tinnitus. Because TNF-α production in the auditory cortex is closely related to the neuroinflammation of tinnitus, it is possible that the immunomodulatory activity of glycogen from honeybee larvae may contribute to the remission of tinnitus-associated depression. However, information on the structure and biological activity of glycogen from honeybee larvae (honeybee glycogen) is not yet understood.

In this study, we found that the molecular weight of honeybee glycogen is higher than that of...
bovine liver and oyster glycogen. In addition, the cytokine production activity of honeybee glycogen was much higher than that of bovine liver and oyster glycogen. These results suggest that glycogen from honeybee larvae is a functional food with immunomodulatory activity.

MATERIALS AND METHODS

Materials

Honeybee larvae (*Apis mellifera*) were obtained from Kurume Bee Farm Co., Ltd. (Fukuoka, Japan). Lyophilized honeybee larvae (sample no. 2012/03/19)—housed at the Faculty of Pharmaceutical Sciences, Tokyo University of Science, Japan—are available upon request. Glycogens from oysters and bovine livers were obtained from FUJIFILM Wako Pure Chemical Co. (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. Shodex STANDARD P-82 containing pullulan polysaccharides (200, 107, 47.1, and 21.1 kDa) were purchased from Showa Denko Co. (Tokyo, Japan). Sugar standards, including L-(+)-rhamnose monohydrate, L-(−)-fucose, L-(−)-galactose, D-(−)-mannose, D-(−)-xylose, and D-(+)glucose were obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Lipopolysaccharides from *Escherichia coli* O55:B5 were purchased from Sigma-Aldrich (St. Louis, MO, USA). The dialysis membrane (MWCO: 3.5 kDa) for desalting was purchased from Repligen (Waltham, MA, USA).

Extraction of polysaccharides from honeybee larvae and fractionation using a diethylaminoethyl (DEAE) Sepharose column

Lyophilized honeybee larvae (10 g) were homogenized with acetone (40 mL) and centrifuged at 6800 ×g for 10 min. The resulting precipitate was proteolyzed for 48 h at 45 °C with actinase E (10 mg/g dry powder) in buffer A containing 50 mM Tris acetate (pH 8.0) and 1% calcium chloride. After incubation, the samples were stirred at 80 °C for 4 h. The debris in the sample solution was removed by filtration with gauze and centrifuged at 10000 ×g for 10 min. The resulting supernatant was mixed with sodium chloride (80 mg/g dry sample) and 9 vol. of ethanol (final 90%) and
incubated at -20 °C overnight. The precipitate obtained after centrifugation at 10000 ×g for 20 min was dissolved in 5 mL of H₂O and dialyzed with 3 L of H₂O to remove NaCl. The resulting crude polysaccharides were obtained by lyophilization. Fractionation using HiPrep DEAE FF (16 mm i.d. × 100 mm, obtained from Cytiva, Tokyo, Japan) was performed as described previously¹¹.

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The molecular weight of the polysaccharides was determined by gel filtration chromatography. Chromatography was performed using an Asahipak 510 HQ column (7.6 mm, i.d. × 300 mm; Showa Denko Co., Tokyo, Japan) that was eluted with 10 mM ammonium bicarbonate at a flow rate of 0.4 mL/min. Separated polysaccharides were detected using a refractive index detector (RID-20A; Shimadzu Corporation, Kyoto, Japan). The gel permeation chromatography (GPC) chromatograms were recorded and analyzed using the Chromato-PRO-GPC data processing software (Run Time Instruments, Kanagawa, Japan). Preparation of partially methylated alditol acetates (PMAAs) from glycogen and GC/MS analysis, and ¹H-NMR at 600 MHz was performed as described previously¹². Neutral sugar analysis by HPLC after hydrolysis of polysaccharides using 2.5 mol/L trifluoroacetic acid, cell culture, and determination of cytokines using an enzyme-linked immunosorbent assay were performed according to the methods described by Suabjakyong et al.¹³.

**Determination of endotoxin**

Endotoxin content in samples was determined using an Endospecy® ES-50M kit (Seikagaku Corp., Tokyo, Japan). The level of endotoxin was 0.77 ng/mL when 10 µg/mL of honeybee glycogen (Fr. 1) was analyzed.

**Statistics**

Values are indicated as the mean ± standard deviation (SD). One-way analysis of variance followed by Dunnett’s test was used to evaluate significant differences between groups treated with LPS or
glycogen. The statistical calculations were carried out using GraphPad Prism version 9.1.1 (GraphPad Software, USA), www.graphpad.com.

RESULTS AND DISCUSSION

Determination of glycan structure of glycogen from honeybee larvae

Crude polysaccharides were extracted from the lyophilized powder of honeybee larvae by acetone treatment, actinase E digestion, and recovered by ethanol precipitation. Dried powder (13.1 mg/g of lyophilized powder) was recovered after desalting and lyophilization. Further fractionation of polysaccharides using the DEAE Sepharose column suggested that crude polysaccharides consisted of neutral sugars (Fig. 1A). Separation of 20 mg crude polysaccharides using the DEAE Sepharose column, resulted in the isolation of 14.2 mg of polysaccharides in fraction (Fr.) 1 (Fig. 1A). Investigation of the neutral sugar composition of Fr.1 polysaccharides indicated that the major sugar of Fr.1 is glucose (Fig. 1B). Anomeric proton peaks corresponding to α (1-4) and α (1-6) linkages were observed in $^1$H-NMR spectra (Fig. 2A), suggesting that the polysaccharide in Fr. 1 is glycogen. Thus, PMAAs from glycogen from honeybee larvae and bovine liver were prepared and subjected to GC/MS. The chromatogram of PMAAs from honeybee larvae was similar to that from the bovine liver (Fig. 2B and 2C). In addition, based on the comparison of peak intensities corresponding to α-1,6 and α-1,4 linkages, it was found that the degree of branching in glycogen from honeybee larvae was slightly higher than that of glycogen from bovine liver. Size-exclusion chromatography of glycogen showed that the weight-average molecular weight ($M_w$) of glycogen from honeybee larvae, oyster, and bovine liver was 790K, 410K, and 200K when pullulan was used as the standard for molecular weight (Fig. 3). Kakutani et al.\textsuperscript{3} reported that the molecular weights of oyster and bovine liver glycogens are 6000K and 2000K, respectively. Considering that the elution time of pullulan P-50 (peak molecular weight: $4.71 \times 10^4$: 8.62 min) obtained by OHpak SB-806M HQ (8.0 mm, i.d. × 300 mm) is almost the same as that of ESG-A ($M_w$: $2.72 \times 10^6$)\textsuperscript{14} (data not shown), the difference in molecular weight might be because of the calculation method used.
Effect of honeybee glycogen on cytokine production activity in RAW264.7 cells

The effect of glycogen from honeybee larvae on the production of TNF-α and IL-6 in RAW264.7 cells was examined in the presence of IFN-γ. Honeybee glycogen strongly stimulated the production of TNF-α and IL-6 (Fig. 4A), whereas commercially available glycogens from oyster and bovine liver failed to produce the cytokines (Fig. 4B), even though RAW264.7 cells were activated using IFN-γ. Considering that the molecular weight of honeybee glycogen is higher than that of glycogen from oysters and bovine liver (Fig. 3), the narrow molecular-weight distributions of glycogen may affect its biological activity. Glycogen structure is influenced by the preparation method because extraction with alkali or acid results in inactive glycogen. Thus, the preparation of glycogen from honeybee larvae is also important to ensure biological activity.

Considering that oral administration of lyophilized powder from honeybee larvae relieves depression associated with tinnitus, and TNF-α production in the auditory cortex is related to neuroinflammation of tinnitus, the immunomodulatory activity of glycogen, at least in part, contributes to the remission of tinnitus-associated depression.

Conflict of Interest: The authors declare no conflict of interest.
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Fig. 1. Glucose is a major component in polysaccharides from honeybee larvae

(A) Fractionation of polysaccharides derived from honeybee larvae. Crude polysaccharides (20 mg) were fractionated using weak-anion exchange chromatography. The fractionated samples were dialyzed, lyophilized, and weighed. Fractionation using a diethylaminoethyl (DEAE) Sepharose column was performed with the following conditions: eluent A, 50 mM sodium phosphate (pH 6.0); eluent B, sodium phosphate (pH 6.0) containing 2.0 mol/L sodium chloride; flow rate, 2.0 mL/min. (B) Neutral sugar analysis of polysaccharides (Fr. 1) using HPLC with post column derivatization.
Fig. 2. Determination of glycan structure of honeybee larvae glycogen

(A) The 600 MHz $^1$H-NMR spectrum of glycogen (1 mg/mL) from honeybee larvae in D$_2$O at 60 °C. Each signal was assigned in comparison with the $^1$H-NMR chemical shift of the HOD signal as 4.77 ppm. (B) Chromatograms of partially methylated alditol acetates (PMAAs) from glycogen of honeybee larvae and bovine liver. (C) Mass fragments ($m/z$) observed in peaks in (B).
Fig. 3. Molecular weight of glycogen from honeybee larvae

(A) Chromatogram of pullulans with different molecular weights and glycogens from different sources. Peak molecular weight (Mp) of pullulan: P-800; $7.08 \times 10^4$, P-400; $3.75 \times 10^4$, P-200; $2.00 \times 10^4$, P-100; $1.07 \times 10^4$, P-50; $4.71 \times 10^3$. (B) Molecular weight of glycogen. $M_w$: weight-average molecular weight, $M_n$: number average molecular weight. (Color figure can be accessed in the online version.)
Fig. 4. Honeybee larva glycogen stimulates the production of TNF-α and IL-6 from RAW264.7 cells

(A) Effect of glycogen on the production of TNF-α and IL-6. RAW264.7 cells (5 × 10^6 cells/well) were treated with glycogen or LPS and incubated for 24 h in the presence of IFN-γ (10 ng/mL). After incubation, the concentrations of TNF-α and IL-6 in the culture medium were determined using an enzyme-linked immunosorbent assay. (B) Effects of glycogen (100 μg/mL) from honeybee, oyster, and bovine liver or LPS (10 ng/mL) on cytokine production in the presence of IFN-γ. Data are expressed as the mean ± SD. *p<0.05, ****p<0.0001 against none; ns, not significant.