**Silkrose: A novel acidic polysaccharide from the silkworm that can stimulate the innate
immune response**

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Abstract

We have identified a novel acidic polysaccharide from silkworm (*Antheraea yamamai*) pupae that activates the mammalian innate immune response. This bioactive polysaccharide was isolated using nitric oxide production in mouse RAW264 macrophages as an indicator of immunostimulatory activity. We named this polysaccharide “silkrose”. It has a molecular weight of 3.15×10^5 and comprises nine monosaccharides. The expression profiles indicated that silkrose induced the expression of proinflammatory cytokines and interferon β that exist downstream of MyD88-dependent and MyD88-independent signaling pathways. Also, the inhibition of Toll-like receptor 4 (TLR4), which exists upstream of the signaling pathways, led to the suppression of NO production by silkrose. Furthermore, this polysaccharide promoted the activation of nuclear factor kappa B in RAW264 cells, indicating that it stimulates the induction of various cytokines in macrophages through the TLR4 signaling pathway. Our results thus suggest that silkrose activates the innate immune response to various pathogenic microorganisms and viral infections.

Keywords:

polysaccharide, nitric oxide, insect, innate immune response, toll-like receptor, macrophage

1. Introduction

Innate immunity is activated by the recognition of pathogen-associated molecules by pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs). Glycoconjugates are considered to be immunologically active molecules of the innate immune system (Akira & Takeda, 2004). Immune cells such as macrophages use PRRs to discriminate species-specific monosaccharide variations and pathogen-derived glycoconjugate structures and activate the innate immune response (Akira, Uematsu, & Takeuchi, 2006). Hence, this mechanism, which is conserved from invertebrates to vertebrates, is important for blocking infection by various pathogens (Akira, Uematsu, & Takeuchi, 2006; Ferrandon, Imler, Hetru, & Hoffmann, 2007; Ishii, Kawasaki, Matsumoto, Tochinali, & Seya, 2007; Li & Xiang, 2013; Rebl, Goldammer, & Seyfert, 2010; Temperley, Berlin, Paton, Griffin, & Burt, 2008).

Various glycoconjugates with a variety of pharmacological properties mediated by PRR recognition have been discovered from plants, sea algae, and fungi, even though they are non-pathogenic organisms, because their exoskeleton is formed by cell walls composed of sugars (Han et al., 2003; Lin et al., 2006; Schepetkin & Quinn, 2006). Chitin and chitin derivatives, which construct the exoskeletons of insects and crustaceans and are polymerized by N-acetyl- β -D-glucosamine, also activate the innate immune response in mammals because they are included in pathogenic fungal cell walls (Lee, Da Silva, Lee, Hartl, & Elias, 2008). Furthermore, immunostimulatory glycoconjugates distinct from chitin and chitin derivatives

have been discovered in insects (Ohta, Ido, Kusano, Miura, & Miura, 2014). Hence, it is possible that insects have various glycoconjugates that stimulate the innate immune response.

In our current study, we isolated a novel acidic polysaccharide from silkworm (*Antheraea yamamai*) pupae that activates the innate immune system in mouse macrophage RAW264 cells. We named this polysaccharide “silkrose”. Surprisingly, the constituent sugars of silkrose included L-rhamnose and D-mannuronic acid, which are mainly present in plant, algae, fungi, and bacteria. We further characterized the mechanisms by which silkrose activates the innate immune system.

2. Materials and methods

2.1. Materials

Dried silkworm pupae were purchased from Tosaka Tensan Kobo (Gunma, Japan). Rat anti-mouse TLR4/MD2 antibody and rat IgG2a were purchased from BioLegend (San Diego, CA) and Invivogen (San Diego, CA), respectively. Mouse anti-human inhibitor kappa B ($\text{I-}\kappa\text{B}$) α antibody was purchased from Cell Signaling Technology (Danvers, MA). Rabbit anti-human nuclear factor kappa B ($\text{NF-}\kappa\text{B}$) p65 antibody and goat anti-human Lamin A/C were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-mouse α -tubulin antibody was purchased from Sigma Aldrich (St. Louis, MO). Alkaline phosphatase-labeled

anti-rabbit, anti-mouse, and anti-goat IgG antibodies were purchased from GE Healthcare (Buckinghamshire, UK). Lipopolysaccharide (LPS) from *E. coli* 0111:B4 was purchased from Sigma Aldrich.

2.2. Preparation of wild silkworm pupal extract

The silkworm pupae were grinded using a mill. The resulting silkworm pupal meal was then added to 10 volumes of ultrapure water and mixed gently for 24 h at 4 °C. The supernatant obtained by centrifugation at $10,000 \times g$ for 1 h was concentrated to about one-tenth of its volume by evaporation in a water bath at 50 °C.

2.3. Purification of a bioactive polysaccharide from silkworm pupal extract

The silkworm pupal extract was added to four volumes of 100% (v/v) ethanol and mixed gently overnight at 4 °C to precipitate the polysaccharides. The precipitate was collected by centrifugation, washed three times with 80% ethanol, and dried under decompression. To dissolve the polysaccharides, the precipitate was added to 20 mM Tris-HCl (pH 8.0) containing 200 mM NaCl and mixed overnight at 4 °C. After the precipitate was removed, crude polysaccharides were obtained.

The crude polysaccharides were applied to a Sephacryl S-500 gel-filtration chromatography column (HiPrep 26/60 Sephacryl S-500 HR; GE Healthcare) pre-equilibrated with 20 mM Tris-HCl (pH 8.0) containing 200 mM NaCl and eluted with the same solution. The flow rate through the column was 1.3 ml/min, and the fractions were collected at regular intervals. The eluted fractions were assayed for the nitric oxide (NO) production activity of RAW264 cells and total carbohydrate by the phenol-sulfuric acid method using glucose as the standard (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956). The fractions containing NO production activity were pooled and precipitated by the addition of four volumes of 100% (v/v) ethanol overnight at 4 °C. The precipitate was then separated by centrifugation and dissolved in 20 mM Tris-HCl (pH 8.0).

The polysaccharides separated using gel-filtration chromatography were fractionated by anion-exchange chromatography on a Q sepharose column (HiPrep Q XL 16/10; GE Healthcare) pre-equilibrated with 20 mM Tris-HCl (pH 8.0). Fractions were prepared with a linear gradient of NaCl from 0 to 1.0 M in turn at a flow rate of 2.0 ml/min. Each fraction was assayed as mentioned above. The active fractions were pooled and precipitated by the addition of four volumes of 100% (v/v) ethanol overnight at 4 °C. The resulting precipitate was then separated by centrifugation and dried in air to obtain the bioactive polysaccharide.

2.4. Determination of the molecular weight of the bioactive silkworm polysaccharide

The molecular weight of the bioactive polysaccharide we isolated from the silkworm was determined by gel-filtration chromatography using a high-performance liquid chromatography system (Hitachi, Tokyo, Japan). The isolated polysaccharide (0.5 mg) was dissolved in 0.2 M phosphate buffer (PB) (pH 7.5) (500 μ l) and filtered using a 0.22- μ m filter applied to a Showdex SB-807 HQ size-exclusion chromatographic column (Showa Denko K.K., Tokyo, Japan). The column was maintained at a temperature of 35 °C, eluted with 0.2 M PB (pH 7.5) at a flow rate of 0.5 ml/min, and detected by a refractive index detector. Preliminary calibration of the column was conducted using Pullulans of different molecular weights (Pullulan P-5, P-10, P-20, P-50, P-100, P-200, P-400, P-800, and P-2500). The molecular weight was calculated from the calibration curve obtained by using various standard Pullulans.

2.5. Monosaccharide composition of the bioactive silkworm polysaccharide

Determination of the monosaccharide composition of the bioactive silkworm polysaccharide was performed in accordance with the method of Sasaki et al. (2008). Briefly, the isolated polysaccharide (100 μ g) was hydrolyzed with 2 M trifluoroacetic acid at 100 °C for 16 h. The hydrolyzed products were then evaporated using N₂ stream and converted to alditol acetates (GC-MS) by successive NaBH₄ reduction and acetylation with Ac₂O-pyridine (1:1, v/v) following the method described by Sasaki et al. (2008). GC-MS analyses were performed on an Agilent 7890A gas chromatography system (Agilent Technologies, Santa Clara, CA)

equipped with a HP-5 capillary column (30 m $\times$ 0.35 mm $\times$ 0.25 μ m) connected to a JEOL MS-1050Q instrument (JEOL, Tokyo, Japan). Helium was used as the carrier gas at a constant flow of 1.0 ml/min. The injector temperature was kept constant at 250 °C and the column temperature was programmed as follows: oven starting temperature of 100 °C, which was gradually increased to 180 °C (10 °C/min, then held for 5 min) and then 320 °C (10 °C/min, then held for 5 min). The MS acquisition parameters included scanning from m/z 50 to 500 in the electron impact mode for routine analysis.

2.6. Cell culture

RAW264 cells (murine macrophage line) were obtained from the Cell Bank RIKEN BioResource Center (Tsukuba, Japan). Cells were grown in MEM supplemented with 10% fetal bovine serum, 0.1 mM non-essential amino acids, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cells were maintained at 37 °C in a 5% CO₂ humidified atmosphere.

2.7. NO assay

The levels of NO in the culture medium of macrophages were measured using a Griess Reagent system kit (Promega, Madison, WI) according to the manufacturer's instructions. Cells were plated at 1×10^5 cells/well in 96-well culture plates and stimulated with varying

concentrations of the bioactive silkworm polysaccharide in the growth medium for 24 h at 37 °C.

The nitrite levels in the culture medium were then measured.

2.8. RNA isolation and real-time quantitative RT-PCR

Total RNA was isolated from RAW264 cells using an RNeasy Plus Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. For the real-time PCR assay, 500 ng of total RNA was reverse-transcribed using the Qiagen QuantiTect Reverse Transcription kit. Real-time PCR analysis was performed on a Bio-Rad CFX96 Real-time PCR Detection system (Bio-Rad Laboratories, Hercules, CA) using SsoFast EvaGreen Supermix (Bio-Rad Laboratories). The gene-specific primers used in this study were the same as those reported by Xia et al. (2012). PCR reactions were performed for 1 min at 95 °C and then 50 cycles of 2 s at 95 °C and 10 s at 60 °C. The comparative CT method was used to determine the amount of the target, which was normalized to an endogenous reference (GAPDH) and relative to a calibrator ($2^{-\Delta\Delta C_t}$) using CFX Manager 2.0 software (Bio-Rad Laboratories). All RT-PCR experiments were performed in triplicate.

2.9. TLR4 blocking experiments

RAW264 cells were plated as described above and then preincubated for 1 h at 37°C (5% CO₂) with blocking antibody against mouse TLR4/MD2 (25 µg/mL) or with an isotype control antibody. Silkrose (10 ng/mL) or LPS (10 ng/mL) was then added to the RAW264 macrophages and the cells were further incubated for 16 h at 37°C in 5% CO₂. The levels of NO in the culture medium of RAW264 cells were measured using the Griess reagent system kit as described above.

2.10. Immunoblot analysis

To prepare cytosolic and nuclear extracts, RAW264 cells treated with the bioactive silkmoth polysaccharide for various times were washed three times with ice-cold PBS, collected with a cell scraper, and harvested by centrifugation. Cytosolic and nuclear proteins were then prepared using a NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Scientific, Rockford, IL) according to the manufacturer's protocol. Aliquots of 10–20 µg of protein samples were separated by 12.5% SDS-PAGE under denaturing conditions and transferred to PVDF membranes (Millipore, Billerica, MA) by electroblotting. Membranes were then blocked with TBS (20 mM Tris-HCl pH 7.5, 500 mM NaCl) containing 0.5% Blocking Reagent (Roche, Mannheim, Germany) for 1 h and then incubated with the appropriate primary antibody diluted in immunoreaction enhancer solution (Can Get Signal Solution 1; Toyobo, Osaka, Japan) overnight at 4 °C. α -tubulin or Lamin A/C was used as a loading control for total or nuclear

proteins, respectively. After the immunoblot was incubated with anti-rabbit, anti-mouse, or anti-goat IgG alkaline phosphatase diluted in immunoreaction enhancer solution (Can Get Signal Solution 2; Toyobo) for 1 h at room temperature, the alkaline phosphatase signal was developed and observed using CDP-star Detection Reagent (GE Healthcare) and an ImageQuant LAS 4000 (Fujifilm, Tokyo, Japan).

2.11. Statistical analysis

All data presented in this study are expressed as the mean $\pm$ SEM. Statistical analyses were performed using a one-way analysis of variance followed by Dunnett or Bonferroni multicomparison test using KaleidaGraph statistical software (Synergy Software, Reading, PA). Significance was set at $p < 0.05$.

3. Results

3.1. Purification of an innate immune system activator present in silkworm pupal extracts

We purified an immunostimulator from silkworm pupal extracts by using it as a marker of NO-producing activity in RAW264 cells. Crude polysaccharides extracted from silkworm pupae were separated by gel-filtration chromatography on a Sephacryl S-500 column.

As shown in Figure 1A, the high-molecular-weight fraction contains the bioactive polysaccharide that induced NO production in RAW264 cells. The fraction containing NO-producing activity was collected and added to four volumes of 100% (v/v) ethanol to precipitate bioactive polysaccharides. The dried ethanol precipitate was dissolved in 20 mM Tris-HCl (pH 8.0) and the resulting supernatant fraction was separated by centrifugation and purified by anion-exchange chromatography on a Q sepharose column eluted with a linear gradient of NaCl from 0 to 1.0 M (Fig. 1B). *In vitro* analysis showed that a single polysaccharide-containing peak stimulated the production of NO in RAW264 cells. We obtained 1.5mg of this bioactive polysaccharide from 500g of silkworm pupae meal.

To investigate whether the bioactive polysaccharide was homogeneous, we performed high-performance size-exclusion chromatography (HPSEC) on a SB-807 HQ column (Fig. 1C). The polysaccharide was detected as a single symmetrical peak, indicating homogeneity. The average molecular weight of the polysaccharide was estimated to be 3.15×10^5 using HPSEC and by reference to Pullulan P-series standard samples of known molecular weights. We named this bioactive silkworm polysaccharide “silkrose”.

3.2. The monosaccharide composition of silkrose

The monosaccharide composition of silkrose was determined by GC-MS analysis of the acetate methyl esters (Fig. 2). GC-MS analysis revealed nine monosaccharides: L-rhamnose,

L-fucose, D-mannuronic acid, D-glucuronic acid, D-mannose, D-glucose, D-galactose, N-acetyl-D-glucosamine, and N-acetyl-D-galactosamine with a molar ratio of 6.2: 2.1: 3.5: 3.5: 9.3: 13.1: 48.9: 5.7: 7.6, respectively.

3.3. Effects of silkrose on activation of the innate immune system *in vitro*

To evaluate the immunostimulatory activity of silkrose, we analyzed the NO production activity of RAW264 cells treated with various concentrations of silkrose. An *in vitro* assay showed that exposure to 1 ng/ml silkrose induced NO production in RAW264 cells (Fig. 3A). Activation of the innate immune response by bioactive polysaccharides is triggered by their recognition by PRRs, such as TLRs, and induces the production of NO and various cytokines through activation of downstream signaling pathways (Akira, Uematsu, & Takeuchi, 2006). We investigated whether silkrose also induces cytokine expression in RAW264 cells (Fig. 3B). The expression of several proinflammatory cytokines, such as tumor necrosis factor (TNF) α , interleukin (IL)1 β and IL6 mRNA that regulated by MyD88-dependent signaling pathway was significantly increased at 6 hours after silkrose treatment in a dose-dependent manner. Silkrose also significantly induced the expression of interferon (IFN) β and IFN-regulatory factor (IRF)7 mRNA that regulated by MyD88-independent signaling pathway, but not IRF3 mRNA, at 6 hours post-treatment.

3.4. *Effect of TLR4 inhibition on NO-producing activity of silkrose in vitro*

We found that silkrose induces proinflammatory cytokines and IFN β gene expression, suggesting that silkrose activates MyD88-dependent and MyD88-independent pathways through the recognition of TLR4 in RAW264 cells. To investigate whether silkrose induces NO production in RAW264 cells via TLR4, we used neutralizing antibodies against TLR4 to inhibit their function and thereby determine their effect on NO production by RAW264 cells (Fig. 4). Anti-mTLR4/MD2 antibodies significantly inhibited silkrose-stimulated NO production.

3.5. *Effect of silkrose on signaling pathways*

We found that silkrose induces cytokine gene expression through TLR4 activation in RAW264 cells. NF- κ B is an important transcription factor functioning downstream of the TLR4 signal transduction pathway. To investigate whether silkrose activities are mediated by NF- κ B, we analyzed the degradation of the NF- κ B inhibitory protein I- κ B α by immunoblotting and assessed the translocation of NF- κ B p65 to the nucleus upon exposure to this polysaccharide. Treatment of RAW264 cells with silkrose induced a rapid degradation of I- κ B α , a response similar to that of LPS treatment (Fig. 5A). Reduced I- κ B α protein levels were observed 15 min after silkrose treatment but recovered to basal levels after 60 min. Furthermore, the translocation of NF- κ B p65 to the nucleus was found to increase at 15 min post-treatment in silkrose-treated

RAW264 cells (Fig. 5B), which correlated with the I- κ B α degradation result. These data indicated that silkrose stimulates the innate immune system through the activation of TLR4.

4. Discussion

Glycoconjugates are one of the active molecules that stimulate the innate immune system. Immune cells, such as macrophages, recognize glycoconjugates derived from various pathogens and activate the innate immune response (Akira, Uematsu, & Takeuchi, 2006). Additionally, a variety of glycoconjugates derived from plants, sea algae, and fungi stimulate the innate immune response, even though they are non-pathogenic organisms (Schepetkin & Quinn, 2006). In insects, chitin and chitin derivatives, which comprise the exoskeleton of insects and are polymerized by N-acetyl- β -D-glucosamine, activate the innate immune response in mammals because they are included in the cell walls of pathogenic fungi (Lee, Da Silva, Lee, Hartl, & Elias, 2008). Recently, we identified a novel water-soluble polysaccharide in melon fly pupae with a molecular weight of 1.01×10^6 (Ohta, Ido, Kusano, Miura, & Miura, 2014). This polysaccharide activates the innate immune response in mammalian macrophage cell lines, and it is suggested that insects have novel bioactive glycoconjugates that are not chitin or chitin derivatives. In our present study, we used macrophage NO production as an immunostimulation indicator to identify a novel polysaccharide from wild silkmoth pupae that activates the innate immune response of a mammalian macrophage cell line. This polysaccharide, with a molecular

weight of 3.15×10^5 and composed of nine monosaccharides, was named “silkrose”.

GC-MS analysis revealed that silkrose contains L-rhamnose and D-mannuronic acid.

Insects, similar to other animals and in contrast to plants, algae, fungi, and bacteria, are unable to synthesize NDP-rhamnose or GDP-mannuronate, an activated monosaccharide (Giraud & Naismith, 2000; Wong, Preston, & Schiller, 2000). NDP-sugars are donors of sugar moieties for substrates that contain glycoconjugates, such as glycoproteins, polysaccharides, and glycolipids (Bar-Peled & O'Neill, 2011; Sousa, Feliciano, & Leitao, 2011; Thibodeaux, Melançon, & Liu, 2008), and are synthesized through *de novo* and salvage pathways. In plants, UDP-rhamnose, which is required for primary cell wall polysaccharides and various L-rhamnose-containing natural organic compounds, is synthesized through a *de novo* pathway from UDP-D-glucose (Kamsteeg, Van Brederode, & Van Nigtevecht, 1978; Oka, Nemoto, & Jigami, 2007; Yonekura-Sakakibara et al., 2008). In insects, L-rhamnose is present in the bioactive polysaccharide which was isolated from melon fly pupae (Ohta, Ido, Kusano, Miura, & Miura, 2014), suggesting that insects have a unique UDP-synthesis pathway, although it remains to be identified (Bar-Peled & O'Neill, 2011).

GDP-mannuronate is also required for cell wall polysaccharides in brown algae and certain bacteria (Sousa, Feliciano, & Leitao, 2011). In some bacteria, this nucleotide sugar is synthesized through a salvage pathway from D-mannose, and GDP-mannose 6-dehydrogenase, which catalyzes the formation of GDP-mannuronate from GDP-mannose, plays a key role in GDP-mannuronate synthesis (Sousa, Feliciano, & Leitao, 2011; Yonekura-Sakakibara et al., 2008). Although GDP-mannose 6-dehydrogenase has not been found in insects, as yet unidentified

enzymes may catalyze the oxidation of GDP-mannose to GDP-mannuronate and synthesize silkrose from these nucleotide sugars.

On the other hand, many phytophagous insects harbor diverse microbial communities in their gut (Behar, Yuval, & Jurkevitch, 2010; Dillon & Dillon, 2004; Schloss, Delalibera, Handelsman, & Raffa, 2006). These microorganisms contribute to larval growth by aiding the feed digestion and nutrients absorption of their host thru providing digestive enzymes, in a synergistic manner, and various nutrients (Anand et al., 2010; Dillon & Dillon, 2004). In plant and bacteria, L-rhamnose and D-mannuronic acid are synthesized in the body and utilized in the moiety of cell wall polysaccharides and natural organic compounds (Bar-Peled & O'Neill, 2011; Sousa, Feliciano, & Leitao, 2011; Thibodeaux, Melançon, & Liu, 2008). Silkmoth is phytophagous insects whose larvae are voracious eaters of leaves of a deciduous oak (*Quercus acutissima* Carruthers) (Oishi, Yokota, Terakoto, & Sato, 2006), suggesting that they harbor stable bacterial communities in their digestive system. Therefore, silkmoth may incorporate the monosaccharides, such as L-rhamnose and D-mannuronic acid, that insects cannot synthesize from digested oak leaves or symbiotic bacterium and utilize as substrates for the synthesis of silkrose.

Various glycoconjugates with a variety of pharmacological properties, such as cytokine induction and NO production by macrophages, have been characterized from plants, sea algae, and fungi, even though they are non-pathogenic organisms, because these species have cell walls that are predominantly composed of various polysaccharides having

species-specific monosaccharides or structures (Schepetkin & Quinn, 2006; Takeuchi & Akira, 2010; Ferreira, Passos, Madureira, Vilanova, & Coimbra, 2015). Immune cells, such as macrophages, activate the innate immune response following treatment with high doses of the bioactive polysaccharides derived from these species (Schepetkin & Quinn, 2006). On the other hand, our current data show that a very low concentration of silkrose can induce NO production in macrophages. The polysaccharide structure is an important determinant of the activation of innate immune cells such as macrophages. Similar to silkrose, low-dose treatments with dipteroose, a bioactive polysaccharide isolated from melon fly pupae, activates the innate immune response via recognition by TLR4 in mammalian macrophages (Ohta, Ido, Kusano, Miura, & Miura, 2014). These insect-derived polysaccharides contain L-rhamnose as their constituent sugar, which is present in plants, fungi, and bacteria, but not in animals. Hence, silkrose may have a characteristic structure comprising L-rhamnose, which is a potent stimulator of mammalian macrophages.

Activation of the innate immune response by polysaccharides is triggered by their recognition by PRRs. PRRs recognize structures that are conserved among various pathogens (Takeuchi & Akira, 2010), and TLR2, TLR4, and dectin-1 have been well characterized as sensors that recognize ligands containing carbohydrate moieties such as peptidoglycans, LPS, β -glucan, and natural polysaccharides (Kumar et al., 2009; Takeuchi & Akira, 2010). The recognition by TLR2, TLR4, and dectin-1 leads to the activation of diverse signaling pathways. Activations of TLR2 and dectin-1 induce the production of proinflammatory cytokines via

activation of NF- κ B, whereas the activation of TLR4 induces the production of not only proinflammatory cytokines, but also IFN β , via the activation of NF- κ B and IRF3 (Kumar et al., 2009; Takeuchi & Akira, 2010). The expression profiles of silkrose-induced cytokines indicates that this polysaccharide induces the expression of not only proinflammatory cytokines, such as TNF α , IL1 β and IL6, but also IFN β , suggesting that silkrose activates MyD88-dependent and MyD88-independent pathway via the recognition of TLR4. Furthermore, we show from our current analyses that the inhibition of TLR4 leads to the suppression of the NO production-stimulating activity of silkrose, and the stimulation of silkrose promoted the degradation of I- κ B α and the translocation of NF- κ B to the nucleus. These results further indicate that silkrose activates the innate immune system via the TLR4 signaling pathway.

In conclusion, we have identified a novel acidic polysaccharide from silkmoth pupae—which we have termed silkrose—that activates the innate immune system of mammalian macrophages. Surprisingly, we found that silkrose is composed of L-rhamnose and D-mannuronic acid, even though insects and higher animals lack the ability to synthesize these compounds, suggesting that insects have unique nucleotide sugar biosynthesis pathways or incorporate these monosaccharides from digested feed or symbiotic bacterium. Furthermore, we show from our current analyses that silkrose induces the production of proinflammatory cytokines and IFN β and that the expression of IFN-inducible genes occurs through the activation of the TLR4 signaling pathway. Our findings also suggest that as silkrose activates the innate immune system in mammalian macrophages, it may boost the immune response to

367 various pathogens.

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Figure legends

Fig. 1. Chromatography profiles of polysaccharides in silkworm pupal extract. (A) Gel-filtration chromatography profile of crude polysaccharide fractions of silkworm pupal extract. Polysaccharides were passed through a Sephacryl S-500 column. Fractions were collected and the sugar content was monitored using the phenol-H₂SO₄ method (filled circle, OD₄₉₀ nm). RAW264 cells were incubated with these diluted fractions for 24 h and the nitrite levels in the culture medium were measured by the Griess assay (open circle, OD₅₄₀ nm). (B) Anion-exchange chromatography profile of the fractions with NO-producing activity in RAW264 cells on a Q sepharose column eluted with linear gradient NaCl solutions. (C) HPLC profiles of acidic polysaccharide of silkworm pupal extract on a Shodex SB-807 HQ column.

Fig. 2. GC-MS chromatogram of silkrose monosaccharides. Peaks: 1, L-rhamnose; 2, L-fucose; 3, D-mannuronic acid; 4, D-glucuronic acid; 5, D-mannose; 6, D-glucose; 7, D-galactose; 8, N-acetyl-D-glucosamine; 9, N-acetyl-D-galactosamine. Myo-inositol which is an internal standard is shown as Myo.

Fig. 3. Silkrose stimulation induces NO production and cytokine expression in macrophages. (A) RAW264 cells were stimulated with various concentrations of silkrose for 24 h and nitrite concentrations in the culture medium were measured as described in the Materials and Methods.

Results are given as means $\pm$ SEM. *** p < 0.001 versus control. (B) RAW264 cells were stimulated with various concentrations of silkrose. The mRNA expression of the indicated cytokines and of IRF3 and IRF7 was measured using real-time PCR at 6 h after silkrose treatment. The results represent the means $\pm$ SEM of three samples for each gene analyzed. ** p < 0.01, and *** p < 0.001 as compared with the control group.

Fig. 4. Silkrose induces NO production in macrophages through TLR4. Macrophages were incubated with neutralizing antibody to TLR4/MD2 (25 μ g/mL) or isotype control IgG for 1 h, followed by 16 h of incubation with silkrose. NO production in the medium was then measured using Griess reagent solution. Results are given as means $\pm$ SEM. *** p < 0.001 versus control.

Fig. 5. Silkrose induces the activation of NF- κ B in macrophages. (A) RAW264 cells were stimulated with silkrose or LPS for 0, 15, 30, 45, or 60 min. I- κ B α was detected by western blot analysis. α -tubulin is shown as a protein loading control. (B) RAW264 cells were treated with silkrose or LPS for 0, 15, 30, or 60 min. NF- κ B p65 translocation into the nucleus was measured by western blot analysis. Lamin A/C was used as a protein loading control.

Figure1

Figure2

Figure3

A

B

Figure4

Figure5