Immunostimulatory activity of snake fruit peel extract on murine macrophage-like J774.1 cells

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Abstract Snake fruit (*Salacca edulis* Reinw.) is a tropical fruit produced in Indonesia. Snake fruit peel is normally discarded as waste. In the present study, it was revealed that snake fruit peel has high bioactivities on stimulation of the immune system. Snake fruit peel extract (SFPE) was prepared by extracting snake fruit peel powder in water for 15 h at 4 °C. SFPE enhanced phagocytotic activity of murine macrophage-like J774.1 cells. Production of cytokine such as tumour necrosis factor (TNF)- α and interleukin (IL)-6 was also stimulated by SFPE. The gene

expression levels for these cytokines were elevated. Immunoblot analysis revealed that SFPE enhanced not only nuclear factor (NF)- κ B but also mitogen-activated protein kinases signalling cascades such as JNK and p38 in macrophage. Overall findings suggested that SFPE has a potential beneficial effect to promote our body health through the stimulation of macrophage.

Keywords J774.1 cells · NF- κ B · Macrophage · MAP kinase · Phagocytosis · Snake fruit

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Introduction

Functional foods are foods that contain bioactive substances which offer beneficial effects on one or more target functions in our body, and are expected to improve the physical function and to reduce the risk of specific pathologies by modulating the immune, secretion, nerve, circulating, or digestive system (Roberfroid 2002; Kumalasari et al. 2013). Several studies have been reported that some foodstuffs can satisfactorily promote the immune response, such as polysaccharides from green tea (Monobe et al. 2008), fungus polysaccharides (Lee and Hong 2011), bulbus arteriosus in tuna (Daifuku et al. 2012), and jellyfish collagen (Putra et al. 2012). Activated immune responses can protect our body and reduce the risks of diseases caused by pathogens (Logambal et al. 2000; Bafna and Mishra 2006).

Macrophages are a member of the innate immune system and play important roles in the host defence system, especially for activation of the adaptive immune system. In the early phase of infection, macrophages phagocytose and degrade invading pathogens (Hume 2006; Katsiari et al. 2010). Macrophages process pathogens, present them as an antigen on its cell surface, and initiate activation of the adaptive immune response. In addition, activated macrophages secrete cytokine such as tumour necrosis factor (TNF)- α and interleukin (IL)-6 to recruit and activate other leukocytes to initiate the adaptive immune response (Aderem and Ulevitch 2000).

Snake fruit (*Salacca edulis* Reinw.) is a tropical fruit cultivated throughout Indonesia and has delicious taste and aroma, which is not only consumed freshly, but also processed into syrup, jam, and chips. Snake fruit mainly consists of carbohydrate. It can be separated into peel, flesh, and seed. Although peel is usually discarded as waste, snake fruit peel exhibits high antioxidant (Deng et al. 2012) and antiproliferative activities on a human cancer cell line (Li et al. 2013). So far, there is no report concerning the effect of snake fruit peel as a functional food on the immune system. The purpose of this study is to evaluate the immunological effect of snake fruit peel on the innate immune system using murine macrophage-like cell line J774.1 cells. The findings would provide a beneficial aspect of snake fruit and to efficiently utilize it as functional foods.

Materials and methods

Materials

RPMI 1640 medium, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). TNF- α and IL-6 ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA) and Biolegend (San Diego, CA, USA), respectively. Goat anti-actin antibody and horseradish peroxidase (HRP)-conjugated anti-goat IgG antibody were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). HRP-labelled anti-rabbit IgG antibody, rabbit antibodies against I κ B α , phosphorylated I κ B α , NF- κ B p65, p38, phosphorylated p38, c-Jun N-terminal kinase (JNK), and

phosphorylated JNK were purchased from Cell Signalling Technology (Danvers, MA, USA).

Cells and cell culture

J774.1 cells, a murine macrophage-like cell line, were obtained from Japanese Collection of Research Bioresource Cell Bank (Osaka, Japan). J774.1 cells were maintained in RPMI 1640 medium supplemented with 100 U/mL of penicillin, 100 μ g/mL of streptomycin, and 10 % FBS at 37 °C in a humidified atmosphere containing 5 % CO₂ and 95 % air.

Sample preparation

Snake fruits at the age of 5 months were harvested from Sleman, Yogyakarta, Indonesia in March 2014. Snake fruit extraction was done as previously described by Leontowicz et al. (2006) with modifications. Briefly, snake fruits were washed with tap water and peel was separated from flesh and seed. Peel was freeze-dried for 48 h, ground, and stored at −20 °C until used. The extraction was done using three different solvents which were deionized distilled water, 70 % aqueous methanol, and 70 % aqueous ethanol. Each solvent has different polarity thus would extract different active substances from snake fruit peel.

Peel extract was prepared by adding 9 mL of solvent into 1 g of the freeze-dried snake fruit peel powder and left for 15 h at 4 °C in rotary shaker. Hence, the initial concentration was 100 mg powder/mL solvent. Low temperature was used to prevent enzymatic browning caused by polyphenol oxidase in snake fruit (Zaini et al. 2013). Insoluble substances were removed by centrifugation at 685 $\times$ g for 30 min. Supernatant was collected and sterilized by a 0.22 μ m membrane filter. Samples were mixed with the equal volume of $\times$ 2 RPMI 1640 medium when treating cells in order to avoid excessive dilution of cell culture media.

Among water, methanolic, and ethanolic extracts, it was revealed that water extract exhibits the most consistent and strongest stimulation on TNF- α and IL-6 production by J774.1 cells (data not shown). Therefore, only water extract was used in present study and called as snake fruit peel extract (SFPE). The amount of the substances contained in SFPE was represented by protein concentration.

Phagocytotic assay

The assay was performed as previously described (Putra et al. 2015) with some modifications. J774.1 cells were seeded at 2.5×10^5 cells/well in an 8-chamber culture slide (Corning, Tewksbury, MA, USA) and incubated at 37 °C. After incubation for 15 h, culture media were replaced and SFPE was added to culture medium at a final concentration of 0.3 and 0.9 mg/mL, and further incubated. Deionized distilled water was used as the control. After incubation for 6 h, culture media were replaced with fresh 10 % FBS-RPMI 1640 medium containing polystyrene latex beads (particle size; 3.0 µm, Sigma-Aldrich) in a ratio of 1:2000 and further incubated for 24 h at 37 °C. J774.1 cells were washed 5 times with phosphate-buffered saline (PBS) to remove excess polystyrene latex beads. The cells were then observed under a conventional light microscope and their photographs were taken by a charge-coupled device (CCD) camera (Moticam 2000, Shimadzu Rika, Tokyo, Japan). Around 100–400 cells were counted in each photograph and classified based on the number of latex beads inside the cells. Relative phagocytotic activity was calculated with Eq. 1.

$$\text{Relative phagocytotic activity (\%)} = 100 \times \frac{\text{Number of J774.1 cells phagocytizing latex beads}}{\text{Number of total J774.1 cells}} \quad (1)$$

Cytokine production assay

J774.1 cells in 10 % FBS-RPMI 1640 medium were seeded into a 48-well culture plate (Corning) at 1.75×10^5 cells/well and cultured at 37 °C for 15 h. After that, culture medium were replaced and SFPE was added to the medium at 0.3 and 0.9 mg/mL. The cells were further incubated at 37 °C for 6 h. Deionized distilled water (DDW) was used for the control treatment. Culture media were collected and cytokine concentrations in the media were measured using ELISA kits.

Real-time RT-PCR

J774.1 cells were seeded at 1.75×10^5 cells/well in a 48-well culture plate (Corning) and cultured for 12 h.

J774.1 cells were treated with SFPE at a final concentration of 0.3 and 0.9 mg/mL, and incubated at 37 °C. After incubation for 6 h, total RNA was isolated from cells using Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan). First-strand cDNAs were synthesized from 3 µg of total RNA using an oligo-dT₂₀ primer (Toyobo, Osaka, Japan) and M-MLV reverse transcriptase (Promega, Madison, WI, USA). Real-time RT-PCR was performed as previously described by Nishi et al. (2011). In brief, a 20-µL of real-time RT-PCR mixture was composed of 0.1 µg of a cDNA sample, 1 µmol/L of a forward primer, 1 µmol/L of a reverse primer, and Thunderbird SYBR qPCR Mix (Toyobo). The thermal cycling conditions were: 20 s at 95 °C; and 40 cycles of 3 s at 95 °C and 30 s at 60 °C. Real-time RT-PCR was performed by StepOnePlus System (Applied Biosystems, Foster City, CA, USA), and the relative gene expression was calculated based on the comparative C_T method using StepOne Software v2.1 (Applied Biosystems). Data were shown as the fold difference normalized to β-actin. Specific PCR primer sequences for each gene are shown in Table 1.

Immunoblot analysis

J774.1 cells were seeded at 1×10^6 cells/dish in a 35 mm culture dish (Corning) and cultivated overnight at 37 °C. The cells were treated with SFPE at a final concentration of 0.3 and 0.9 mg/mL and further incubated for 6 h at 37 °C. Cytosolic and nuclear proteins were each isolated using a CellLytic NuCLEAR Extraction kit (Sigma-Aldrich) according to the manufacturer's instructions. Protein concentration was quantified using a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA), which is a

Table 1 Sequences of primers for real-time RT-PCR

Primer	Sequences (5'–3')
β-actin	CATCCGTAAAGACCTCTATGCCAAC (sense) ATGGAGCCACCGATCCACA (antisense)
TNF-α	CTACTCCCAGGTTCTCTTCAA (sense) GCAGAGAGGAGGTTGACTTTC (antisense)
IL-6	AAGCCAGAGTCCTTCAGAGAGAT (sense) TTGGATGGTCTTGGTCCTTAGC (antisense)

colorimetric assay based on Lowry method. The standard was prepared from bovine serum albumin and the absorbance was measured at 655 nm with a microplate reader (Model 550; Bio-Rad Laboratories). Heat-denatured proteins (10–15 µg) were separated on a 10 % polyacrylamide gel. Immunoblot analysis using various antibodies was performed as described previously (Putra et al. 2014). Bands were analysed using a ChemiDoc XRS Plus apparatus (Bio-Rad Laboratories) and the chemiluminescent intensity was quantified using the Quantity One software (Bio-Rad Laboratories).

Statistical analysis

Data are expressed as mean $\pm$ standard deviation (SD). Statistical significance was evaluated by one-way ANOVA followed by Tukey–Kramer post hoc test. p values <0.05 were considered statistically significant. Significant differences are represented as * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$.

Results and discussion

Effect of SFPE on phagocytotic activity

Phagocytes such as neutrophils, monocytes, and macrophages are important members in the innate immune system. These cells respond to invasion of pathogenic organisms in the early phase by directly eliminating pathogens and coordinating the inflammatory responses (Janeway and Medzhitov 2002; Tosi 2005). In order to study the immunostimulatory activity of SFPE, firstly, the effect of SFPE treatment on phagocytotic activity of J774.1 cells was evaluated. As a result, SFPE enhanced the phagocytotic activity of J774.1 cells. SFPE increased number of the beads inside the cells as indicated in Fig. 1a. Relative phagocytotic activity is described as the percentage of J774.1 cells with phagocytosed beads compared to total number of J774.1 cells. In addition, SFPE stimulated resting J774.1 cells to phagocytose the beads, and increased relative phagocytotic activity of J774.1 cells as shown in Fig. 1b. Relative phagocytotic activity was increased from 82.4 % (control) to 96.2 % (SFPE 0.3 mg/mL) and 99.3 % (SFPE 0.9 mg/mL), respectively. These findings suggested that

SFPE possesses the immunostimulatory activity to activate phagocytotic activity of J774.1 cells. Phagocytosis is a process to remove and to degrade invading microbes. It requires recognition through receptors on the surface of the cells. Toll-like receptor (TLR) 4 is involved in this recognition stage and initiates the process (Anand et al. 2007).

Effect of SFPE on cytokine production and gene expression levels

The effect of SFPE on TNF- α and IL-6 production was examined. Results revealed that TNF- α and IL-6 production was stimulated by SFPE as shown in Fig. 2. SFPE stimulated TNF- α production by 2.7- and 2.5-folds at 0.3 and 0.9 mg/mL, respectively. IL-6 production was more strongly stimulated by SFPE treatment. SFPE also enhanced IL-6 production by 78- and 48-folds at 0.3 and 0.9 mg/mL of SFPE, respectively. In addition, SFPE also enhanced gene expression levels of both cytokines in a similar manner with cytokine production (Fig. 2). Macrophage activation process includes the generation of pro-inflammatory cytokines such as TNF- α , IL-1, IL-6, and IL-12. TNF- α rapidly takes place to activate and recruit other lymphocytes, to enhance various functional responses, and to induce the expression of immunoregulatory mediator genes (Feuerstein et al. 1994). IL-6 stimulates the terminal differentiation of B cells into plasma cells and plays a central role in fever and acute phase responses (Muraguchi et al. 1988; Sica et al. 1990).

SFPE was prepared by water extraction, indicating that the active substance was water-soluble. Heat and proteinase treatment did not cancel the stimulatory effect of SFPE on TNF- α and IL-6 production by J774.1 cells (data not shown). In addition, dialysis treatment of SFPE by dialysis membranes with molecular weight cut off (MWCO) of 14 kDa did not affect the stimulatory activity (data not shown). It is supposed from this fact that the active substance in SFPE is heat stable and not a protein. The active substance in SFPE seemed to be water-soluble carbohydrates such as pectin.

Optimum stimulation for cytokine production and gene expression level was given at 0.3 mg/mL of SFPE. Hence, intracellular signalling cascade analysis was done at 0.3 mg/mL of SFPE.

Fig. 1 Effect of SFPE on phagocytotic activity of J774.1 cells. J774.1 cells were cultured in 10 % FBS-RPMI 1640 medium containing 0.3 or 0.9 mg/mL of SFPE, and incubated for 6 h at 37 °C. Control cells were cultured in the medium containing deionized distilled water instead of SFPE. Then, culture media

were replaced with 10 %FBS-RPMI 1640 medium containing polystyrene latex beads and further incubated for 24 h at 37 °C. After taking pictures, the number of J774.1 cells was counted and classified based on the number of latex beads inside the cells (*bar graph*)

Effect of SFPE on NF-κB and mitogen-activated protein (MAP) kinase signalling cascades

Intracellular signalling pathway via Toll-like receptor (TLR) 4, including NF-κB and MAP kinase signalling cascades, is a major pathway for activation of macrophage. The effect of SFPE on TLR4 signalling was evaluated by immunoblot analysis. As shown in Fig. 3, SFPE elevated IκBα phosphorylation and NF-κB translocation into the nucleus. SFPE also activated two of MAP kinase signalling cascades by enhancing phosphorylation of JNK and p38 in J774.1 cells as indicated in Fig. 4. These findings indicated that the

active substance in SFPE may elevate cytokine gene transcription levels by activating NF-κB, JNK, and p38 signalling cascades, and facilitated cytokine production by J774.1 cells.

Macrophages are activated through TLR4, CD14, scavenger receptor, dectin-1, complement receptor 3, or mannose receptor (Brown et al. 2002; Herre et al. 2004; Schepetkin and Quinn 2006). Some studies defined that activation of the TLR4 signalling pathway induces production of proinflammatory mediators such as TNF-α and IL-6 (Gorina et al. 2011; Guijarro-MuñozI et al. 2014; Kumalasari et al. 2014). Activation of TLR4 by specific molecules

Fig. 2 Effect of SFPE on cytokine production and their genes expression level in J774.1 cells. J774.1 cells were cultured in 10 % FBS-RPMI 1640 medium containing 0.3 or 0.9 mg/mL of SFPE for 6 h at 37 °C. Culture media and cells were used for measuring cytokine concentrations by ELISA and for evaluating gene expression levels by real-time RT-PCR, respectively. Control cells were cultured in the medium containing deionized distilled water instead of SFPE. Data are represented as mean $\pm$ standard deviation ($n = 3$). Statistical significance was evaluated by one-way ANOVA followed by Tukey–Kramer post hoc test. Significant differences against control are represented as ** $p < 0.01$ or *** $p < 0.001$

leads to the subsequent signal transduction such as NF- κ B and MAP kinase signalling. This activation process leads to the initiation of cytokine gene expression (Zhang and Ghosh 2001; Lu et al. 2008). NF- κ B is an important transcription factor with a wide range of target genes to regulate immune responses including inflammation (Hayden and Ghosh 2004). In TLR4 signalling pathway, MAP kinase family is also responsible for the immune responses. MAP kinase family consists of extracellular signal-regulated kinases (ERKs), JNK, ERK5/big MAP kinase 1 (BMK1), and p38 group of protein kinases (Ono and Han 2000). SFPE activated phosphorylation of JNK and p38 as shown in Fig. 4. The activation of JNK contributes to proinflammatory cytokine production, cell growth, differentiation, survival, and apoptosis (Davis 2000; Putra et al. 2014; Weston and Davis 2002). Activation of p38 also enhances the production of proinflammatory

cytokines (New and Han 1998). Taken together, our results suggest that SFPE induces cytokine production via not only NF- κ B, but also JNK and p38 signalling cascades.

Immunostimulatory effects of SFPE were evaluated with mouse J774.1 cells in the present study. For the application of SFPE to human health, its effects should be evaluated with human macrophages. However, it is not easy to obtain adequate macrophages from human. Because Pantic et al. (2014) found that cyclic peptide tigerinins stimulate cytokine production not only by mouse peritoneal macrophages but also human peripheral blood mononuclear cells including macrophages, it is possible to consider that SFPE might exert the immunostimulatory effects on human macrophages as well as mouse macrophages. Detailed evaluation of the immunostimulatory effects of SFPE on human is required for the practical application of snake fruit as a functional food.

Fig. 3 Immunoblot analysis of NF-κB signalling in J774.1 cells treated with SFPE. J774.1 cells were cultured in 10 % FBS-RPMI 1640 medium with or without 0.3 mg/mL of SFPE for 6 h at 37 °C. Actin and histone were used as a loading control for normalization of proteins in the cytosol and nucleus,

respectively. The p-IκBα represents phosphorylated IκBα. *Bar graph* shows the fold induction of the p-IκBα over the whole IκBα. A representative blot from three independent experiments is shown

Fig. 4 Immunoblot analysis of MAP kinase signalling in J774.1 cells treated with SFPE. J774.1 cells were cultured in 10 % FBS-RPMI 1640 medium with or without 0.3 mg/mL of SFPE for 6 h at 37 °C. Actin was used as a loading control for normalization of proteins. The p-p38 and p-JNK represents

phosphorylated p38 and JNK, respectively. *Bar graphs* show the fold induction of the phosphorylated target protein over its whole protein. A representative blot from three independent experiments is shown

Conclusion

In this study, novel immunological effect of snake fruit peel extract (SFPE) to stimulate macrophages activation was evaluated. SFPE increased phagocytotic activity of J774.1 cells. TNF-α and IL-6 production as well as their genes expression level were increased by SFPE. It was supposed that SFPE increased cytokine production by elevating gene

expression level through the activation of NF-κB, JNK, and p38 signalling cascades.

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