Evaluation of immunostimulatory effect of the arrowroot (*Maranta arundinacea*. L) in vitro and in vivo

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Abstract Arrowroot (*Maranta arundinacea*. L) is an underutilized local crop potentially to be developed as carbohydrate source and functional food in Indonesia. The objectives of this research are to evaluate the immunostimulatory effects of arrowroot extracts in vitro by using animal cell culture techniques, and in vivo by using BALB/c mice. The arrowroot tuber extracts were prepared by heat-treatment at 121 °C for 20 min in distilled water. The IgM production

stimulatory activity of arrowroot tuber extracts against human hybridoma HB4C5 cells and mouse splenocytes was assessed. The result indicated that the arrowroot tuber extract stimulated IgM production by HB4C5 cells and immunoglobulin (IgG, IgA and IgM) production by splenocytes in vitro. In addition, the arrowroot tuber extracts strongly enhanced interferon γ production by splenocytes. In vivo study indicated that the diet containing arrowroot extracts increased the serum IgG, IgA and IgM levels in mice. These results revealed that the arrowroot tuber extracts have immunostimulatory effects in vivo as well as in vitro.

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Abbreviations

Con A	Concanavalin A
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
GALT	Gut-associated lymphoid tissue
HRP	Horseradish peroxidase
IFN- γ	Interferon- γ
Ig	Immunoglobulin
OF	Oligofructose
PBS	Phosphate-buffered saline
RS	Retrograded starch
SCFA	Short chain fatty acid

Introduction

There are increasing evidences that diet can modulate immune function in body. Activation of the immune response in our body contributes to our health by reducing the risk of chronic disease or boosting natural immune protection. There are many substances that promote an immune response. The immunoglobulin production stimulating factors in foodstuffs contributing to development of functional food is of recent high interest (Sugahara et al. 2005).

Immune response is activated by variety of substances including carbohydrates. Diet modulates immune functions in different ways and affects host resistance to infections. In addition to the essential nutrients in food, nonessential food constituents such as nondigestible carbohydrates also affect the immune system. Non-digestible carbohydrates such as inulin and oligofructose (OF), including their intestinal fermentation products, may modulate the gut-associated lymphoid tissue (GALT) as well as the systemic immune system. Inulin and OF are classified as prebiotics which occur naturally as plant storage carbohydrates in vegetables, cereals, and fruits (Seifert and Watzl 2007).

The use of prebiotics such as oligosaccharide has been considered as a means of influencing the gut microbiota and risk of allergy. The gut is a complex environment influenced largely by the microbial content, bacterial secondary metabolites, immune-modulators and quorum-sensing molecules, and host factors including secretions (Louis et al. 2007). Simple molecules such as mono-, di- and oligosaccharides derived from complex carbohydrate during digestion, may exert these biological effects. The complex carbohydrates exert an effect on gut-associated immunity before absorption, which is then transferred to the systemic immune responses via lymph nodes and Peyer's patches. Studies on the immunostimulatory effect of carbohydrates, especially oligosaccharides in humans, are very important.

Arrowroot (*Maranta arundinacea*. L) is a locally grown tuber crop in Indonesia. The arrowroot starch has high digestibility and is commonly used as a thickener in many foods such as puddings and sauces, cookies and other baked goods. Arrowroot is bland, making it suitable for neutral diets, especially for people who are feeling nauseous. Some people believe that arrowroots help to soothe upset stomachs, which is the reason why many health food stores in Indonesia display arrowroot

cookies. The arrowroot tuber contains plenty of starch and other compounds. The starch from arrowroot flour has a nutrition composition of 11.9% water, 0.58% ash, 25.9% amylose, 0.14% protein, 0.84% fat, 8.7% insoluble dietary fiber, and 5.0% soluble dietary fiber. Recent study suggested that the arrowroot flour is a potential source of prebiotics (Harmayani et al. 2011). However, information on the immunostimulatory effects of polysaccharides from arrowroot is limited. The objective of this research is to evaluate the immunostimulatory effects of the arrowroot tuber extracts and various oligosaccharides in vitro and in vivo.

Materials and methods

The arrowroot tuber extracts

Arrowroot tubers were grated and suspended in distilled water. The suspension was settled down overnight to collect the precipitate. The precipitate was air-dried and ground to make arrowroot powder. The arrowroot tuber powder was suspended in distilled water at 5 g/50 mL. The suspension was heated at 121 °C for 20 min, and centrifuged at 15,600×g for 20 min to remove insoluble substances. The supernatant was sterilized by filtration and used as the arrowroot extracts.

The arrowroot extract solution was also prepared at room temperature. The arrowroot tuber powder was suspended in distilled water and stirred at 25 °C for 12 h, and centrifuged at 15,600×g for 20 min to remove insoluble substances. The supernatant was sterilized by filtration before use. The arrowroot tuber powder was also extracted in 100% ethanol. The arrowroot tuber powder was suspended in 100% ethanol at 5 g/10 mL, and stirred at 25 °C for 12 h. Following centrifugation at 15,600×g for 20 min, supernatant was collected and sterilized by filtration.

Cell and cell culture

Human-human hybridoma HB4C5 cells producing monoclonal IgM were used for the assay of the Ig production stimulating activity (Sugahara et al. 2005). The HB4C5 cell was a fusion product of a human B lymphocyte from a lung cancer patient and a human fusion partner, NAT-30 (Murakami et al. 1985). HB4C5 cells were maintained in ERDF medium

(Kyokuto Pharmaceutical, Tokyo, Japan) supplemented with $10 \mu\text{g mL}^{-1}$ of insulin (Sigma, St. Louis, MO, USA), $20 \mu\text{g mL}^{-1}$ of transferrin (Sigma), $20 \mu\text{M}$ ethanolamine (Sigma), and 25 nM sodium selenite (Sigma) (ITES-ERDF) at 37°C under humidified $5\% \text{ CO}_2$ – $95\% \text{ air}$ (Murakami et al. 1982).

Ig production stimulating effect of the arrowroot extracts on HB4C5 cells

The Ig production stimulating activity *in vitro* was examined by measuring the amount of IgM secreted by HB4C5 cells in culture medium. The arrowroot extracts were solubilized by heat-treatment at 121°C for 20 min and assayed for the Ig production stimulating activity on human hybridoma HB4C5 cells. HB4C5 cells were inoculated in ITES-ERDF medium containing various concentrations of the arrowroot extracts at $5.0 \times 10^4 \text{ cells mL}^{-1}$. An assay of Ig production stimulating activity was performed in a 96-well culture plate. After cultivation in a CO_2 incubator at 37°C for 6 h, the amount of IgM secreted into the culture medium was determined by enzyme-linked immunosorbent assay (ELISA) using anti-human IgM (Sugahara et al. 2005). Briefly, goat anti-human IgM antibody (Biosource International, Camarillo, CA, USA) at $1.0 \mu\text{g mL}^{-1}$ was added to a 96-well plate at $100 \mu\text{L well}^{-1}$, and incubated for 2 h at 37°C . After washing with 0.05% Tween 20-phosphate-buffered saline (T-PBS) 3 times, each well was blocked with 5% skim milk-PBS solution for 2 h at 37°C . Following blocking reaction, each well was treated with $50 \mu\text{L}$ of culture supernatant for 1 h at 37°C . After washing, wells were then treated with $100 \mu\text{L}$ of horseradish peroxidase (HRP)-conjugated anti-human IgM antibody (Biosource International) diluted 2,000 times with 5% skim milk-PBS for 1 h at 37°C . Then, 0.6 mg mL^{-1} of 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) dissolved in $0.03\% \text{ H}_2\text{O}_2$ – 0.05 M citrate buffer (pH 4.0) was added to the wells at $100 \mu\text{L}$, and the absorbance was measured at 415 nm after the addition of μL of 1.5% oxalic acid to terminate the coloring reaction. Ig production assays were triplicated.

Immunostimulatory effect of the arrowroot extract on mouse splenocytes *in vitro*

Two 6-week-old female BALB/c mice (Japan SLC, Shizuoka, Japan) were housed with a pelleted basal

diet and water *ad libitum*, in an animal room under 12 h light/dark cycle at a temperature of $24 \pm 1^\circ\text{C}$ and a humidity of $55 \pm 5\%$. Mice were sacrificed by cervical dislocation and spleens were excised from mice. Suspension of splenocytes was made by gently passing the minced organ through a mesh with a pore size of $40 \mu\text{m}$ into a culture dish. Splenocytes were centrifuged at $190 \times g$ for 5 min and hemolyzed 2 times using hemolysis buffer ($155 \text{ mM NH}_4\text{Cl}$, 15 mM NaHCO_3 , 1 mM EDTA , pH 7.3). Splenocytes were then washed with PBS and centrifuged at $190 \times g$ for 5 min, and finally suspended in 5% fetal bovine serum (FBS) (SAFC Biosciences, Lenexa, KS, USA)-RPMI 1640 (Sigma) medium supplemented with 100 U L^{-1} of penicillin and 100 mg L^{-1} of streptomycin. All animal experiments described herein were carried out in accordance with protocols approved by the Ehime University Animal Care and Use Committee and were performed in accordance with applicable guidelines and regulations.

Mouse splenocytes were inoculated in 5% FBS-RPMI 1640 medium. The assay of the IgM, IgA and IgG production-stimulating activity was performed in a 96-well culture plate as mentioned previously (Nishimoto et al. 2009). The cell density of mouse splenocytes was $1 \times 10^6 \text{ cells mL}^{-1}$. After cultivation in a CO_2 incubator at 37°C for 48 h, the amounts of IgM, IgA and IgG secreted into culture medium were determined by ELISA. Goat anti-mouse IgM (Invitrogen, Carlsbad, CA, USA), goat anti-mouse IgG (Invitrogen) or rabbit anti-mouse IgA (Invitrogen) were used at $1.0 \mu\text{g mL}^{-1}$. After incubation for 2 h at 37°C , each well was blocked with 5% skim milk-PBS solution for 2 h at 37°C . Following blocking reaction, each well was treated with $50 \mu\text{L}$ of culture supernatant for 1 h at 37°C . The wells were then treated with $100 \mu\text{L}$ of HRP-goat anti-mouse IgM (Invitrogen), HRP-goat anti-mouse IgG (Invitrogen) or HRP-goat anti-mouse IgA (Invitrogen) diluted 1,000 times with 5% skim milk-PBS for 1 h at 37°C . After washing the wells, 0.6 mg mL^{-1} of ABTS dissolved in $0.03\% \text{ H}_2\text{O}_2$ – 0.05 M citrate buffer (pH 4.0) was added to the wells at $100 \mu\text{L}$, and the absorbance at 415 nm after the addition of 1.5% oxalic acid to terminate the coloring reaction at $100 \mu\text{L}$. The assays were triplicated. In addition, IFN- γ level in culture medium was also measured by ELISA using rat anti-mouse IFN- γ antibody and biotin-labeled rat anti-mouse IFN- γ antibody (Thermo Fischer Scientific, Waltham, MA, USA).

Table 1 Composition of animal diet

Composition	AIN-93 standard diet (g kg ⁻¹)	AIN-93 arrowroot diet (g kg ⁻¹)
Casein	140	140
L-cystine	1.8	1.8
Sucrose	100	100
Corn starch	620.7	–
Arrowroot powder	–	620.7
Cellulose	50	50
Soybean oil	40	40
Coline bitartrat	2.5	2.5
AIN-93 mineral mix	35	35
AIN-93 vitamin mix	10	10
Total	1,000	1,000

Animals and diets

BALB/c mice (6-week-old) were assigned into 2 groups ($n = 8$), the control group and the arrowroot group. The control group was fed with AIN93 standard diet (Reeves et al. 1993) and the arrowroot group was fed with AIN93 standard diet containing the arrowroot powder instead of cornstarch for 14 days. Diet composition is summarized in Table 1. On day 15, the blood was collected and centrifuged at $18,000 \times g$ for 30 min at 4 °C to prepare the serum (LeBlanc et al. 2004). The IgM, IgG, and IgA levels in the serum were measured by ELISA.

Measurement of short chain fatty acid (SCFA)

Fecal samples were centrifuged at $5,000 \times g$ for 20 min and the supernatant was collected and analyzed by gas chromatography (GC-8A, Shimadzu, Kyoto, Japan) for SCFA content using a capillary column (4 mm $\times$ 180 cm) (Delzenne and Roberfroid 1994).

Statistical analysis

All results are expressed as the means $\pm$ standard deviation (SD). Tukey's t test was used to assess the statistical significance of the difference.

Results and discussion

Effect of the arrowroot extracts on IgM production by HB4C5 cells

Immunoglobulin production stimulatory effect of arrowroot extracts was evaluated. As indicated in Fig. 1a, the arrowroot heat-extracts stimulated IgM production by HB4C5 cells in a dose-dependent manner. Interestingly, the arrowroot extracts prepared at room temperature did not enhance IgM production at all (Fig. 1a). It is supposed from this result that heating process solubilized the active substance in the arrowroot tuber powder or activated the inactive substances in the arrowroot extracts prepared at room temperature. In addition, arrowroot extracts in 100% ethanol did not activate IgM production by HB4C5 cells (Fig. 1b).

Then, the arrowroot extracts prepared at 25 °C was heated at 121 °C for 20 min, and the stimulatory activity was assessed. As a result, the solution did not stimulate IgM production by HB4C5 cells (data not shown). These results suggest that the active substance in arrowroot tuber is insoluble in water and ethanol. Since the active substance in the arrowroot tuber was insoluble in ethanol, it may not be low molecular weight phytochemicals such as polyphenols. In addition, the active substance in the arrowroot tuber was solubilized by heat treatment, and the activity of the substance is heat-stable. It is supposed from these facts that the active substance is a sort of polysaccharides such as starch. Haralampu (2000) indicated that retrograded starch, particularly retrograded amylose is the most stable form. Retrograded starch (RS) is of particular interest because of its thermal stability. The general behavior of RS is physiologically similar to soluble, fermentable fiber (Haralampu 2000).

Effect of the arrowroot extracts on mice splenocytes in vitro

The heat-solubilized arrowroot extracts stimulated Ig production by HB4C5 cells. Therefore, further immunostimulatory effect of the arrowroot extracts was examined by using mouse primary splenocytes. Splenocytes were collected from the spleen of 6-week-old female BALB/c mice, and the effect of arrowroot

Fig. 1 Effect of the arrowroot extracts on IgM production by HB4C5 cells. **a** Arrowroot powder was suspended in distilled water and extracted at 121 °C for 20 min (*open circle*) or at 25 °C for 12 h (*open triangle*). HB4C5 cells were inoculated in ITES-ERDF medium containing various concentrations of each arrowroot extract at 5.0×10^4 cells mL^{-1} , and cultured for 6 h. The control (*filled circle*) was supplemented with distilled water instead of the arrowroot extracts. Each result is presented as the mean $\pm$ SD of three independent measurements. **b** Arrowroot

powder was suspended in 100% ethanol and extracted at 25 °C for 12 h (*open circle*). HB4C5 cells were inoculated in ITES-ERDF medium containing various concentrations of each arrowroot ethanol extracts at 5.0×10^4 cells mL^{-1} , and cultured for 6 h. The control (*filled circle*) was supplemented with 100% ethanol at 1% of culture volume instead of the arrowroot extracts. Each result is presented as the mean $\pm$ SD of three independent measurements

Fig. 2 Effect of the arrowroot extracts on Ig production by mouse splenocytes in vitro. Mouse splenocytes were inoculated in RPMI 1640 medium supplemented with 5% FBS and various concentrations of the arrowroot starch extracts, and cultured for

48 h. The control (*filled circle*) was supplemented with distilled water instead of the arrowroot extracts. Each result is presented as the mean $\pm$ SD of three independent measurements

extracts on splenocytes in vitro was evaluated. As shown in Fig. 2, the arrowroot extracts stimulated IgG, IgA and IgM production by splenocytes in vitro fourfold, sevenfold, and twofold, respectively.

In addition, the arrowroot extracts strongly enhanced $\text{IFN-}\gamma$ production by splenocytes treated with $10 \mu\text{g mL}^{-1}$ of Con A (Fig. 3). This result suggests that the arrowroot extracts stimulate not only

Fig. 3 Effect of the arrowroot extracts on IFN- γ production by mouse splenocytes *in vitro*. Mouse splenocytes were inoculated in RPMI 1640 medium supplemented with 5% FBS, $10 \mu\text{g mL}^{-1}$ of Con A and the various concentrations of the arrowroot extracts, and cultured for 48 h. The control (filled circle) was supplemented with distilled water instead of the arrowroot extracts. Each result is presented as the mean $\pm$ SD of three independent measurements

antibody production by B lymphocytes, but also cytokine production by T lymphocytes.

Effect of the arrowroot powder *in vivo*

BALB/c mice were fed the arrowroot powder to examine the immunostimulatory effect *in vivo*. As summarized in Fig. 4, the feeding of the arrowroot tuber powder for 14 days significantly enhanced IgG, IgM, and IgA levels in serum. The arrowroot tuber powder might contain resistant starch acting as dietary fibers that could stimulate the immune system. The immunostimulatory effects of dietary fibers and prebiotics have been extensively studied (Schley and Field 2002). Glucose and some oligosaccharides are released from RS over a sustained period through the normal digestive process. The indigestible oligosaccharides may also play an important role in modulating the immune system. Nakamura et al. (2004) revealed the effect of OF intake (50 g kg^{-1} diet) in infant BALB/c mice on IgA. The expression of the secretory component, as well as IgA was elevated in the small and large intestinal tract. Although the mechanism of poly- and oligosaccharide in inducing

Fig. 4 Effect of the arrowroot diet on Ig level in serum. BALB/c mice (6-week-old) were fed AIN-93 standard diet as control (C) or AIN-93 arrowroot diet (A) for 14 days to examine the immunostimulatory effect *in vivo*. Each circle represents the Ig level in serum from the independent mouse, and bars represent average of each group ($n = 8$). Statistically significant differences from control are represented as * $p < 0.05$, or ** $p < 0.001$

immunomodulation is not clear yet, it was proposed that *in vivo* mechanism of probiotics such as inulin or fructooligosaccharides inducing immunomodulation was done through: (1) selective increase/decrease in specific intestinal bacteria that modulate local cytokine and antibody production; (2) increasing in intestinal SCFA production and enhanced binding of SCFA to G-coupled protein receptors on leukocytes; (3) interacting with carbohydrate receptors on intestinal epithelial cells and immune cells; and (4) partial absorption of inulin or OF resulting in local and systemic contact with the immune system (Seifert and Watzl 2007). The Ig production stimulatory activity of the arrowroot extracts was depressed by amylase treatment (data not shown). This means that the active substance in the arrowroot extracts is a kind of starch. However, the activity was not completely inactivated by amylase treatment. On the other hand, when the amylase-treated arrowroot extracts was treated with trypsin, the activity was completely inactivated (data not shown). This suggests that there are two kinds of active substances in arrowroot extracts, one is kind of starch and the other one is protein.

SCFA profile in cecal content

Molar proportion of SCFA in caecum is shown in Table 2. Caecum molar proportion of the arrowroot diet group tended to have a high amount of butyrate compared with the control group. The gut microflora

Table 2 Molar proportion of SCFA in caecum

Group	Acetate (%)	Propionate (%)	Butyrate (%)
Control	71	16	13
Arrowroot	65	14	21

may modulate immune cells through fermentation of dietary fibers to SCFA (Schley and Field 2002; Gibson and Roberfroid 1995). Butyrate production in the colon may reduce the requirement of epithelial cells for glutamine, thereby sparing it for other cells, such as those of the immune system (Jenkins et al. 1999). Although further work is needed to better define the mechanisms for immunomodulation, there are preliminary data to suggest that the consumption of prebiotic fibers can modulate parameters in GALT, secondary lymphoid tissue and peripheral circulation (Schley and Field 2002).

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