



Inhibiting or enhancing effect of sulfuric acid-treated wheat starch on antibody production induced by two types of adjuvant

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ABSTRACT

Glycogen has been reported to have immune-regulating activity. We examined in this study the immune-regulating activity of wheat starch of various molecular weights, because both starch and glycogen are made from glucose components linked by α -1,4 and α -1,6 glycoside bonds. Wheat starch was treated by sulfuric acid to prepare starch samples with differing molecular weight. The acid-treated starch inhibited cytokine production from murine splenocytes when the splenocytes were incubated with the antigen and a starch sample. The activity depended on the treatment time by sulfuric acid. Mice were then i.p. immunized with some antigens and the starch mixed with two types of adjuvant. The starch also inhibited the *in vivo* antibody production when administered with an alum adjuvant. In contrast, the starch enhanced the antibody response when administered with complete Freund adjuvant, indicating that the starch regulated immune responses depending on the molecular weight and surrounding circumstances.

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1. Introduction

Allergic diseases of hay fever and asthma, as well as autoimmune diseases of colitis and rheumatoid arthritis are of immunological type induced by unbalanced or hyper immune responses. The increase of these immunological diseases has recently become one of the most severe health problems throughout the world, because the quality of life (QOL) of the patients is generally low. Many groups have tried to use the immune regulating function of food factors to improve QOL of these patients, for example with some kind of poly- or oligo-saccharides, tea catechins, and lactic acid bacteria.

Recent studies have shown that allergy is induced by Th2 type of immune response, while some autoimmune diseases, for example rheumatoid arthritis and multiple sclerosis, are induced by Th1-type responses (Finkelman & Urban, 2001). The Th17 type of response has also been considered to be involved in some inflammatory diseases (Kimura & Kishimoto, 2011). Food factors, such as lactic acid bacteria, have been found to inhibit allergy by suppressing the Th2 response (Enomoto et al., 2009; Yoshida, Hirano, Wada, Takahashi, & Hattori, 2004), while there are few foods known to inhibit Th1 or Th17. Such activity of food factors could also be useful to improve QOL of the patients with those diseases. However, such Th1, Th2 and Th17 immune responses are also necessary to protect us

from infections. The complete inhibition of these immune response by some medicines is therefore not always the best way to treat allergy or autoimmune diseases. A method to specifically inhibit an unbalanced or needlessly strong response is necessary. Foods do not generally make a major or rapid difference to our health, but a daily intake of functional foods might gradually improve our health without any serious side effects. Controlling the intensity and balance of such immune responses as Th1, Th2 and Th17 by some food factors can be expected to maintain a healthy life.

Amylopectin, a type of starch, consists of polymerized glucose units linked by α -1,4 and α -1,6 glycoside bonds and are widely contained in grain. Starch in foods is not only necessary as a source of energy, but also plays an important role in processing food products. In contrast, glycogen, which also consists of polymerized glucose units, provides a stock of glucose in animals, and the structure and character of glycogen are known to be different from those of starch. Glycogen has more branches, which are formed by α -1,6 glycoside bonds, and higher water solubility than starch. Recent studies have revealed glycogen to possess several immune-regulating functions (Ryoyama, Kidachi, Yamaguchi, Kajiura, & Takata, 2004; Takaya et al., 1998). The effect of glycogen obtained from shellfish, for example scallops and oysters, on the anti-tumour response was initially studied, and it had been thought that the activity of glycogen strongly depended on its structure (Takaya et al., 1998). However, Kakutani et al. (2007) have demonstrated that some enzymatically synthesized types of glycogen also had strong activity, like the glycogen obtained from shellfish, and that this activity strictly depended on the molecular weight and not the

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structure. This finding implies that starch may have similar activity if its molecular weight can be appropriately adjusted.

The immune regulating function of starch has hardly been reported, because the molecular weight of amylopectin in foods is in the range of $3.1\text{--}5.2 \times 10^8$ (Yoo & Jane, 2002). According to the report by Kakutani et al. (2007), glycogen with a molecular weight of about 5×10^6 demonstrated the strongest activity, indicating that some type of treatment would be required for starch to provide such activity. Treating starch with sulfuric acid is widely used by the food industry to imbue starch with appropriate properties such as viscosity and water solubility. Although there is no reported study examining the immune regulating function of sulfuric acid-treated starch, this or other methods to reduce the molecular weight of starch would be useful to generate special properties for food processing and also to develop novel food materials with immune regulating functions; it is therefore necessary to confirm whether or not sulfuric acid-treated starch can regulate immune responses.

Any examination of the immune-regulating activity of acid-treated starch requires samples of starch with varying molecular weight. We therefore prepared three types of starch by treating with sulfuric acid for different periods of time. The differing effects of these different types of starch on the T cell response, especially on the cytokine production, were first examined. The effects of these starch samples on the antibody production were then investigated *in vivo*. Our findings will help to provide novel functions of starch for immune regulation, and expand its application to various food products.

2. Materials and methods

2.1. Mice

Six-week-old BALB/c mice were purchased from Clea Japan (Tokyo, Japan). Ovalbumin (OVA)-specific T cell receptor transgenic mice (TCR-Tg mice; DO11.10) were used at 6–14 weeks old, the T cells of DO11.10 mice recognizing OVA_{323–339} restricted to I-A^d. All the mice were maintained and used in accordance with the guidelines for the care and use of experimental animals of Tokyo University of Agriculture and Technology.

2.2. Antigens and antibodies

Crude bovine β -lactoglobulin (β -LG; genotype AA) was prepared from fresh milk of a Holstein cow according to the method of Armstrong, McKenzie, and Sawyer (1967). This crude β -LG was purified in a DEAE-Sephacrose Fast Flow column (3.0 ID $\times$ 40 cm; Amersham Pharmacia Biotech, Buckinghamshire, UK) by the method described previously (Hattori, Nagasawa, Ametani, Kaminogawa, & Takahashi, 1994), the degree of purity being confirmed by polyacrylamide gel electrophoresis (PAGE) performed by the method of Davis (Davis, 1964). OVA was purchased from Seikagaku Kogyo (Tokyo, Japan). Concanavalin A (Con A; Sigma, Tokyo, Japan) was used for antigen-independent T cell activation.

Purified rat anti-mouse IFN- γ mAb (R4-6A2, BD Pharmingen, San Diego, CA), purified rat anti-mouse IL-4 mAb (11B11, BD Pharmingen), biotinylated rat anti-mouse IFN- γ mAb (XMG1.2, BD Pharmingen), biotinylated rat anti-mouse IL-4 mAb (BVD6-24G2, BD Pharmingen), biotinylated rat anti-mouse IgG1 Ab (Zymed, San Francisco, CA), biotinylated rat anti-mouse IgG2a Ab (Zymed) and biotinylated rat anti-mouse IgE mAb (R35-118, BD Pharmingen) were used for ELISA.

2.3. Preparation of sulfuric acid-treated starch

Wheat starch (Ukiko, Manto, Tokyo, Japan) was suspended in an 18% sulfuric acid solution and kept at 38 °C for 0, 4, 8 and 12 days.

Each sample was collected by a centrifugation (800g), the supernatant being used for analyzing the soluble sugar amount to evaluate the progress of the acid treatment. After removing the supernatant, the precipitate was washed by ice-cooled water. We used ice-cooled water to avoid the gelatinization of the starch caused by heat which occurred by mixing the water and the residual acid. The gelatinization results in loss of yield after re-centrifugation. This procedure was conducted at least five times until the supernatant showed a neutral pH value. A part of each of these samples was subdivided and dried at 110 °C to estimate the weight of the sample obtained. The starch samples were used for the experiments after suspending in PBS and autoclaving. The progress of the sulfuric acid treatment was evaluated by measuring the amount of soluble saccharide in the supernatant by the phenol–sulfuric acid method. The amount of insoluble saccharide was calculated by subtracting the soluble saccharide amount from the total starch amount. The difference in the amount of insoluble saccharide between the starch treated for 0 days and each other treated sample is shown in Table 1 as dissolved saccharides. The percentage of the amount of dissolved saccharide in each treated sample compared with the amount of insoluble saccharide in the starch treated for 0 days is also shown in Table 1 as the acid hydrolysis rate. Untreated starch was used as a control in the Figs. 1–3, while 0 day-treated starch was used in Figs. 4 and 5. We confirmed those two starch samples had similar activity by performing the same experiment as that shown in Fig. 1 (data not shown).

2.4. Cell culture

The effects of the starch on a splenocyte culture were evaluated by incubating spleen cells (2×10^6 cells/ml) of DO11.10 with OVA (0 and 10 μ M) and each starch (0.1 and 1 mg/ml) in an RPMI1640 medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10% heat-inactivated fetal calf serum (JRH Bioscience, Lenexa, KS). Each supernatant was collected after 2 days of incubation for use in subsequent cytokine and cytotoxicity assays.

The results shown in Fig. 3 were obtained from spleen cells of DO11.10 (2.5×10^6 cells/ml) and CD90-positive spleen cells (8×10^5 cells/ml) that had been incubated with ConA (2 μ g/ml) and each starch (1 mg/ml). The CD90-positive cells (T cells) in this experiment were isolated from spleen cells by magnetic separation, using CD90 MACS beads and a MACS separation column (Milt-ny Biotec, Bergish Gladbach, Germany).

2.5. Immunization and preparation of the sera

BALB/c mice (5 mice/group) were i.p. immunized with β -LG. The immunization was carried out after, respectively, dissolving β -LG and each starch in PBS at 2 mg/ml and 20 mg/ml. This solution was mixed with an equal volume of an alum adjuvant (20 mg/ml), and 100 μ l was injected into each mouse. In the other experiment, BALB/c mice (5 mice/group) were i.p. immunized with OVA. In this case, OVA and each starch were, respectively, dissolved in PBS at 2 and 20 mg/ml. This solution was then emulsified with an equal volume of complete Freund adjuvant (CFA; Difco Laboratories, Detroit, MI), and 100 μ l was injected into each

Table 1
Progress of the acid treatment.

Treatment	Dissolved saccharides (mg/ml)	Acid hydrolysis rate (%)
0-day	–	–
4-day	2.9	24.8
8-day	3.6	30.8
12-day	8.6	73.5

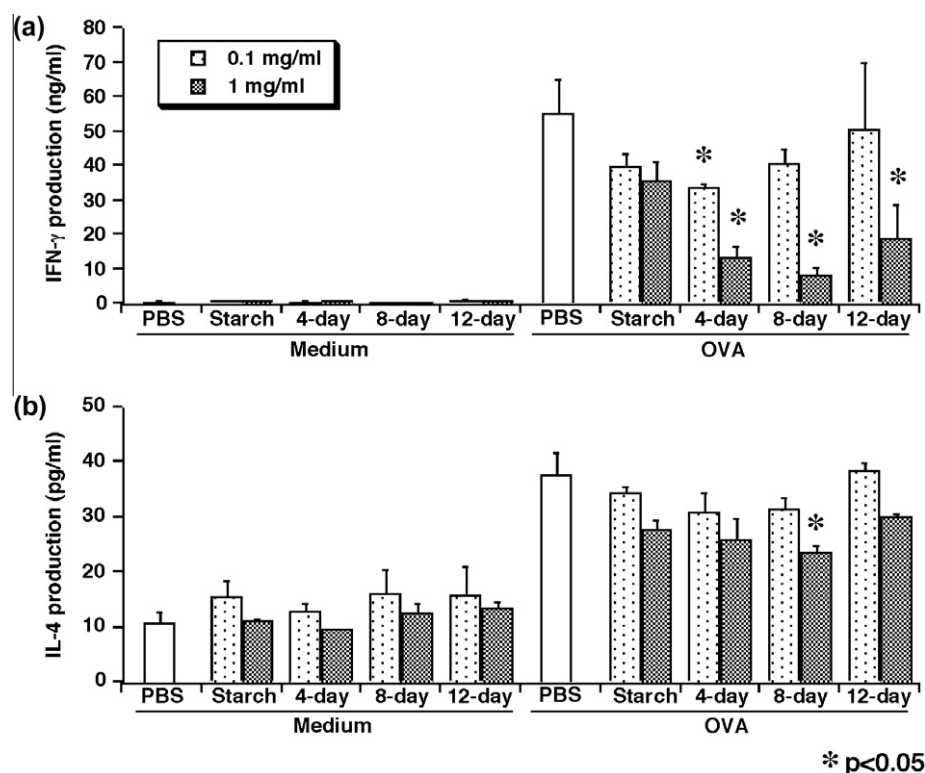


Fig. 1. Effect of acid-treated starch on splenocyte cytokine production *in vitro*. Splenocytes of DO11.10 were cultured with OVA and each starch sample. Each supernatant was collected 2 days later, and the amounts of IFN- γ (a) and IL-4 (b) were measured by ELISA. The results are representative of two independent experiments. *Significant difference compared with the control (PBS) value ($p < 0.05$).

mouse. Two weeks after the immunization, sera were prepared and analyzed for the β -LG- or OVA-specific IgG1, IgG2a and IgE titers by ELISA.

2.6. ELISA

The amounts of IFN- γ and IL-4 in each supernatants were detected by ELISA. In brief, a 96-well plate (Maxisorp, Nunc, Roskilde, Denmark) was coated overnight at 4 °C with purified rat anti-mouse IFN- γ mAb or purified rat anti-mouse IL-4 mAb. Each plate was blocked by 1% bovine serum albumin (Seikagaku Kogyo) in PBS after washing, and the culture supernatant and standard were added to the plate after further washing. After another washing, the bound cytokines were detected by the biotinylated rat anti-mouse IFN- γ mAb or biotinylated rat anti-mouse IL-4 mAb, before being incubated with alkaline phosphatase-streptavidin (Zymed). A *p*-nitrophenyl-phosphate substrate was added after a final washing, and the absorbance was measured at 405 nm.

The amounts of the β -LG- or OVA-specific IgG1, IgG2a and IgE were also measured by ELISA. A 96-well plate was, respectively, coated overnight at 4 °C with 0.01% β -LG or OVA in PBS. Each plate was blocked by 1% OVA or bovine serum albumin (BSA) in PBS after washing. The serum and standard were added to the plates after further washing. After another washing, the bound cytokines were detected by the biotinylated rat anti-mouse IgG1 Ab, biotinylated rat anti-mouse IgG2a Ab or biotinylated rat anti-mouse IgE mAb, before being incubated with alkaline phosphatase-streptavidin. A *p*-nitrophenyl-phosphate substrate was added after a final washing, and the absorbance was measured at 405 nm.

2.7. Cytotoxicity

We measured the lactate dehydrogenase (LDH) activity in the culture supernatant to evaluate the cytotoxicity of the starch to

spleen cells. LDH in the culture supernatant was alienated from those cells with an injured cell membrane. We used cells cultured in the presence of 0.2% Tween 20 as the positive control, and an LDH-Cytotoxic test kit (Wako, Osaka, Japan) was used for this assay.

2.8. Statistics

Differences between the control and starch-treated groups were evaluated by Student's *t*-test, differences being considered as significant at $p < 0.05$.

3. Results

3.1. Sulfuric acid-treated starch inhibited IFN- γ and IL-4 produced by antigen-stimulated spleen cells

Splenocytes of DO11.10 were incubated with OVA and the starch samples to investigate the influence of the sulfuric acid treatment on cytokine production. As shown in Fig. 1a and b, the amounts of IFN- γ and IL-4 in the supernatant were significantly suppressed by the addition of starch to the culture. The effect strongly depended on the time of the sulfuric acid treatment. The activity initially increased as the treatment time was extended, but had decreased in the sample treated for 12 days, indicating that there was an appropriate treatment time to produce starch with strong immune-regulating activity.

3.2. Starch showed hardly any cytotoxic effect

Fig. 2 shows that the LDH activity hardly changed in each supernatant from the culture in which splenocytes had been incubated with each starch sample when compared with the control (no starch), while the LDH activity was significantly increased by a

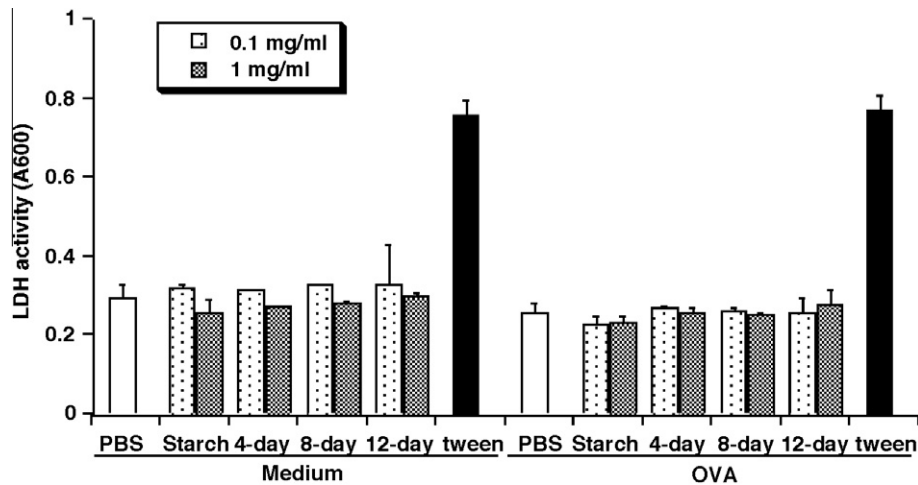


Fig. 2. Cytotoxic effect of acid-treated starch on a splenocyte culture. Splenocytes of DO11.10 were cultured with OVA and each starch sample. Tween 20 (0.2%) was added to the culture instead of the starch as a positive control for the injured cells. Each supernatant was collected 2 days later, and the LDH activity was measured by an LDH–Cytotoxic test kit. The results are representative of two independent experiments.

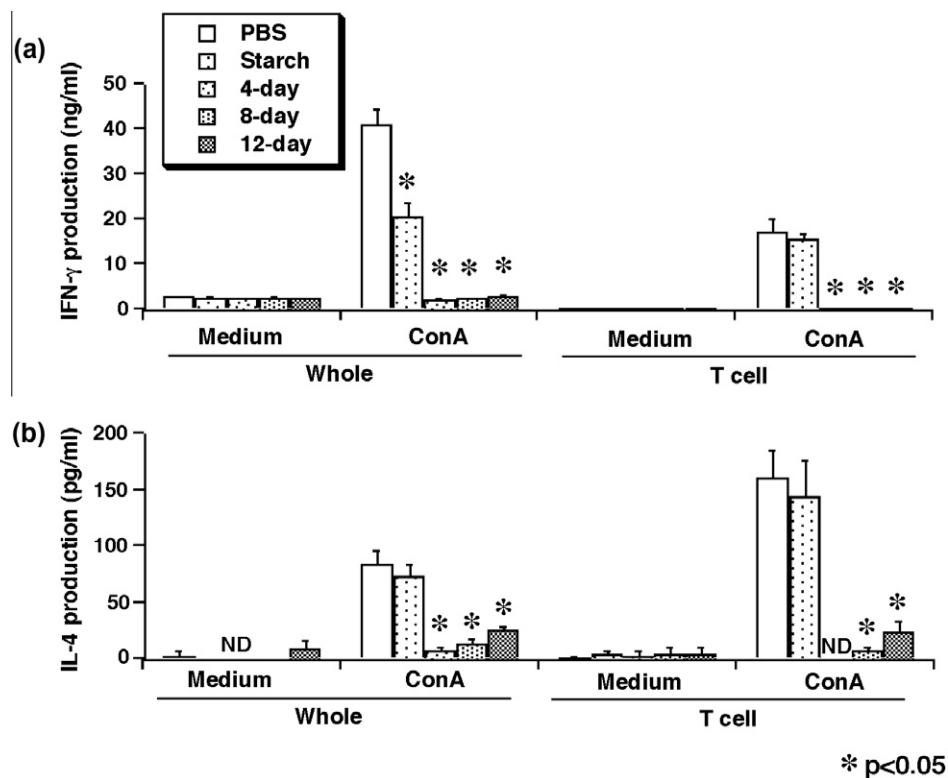


Fig. 3. Effect of acid-treated starch on T cell cytokine production. T cells were purified from the splenocytes of DO11.10 and cultured with ConA and each starch sample. Each supernatant was collected 2 days later, and the amounts of IFN- γ (a) and IL-4 (b) were measured by ELISA. The results are representative of two independent experiments. *Significant difference compared with the control value ($p < 0.05$).

treatment with Tween 20 (positive control). We interpret from this result that the inhibitory effect of the starch on the immune responses could be evaluated by our system without needing to consider any cytotoxic effect.

3.3. Starch directly affected the T cells

We examined the mechanism for the effect of the starch in detail. To investigate the target cells of the starch, T cells were purified from the spleen cells of DO11.10 by the MACS method and then incubated with ConA and the starch. The starch significantly

inhibited the cytokine production from purified T cells as well as that from whole spleen cells (Fig. 3). The activity of the starch depended on the time for the sulfuric acid treatment. We consider from this result that the starch directly affected the T cell response.

3.4. Starch inhibited IgG2a and IgG1 production but not IgE induced by the alum adjuvant, while it enhanced IgG2a and IgG1 production induced by CFA *in vivo*

We then investigated the influence of the starch on antibody production *in vivo*. BALB/c were immunized with the antigen

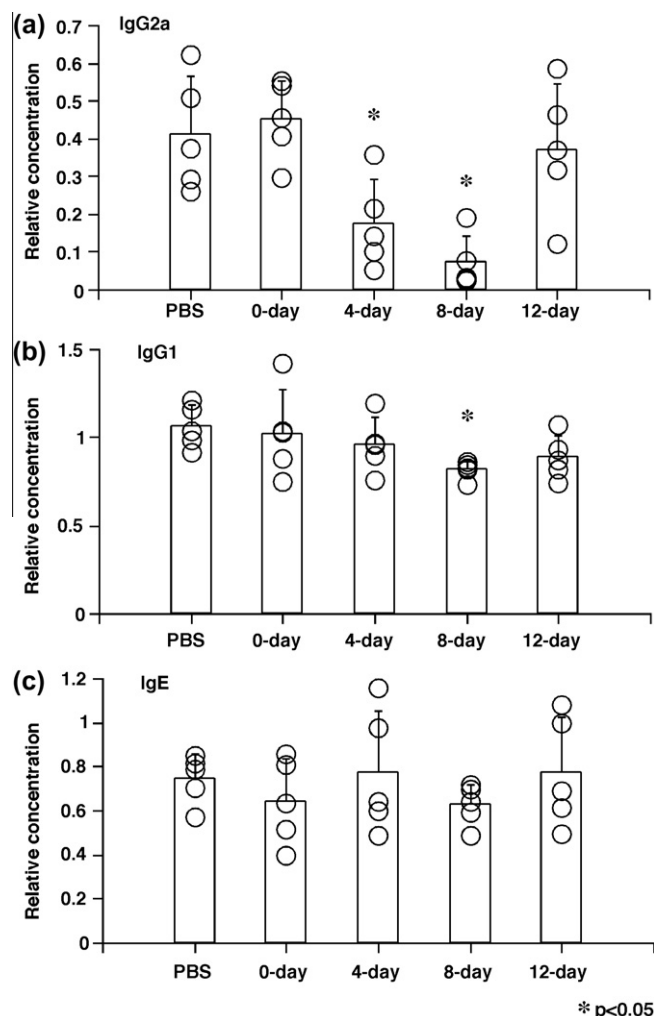


Fig. 4. Effect of acid-treated starch on antibody production when administered with an alum adjuvant *in vivo*. BALB/c mice were immunized with β -LG mixed with each starch sample and the alum adjuvant. Anti-sera were prepared from those mice 2 weeks later. The β -LG-specific IgG2a (a), IgG1 (b) and IgE (c) antibody titers were measured by ELISA. The results are representative of two independent experiments. *Significant difference compared with the control value ($p < 0.05$).

(β -LG) mixed with the starch and alum adjuvant. We only used β -LG as the antigen in this experiment, because we had found that steadier IgE production could be induced by this antigen rather than OVA. The β -LG-specific antibody titer in the serum was measured 2 weeks later. The production of β -LG-specific IgG2a was significantly inhibited by co-administration with the starch (Fig. 4a). IgG1 production was also slightly inhibited (Fig. 4b). The activity of the starch depended on the time for the acid treatment, just like the *in vitro* observation, showing high activity in the samples that had been treated for 4–8 days. In contrast, the production of IgE was not influenced by any of the starch samples (Fig. 4c).

We next tested the influence of the starch on the antibody production induced by another adjuvant. BALB/c were immunized with the antigen (OVA) mixed with the starch and CFA. The OVA-specific antibody titer in the serum was measured 2 weeks later. Interestingly, the production of OVA-specific IgG2a and IgG1 were both enhanced by co-administration with the starch (Fig. 5a and b). This activity was opposite from that obtained when the starch was immunized with the alum adjuvant. The activity of the starch also depended on the treatment time, like the cases already mentioned.

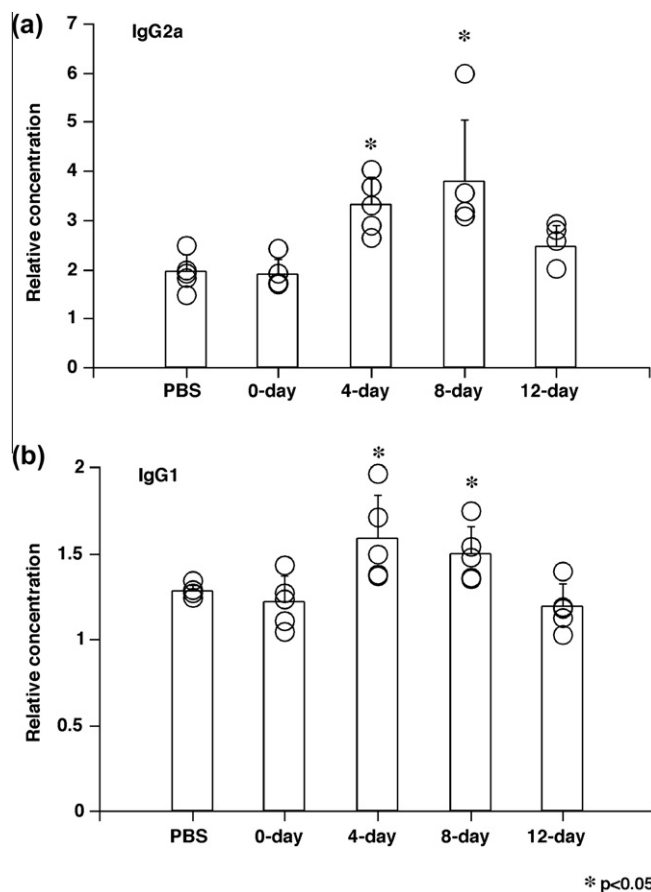


Fig. 5. Effect of acid-treated starch on antibody production when administered with CFA *in vivo*. BALB/c mice were immunized with OVA mixed with each starch sample and CFA. Anti-sera were prepared from those mice 2 weeks later. The OVA-specific IgG2a (a) and IgG1 (b) antibody titers were measured by ELISA. The results are representative of two independent experiments. *Significant difference compared with the control value ($p < 0.05$).

4. Discussion

The objective of this study was to identify whether or not sulfuric acid-treated starch could control the immune responses. We examined in detail the effects of the starch on the T cell response *in vitro* and the antibody production *in vivo*. Our results confirm the data reported in previous studies, showing that α -glucans can regulate immune responses and that the activity depends on the molecular weight. Several sorts of glycogen had been used as α -glucans in those studies, while this present study revealed that starch also has an immune-regulating function depending as well on the molecular weight. We also found in this study that the immune-regulating activity was influenced by the surrounding circumstances. These findings suggest the potential use of a sulfuric acid treatment to generate a novel functional food material with immune-modulating activity.

Recent studies have shown that several food factors can regulate immune responses, and they are expected to be applied for inhibiting allergy, autoimmune diseases, infections and cancer. Some strains of lactic acid bacteria have been reported to inhibit allergic responses and to enhance anti-tumour and anti-virus responses by enhancing the Th1 response (Shida & Nanno, 2008; Yasui, Shida, Matsuzaki, & Yokokura, 1999). Some types of polyphenols, including catechins and isoflavones, have been reported to have anti-allergy or anti-tumour activity by mechanisms different from the action of lactic acid bacteria (Fujimura et al., 2007; Lee, Kishikawa, Kumazoe, Yamada, & Tachibana, 2010; Yuan-jing, Nan-shan, & Lian,

2009). There have also been some studies demonstrating the immune-regulating activity of poly- or oligo-saccharides; for example, β -glucan contained in some mushrooms or fungi has been reported to have anti-tumour activity (Rop, Mlcek, & Jurikova, 2009). Tsuji et al. (2003) have also reported that λ -carageenan had an anti-allergic function, and we have also shown that oligosaccharides of alginic acid obtained from seaweed had anti-allergic activity by enhancing the Th1 response (Yoshida et al., 2004). Glycogens are also one of the poly-saccharides that have been reported to have anti-tumour activity (Takaya et al., 1998). In initial studies, the activity of glycogen had been reported to depend on the origin from which the glycogen had been prepared, considering that the differing activity might have been derived from differences in its structure (Takaya et al., 1998). However, Kakutani et al. (2007) have reported that the activity of glycogen depended on its molecular weight and not its structure. We were particularly interested in the report, because no immune-regulating activity of starch had previously been reported, although both glycogen and starch consist of polymerized glucose. We considered that this difference was due to inappropriate molecular weights, according to Kakutani's report. We therefore decided to examine the activity of starch of differing molecular weight and found that starch with an appropriate molecular weight has an immune-regulating function, like glycogen.

Our present study has demonstrated that the sulfuric acid-treated starch inhibited the T cell response *in vitro*, while it inhibited or enhanced antibody production *in vivo*, depending on the surrounding circumstances. It has been shown in previous reports that glycogen exhibited anti-cancer activity by enhancing immune responses (Ryoyama et al., 2004; Takaya et al., 1998). Kakutani et al. (2007) have reported that glycogen enhanced the production of NO, TNF- α and IL-6 from macrophages *in vitro*. Our result is quite different from theirs, however. Although we need further work to clarify the difference between these two studies, we hypothesize here that both starch and glycogen would have distinct effects on the immune system depending on the surrounding circumstances. Kakutani et al. (2007) used a macrophage cell line by stimulating with IFN- γ , and reported that this treatment was necessary to observe the activity of glycogen.

Our results indicate that starch with an appropriate molecular size preferentially inhibited the Th1 type of antibody *in vivo* when administered with an alum adjuvant. This effect was also apparent on cytokine production when tested *in vitro*. These results are particularly interesting, because food materials which can selectively inhibit the Th1 response have rarely been reported. The Th1 response is important for protecting us from infection, although an over-response of Th1 causes such autoimmune diseases as multiple sclerosis and rheumatoid arthritis. Rheumatoid arthritis in particular is considered to be caused by the collagen-specific IgG2a antibody. Our results lead to the expectation that starch with an appropriate molecular weight might be useful for treating these diseases without having any prominent effect on Th2-type immune responses. We need to study the mechanism for this in more detail.

We have shown in this report that the starch directly inhibited the cytokine production from T cells. A previous study has reported that there was no difference in the adhesion level to the macrophage cell surface among some glycogens with differing molecular weight, although their activities were quite different (Kakutani et al., 2007). It is considered, from these observations, that the adhesion to the cell surface might be *via* some kind of specific receptors not involved in their activities. Further studies are needed to identify the mechanism for starch and glycogen to regulate immune responses, and how the molecular weight affects the activity.

Our results have demonstrated that the activity of the starch strongly depended on the molecular weight, like the case of glycogen. The sulfuric acid treatment of the starch was found to be a

useful method for generating starch with the immune-regulating activity. However, we could not determine the particular size to exhibit the strongest activity. Further work is required to confirm whether the particular size would be common between starch and glycogen. To achieve that, a study to fractionate the starch included in each sample based on the molecular weight is in progress in our laboratory. We considered it would be difficult to determine the particular molecular weight of unfractionated samples, because each sample consists of the starch with wide ranges of molecular weight. We had therefore decided to determine the molecular weight of the starch which has high immune-regulating activity, after fractionating it. In addition, we are planning to prepare the starch samples by enzyme treatment. The acid treatment of starch specifically hydrolyzes amorphous domains of starch, while the effects of enzyme treatment depends on the kind of enzyme. We can therefore obtain the starch with various structure and molecular size by using several kinds of enzyme, although it would be difficult to prepare the same molecule with acid treatment. The study on the immune-regulating activity of those samples would reveal the importance of the molecular size and structure of starch for their function, which has been already investigated about glycogen.

The most interesting finding in this study is the variability of the starch activity according to the surrounding circumstances. The acid-treated starch inhibited immune responses, especially those of the Th1 cytokine and antibody. On the other hand, it enhanced the antibody production when immunized with CFA. An undesired response of Th1 leads to autoimmune diseases or inflammation, while the Th1 response is necessary to protect us from infection and cancer. Our results suggest that starch might inhibit the Th1 response in the steady state, while enhancing the Th1 response cooperating with danger signaling. Kaji, Kiyoshima-Shibata, Nagaoka, Nanno, and Shida (2010) have reported that one of the strains of lactic acid bacteria induced IL-12 production in macrophages, but this activity changed to inducing IL-10, instead of IL-12, when the macrophages were stimulated with the bacteria in the presence of another strain of lactic acid bacteria. This report indicated that the signals from some pattern recognition receptors (PPRs) could be modified by the signals from other PPRs, resulting in completely different responses. Another possibility about the flexibility of the starch activity is more simple. Figs. 1 and 2 show that the effect of starch on the splenocyte responses seemed much stronger when the splenocytes were stimulated by ConA than by OVA. This observation might mean that starch suppressed the T cell responses, while enhancing the antigen-presenting cell responses. Activation of the antigen-presenting cells might therefore be needed for the enhancing effect of starch to overcome the suppressing effect, because Kakutani et al., (2007) have reported that the immune-activating effect of glycogen was only apparent when the macrophage cells were activated by IFN- γ , as already mentioned. The activation of antigen-presenting cells would be not enough to demonstrate an enhancing effect in the case of the *in vitro* experiment and alum experiment. In any case, the mechanism for starch in regulating immune responses should be clarified in order to use it as a novel food material with safety and effectiveness.

We used the starch after autoclaving in this study, so it retained its activity when used for foods, such as bread and pasta. Our results would contribute to increasing the consumption of these foods. However, one major aspect needs to be elucidated. We used in this study a starch sample by mixing with an antigen and adjuvant, and injected i.p. It is considered that starch can be easily digested by enzymes when orally administered; to demonstrate the function as a food material, starch needs to be absorbed from the intestines with maintaining an appropriate molecular weight, thus avoiding this digestion. The activity of starch after its oral

administration must therefore be examined in the next study. Further studies will also be required for determining the appropriate treatment to generate a starch that is effective when orally administered or to develop the delivery system.

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