

**Oral administration of a fruiting body extract of *Boletopsis leucomelas* enhances intestinal IgA production in LPS-challenged mice**

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**Running head: *B. leucomelas* extract enhances IgA**

## **Abstract**

The present study showed that a hot water extract of the fruiting body of the edible mushroom *Boletopsis leucomelas*, known as “*Kurokawa*” Japanese, strongly stimulated IgA-production in mouse spleen cells in our screening experiment. The *in vivo* study was also conducted with the objective of enhancing adaptive immune response by oral administration of the hot water extract of *B. leucomelas* (BLE) in LPS-challenged mice. The mice were fed a standard diet with or without 0.16% BLE. The mice were also orally administered PBS or LPS weekly at days 7, 14 and 21. Results indicated that LPS-specific serum IgG, IgM and IgA were increased in the BLE diet group compared to the standard diet group. Interestingly, intestinal total IgA and LPS-specific IgA were significantly increased in the BLE diet group. Moreover, the <1,000 Da, 5,000-10,000 Da phenol sulfate-positive molecules from BLE showed significant IgA-producing activity.

**Keywords;** *B. leucomelas*, *Kurokawa*, LPS challenge, IgA, adaptive immunity

## Introduction

Edible mushrooms are used as a flavorful food and health-promoting food supplement, and as a drug in limited geographic regions. Numerous studies have shown that certain mushrooms contain bioactive compounds that exhibit anti-tumor properties [Jeong et al., 2012; Lavi et al., 2012]. Recently, with the remarkable increase in research on the effects of mushrooms on immunity, new tools have become available enabling novel approaches in cancer treatment, which exploit the specificity of the immune system. However, little data is available on the efficacy of mushrooms in mucosal immunity. The adaptive mucosal immune defense is largely mediated by secretory IgA (sIgA), which is the predominant class of immunoglobulins [Macpherson et al., 2011]. The function of sIgA in mucosal defense is to perform immune exclusion of potentially harmful pathogens and antigens, and contribute to immunologic homeostasis [Renz et al., 2011]. In particular, enhancement of the adaptive immune response and intestinal IgA production could be helpful in protecting against infection and pathogenic antigens such as lipopolysaccharide (LPS), a component of the Gram-negative bacterial cell wall [Renz et al., 2011]. The development of such a common mucosal immune system appears to be supported by experimental studies demonstrating that oral intake of heat-killed lactic acid bacteria [Shimosato et al., 2011; Tobita et al., 2010] and a dietary casein phosphopeptide [Otani et al., 2003] can lead to the induction of sIgA in the gut. However, no studies have been conducted to determine the effect of edible mushroom consumption on IgA production. The aim of the present study was to investigate intestinal immune regulation in LPS-challenged mice fed a diet with or without the edible mushroom *Boletopsis leucomelas* (known as “Kurokawa” in Japanese) extract (BLE).

## **Materials and methods**

### **Materials**

LPS derived from *Salmonella typhimurium* endotoxin was purchased from Difco Laboratories (Detroit, MI). Horseradish peroxidase (HRP)-labeled anti-mouse IgA, IgG, IgM and IgE were obtained from Bethyl Laboratories (Montgomery, TX). Defined fetal bovine serum (FBS) was obtained from HyClone Laboratories (Road Logan, UT). Penicillin was purchased from MP Biomedicals (Costa Mesa, CA). RPMI-1640 was purchased from Nissui Pharmaceutical (Tokyo, Japan). TMB (tetramethyl benzidine) was purchased from KPL (Gaithersburg, MD). All chemicals used in this study were of the highest analytical grade commercially available.

### **Hot water mushroom extracts**

Supplemental Table 1 lists the mushroom strains used in this study. The wild-type mushrooms were collected from the forests of Nagano Prefecture, Japan. The cultivated mushrooms were purchased from the market in Kamiina, Japan or a mushroom company (JA Kamiina, Kamiina, Japan). The mushrooms (100 g) were cut into approximately 10 x 10 mm pieces and were subsequently ground with sea sand (30 g per 1 g of mushrooms) for 20 min, after which the ground mushrooms were suspended in 500 mL boiled distilled water for 1 min. After filtering the extract through a layer of cloth, the filtrate was centrifuged **at 5,000 x g for 30 min**. The resulting supernatant was freeze-dried to obtain the hot water mushroom extracts.

### **Spleen cell suspensions and cell cultures**

Pathogen-free male C3H/HeN mice (4 weeks of age) were purchased from

Japan SLC (Shizuoka, Japan). After preliminary breeding for 2 weeks, mouse spleen cells were prepared using standard methods [Shimosato et al., 2010]. The spleen tissue was then homogenized in RPMI-1640 medium containing 5% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. The resulting cell suspension was washed three times in this medium and adjusted to  $1 \times 10^6$  viable cells/mL. The cell suspension (1 mL) was then plated into the wells of a 24-well flat-bottom plate (Sarstedt, Inc., Newton, NC) and the *B. leucomelas* extract (BLE) solution was added at a final concentration of 0, 10, 50 or 100 µg/mL. The cells were cultured at 37 °C in a humidified 5% CO<sub>2</sub> incubator for 72 hr for immunoglobulin ELISA.

### **Mitogenicity assay**

BLE-mediated mitogenic activity was assessed using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays [Ito et al., 2013]. Cells were seeded in 96-well plates at a density of  $6 \times 10^5$  cells/well. The cultures were then exposed to BLE (0, 10, 50, and 100 µg/mL) with or without 50 ng/mL LPS. An MTT assay was performed on cells that had been cultured with BLE as described above. Briefly, 100 µL of media containing MTT (Sigma-Aldrich, St. Louis, MO, USA; 0.5 mg/ml) was added to the cells for 2 hr. Non-internalized MTT was then washed away, and the cells lysed by the addition of 50 µL DMSO. This released the MTT internalized by viable cells. MTT concentration was measured colorimetrically, and cell viability was determined as the OD at 570 nm of treated/untreated cultures.

### **Feeding procedure**

Pathogen-free male C3H/HeN mice (4 weeks of age) were purchased from

Japan SLC. The mice were housed at  $23 \pm 2$  °C under a standard 12 h light/dark cycle. Mice were given a commercial mouse pellet feed (MF, Oriental Yeast Company, Tokyo, Japan) for a week. Then, mice were given a defined protein-free diet, 'PM15765' (Purina Mills, St. Louis, MO, USA) supplemented with approximately 25% ovalbumin (OVA) as a protein source and sterile water *ad libitum* [Shimosato et al., 2011, Shigemori et al., 2013]. After preliminary breeding for 1 week, mice were fed standard diet with or without 0.16% BLE (Supplemental Table 2). The mice were also orally administered 200  $\mu$ L PBS or 200  $\mu$ L PBS containing 10  $\mu$ g LPS weekly at days 7, 14 and 21. Five weeks later, all mice were sacrificed by separating the cervical vertebrae and organs were extracted for analysis of immune responses. The treatment schedule is presented in **Fig. 2**. Blood samples were successively collected from the heart of sacrificed mice. The composition of the test diets used is listed in Supplemental Table 1. Control diet+PBS (CP group,  $n=4$ ); Control diet+LPS (CL group,  $n=4$ ); BLE diet+LPS (BL group,  $n=4$ ).

### **Preparation of intestinal extract**

Samples (1 g) of intestinal tract washes (duodenum to rectum) were ground using a pestle for 20 min at  $2 \pm 1$ °C with sea sand (1 g) in 2.5 mL of 0.01 M sodium phosphate buffer (PBS, pH 7.2) containing 0.15 M sodium chloride. The ground material was then centrifuged at **1,200 x g for 30 min** at 4 °C and the supernatant collected.

### **Immunoglobulin assay**

Total IgA level was measured using a mouse enzyme-linked immunosorbent

assay (ELISA) quantitation kit (Bethyl Laboratories) as described previously [Macpherson et al., 2011]. IgA-producing activity (%) was calculated using the following formula: (%) =  $100 \times \frac{[\text{ELISA value (A490 nm) treated with mushroom extract (100 } \mu\text{g/mL)} - \text{ELISA value (A490 nm) treated with PBS}]}{\text{ELISA value (A490 nm) treated with PBS}}$ . The indirect ELISA previously reported by Otani et al. was used to determine the levels of IgG, IgM, IgA, and IgE specific to LPS from *S. typhimurium* [Shimosato et al., 2012]. The antibody level was represented as an antibody index calculated by the following formula: antibody level = ELISA value (A450 nm) x dilution-fold of the test sample.

### **Ultrafiltration and fractionation of BLE**

An aliquot (100 ml) of the hot water extract (derived from 100 g of raw *Kurokawa*) was concentrated by ultrafiltration using 1,000, 5,000, and 10,000 cut-off membrane disc filters (Ultrafiltration disk membranes, Millipore Co., Billerica, MA, USA). The BLE was chromatographed on a DEAE-Sephadex A-50 column (either in the phosphate or chloride ion form; Amersham Pharmacia Biotech AB, Uppsala, Sweden) at 2-4°C. The Sephadex column had been pre-equilibrated with 0.1 M phosphate buffer (pH 8.0) at 2-4°C before application of the BLE. After adsorption of the sample and washing with the above buffer, the bound proteins were eluted with 200 mM NaCl in 0.1 M phosphate buffer (pH 8.0). Flow rates of 25 mL/hr were obtained for  $\phi 3.0 \times 31$  cm columns. The elution was monitored by absorbance at 280 nm by UV1200 UV-VIS Spectro-Photo-Meter (Shimadzu Co., Kyoto, Japan). Fractions (5.0 mL) were collected using an automatic fraction collector. The dialyzed solution was freeze-dried, stored at -35°C and used within a week for IgA production assays (sample:

10 µg/mL).

### **Gel filtration of F-3 fraction**

**The obtained F-3 fraction by DEAE column was applied to a Bio-Gel P-10 column (φ2.5×100 cm) and eluted with distilled water at 2-4°C.** Fractions (5.0 mL) were collected with an automatic fraction collector, at a flow rate of 20 mL/hr. Absorbance at 220 nm or 490 nm, after color development with the phenol-sulfuric acid reaction, was determined for alternate fractions with a UV1200 UV-VIS Spectro-Photo-Meter. Fractions were stored at -35°C in plastic tubes and used within a week for IgA production assays (sample: 10 µg/mL).

### **Statistical analysis**

Statistical analyses were performed using MedCalc, version 9.3.7.0 (MedCalc Software, Mariakerke, Belgium) and S-Plus (Version 7, Insightful Corp, Miami, FL, USA). Differences between groups were assessed using a one-way analysis of variance (ANOVA) with *Bonferroni's* post-hoc test to control for type I error. All tests were two-sided; probability values less than 0.05 were considered significant.

## **Results**

### **IgA-producing activity and mitogenic activity of BLE**

Supplemental Table 1 shows that the hot water extract of *B. leucomelas* exhibited strong IgA-producing activity in mice spleen cell cultures compared to the other mushrooms. To investigate the immunostimulatory activity of BLE, we examined its mitogenic activity in mouse spleen cell cultures. The MTT values of 50 and 100

µg/mL BLE were significantly higher than that of medium control (**Fig. 1A**). We also examined its mitogenic activity in LPS-treated cells. The result shows that 50 and 100 µg/mL BLE can induce a strong immune response in LPS-treated cells (Fig. 1 B).

### **Body weight changes**

We subsequently examined IgA-producing activity in LPS-challenged mice fed the standard diet with or without 0.16% BLE for 35 days. The mice were also orally administered PBS or PBS containing LPS weekly at days 7, 14 and 21. The body weight of mice was monitored during the experiment in order to investigate the effect of the test diets on changes in body weight. BLE and LPS shows no influence on nutritive function. Acute toxicity was not observed in this study. There were no significant differences in the body weight of mice fed approximately the same level of protein, whether or not they received BLE and LPS (data not shown).

### **Serum and intestinal LPS-specific antibody production in LPS-challenged mice orally treated with BLE**

LPS-specific IgA, IgG, IgM and IgE levels were measured by indirect ELISA. The LPS-specific IgG, IgA from serum (**Supplemental Fig. 1A, Fig. 3**), total IgA, LPS-specific IgA, IgG and IgM from the intestinal extract (**Fig. 4A, B, Supplemental Fig. 2A, B**) of CL and BL group mice that were orally challenged with 10 µg LPS/mouse were significantly ( $P<0.01$ ) higher than the CP group. Interestingly, serum LPS-specific IgG, IgM, IgA (**Supplemental Fig. 1A, B, Fig. 3**), and intestinal LPS-specific IgA of the BL group were significantly ( $P<0.01$ ) higher than in the CL group (**Fig. 4B**). Total IgA was also significantly ( $P<0.01$ ) increased in the BL group compared with the CL

group (**Fig. 4A**), whereas no differences were found in intestinal LPS-specific IgG and IgM levels with or without BLE (**Supplemental Fig. 2A, B**). Intestinal IgA plays a principal role in the intestinal immune system; it prevents infection at the early stages by excluding bacteria and viruses from the gastrointestinal tract [Hocini & Bomsel 1999; Geuking et al., 2012; Santaolalla & Abreu 2012]. These results suggest that the BLE diet induces more LPS-specific IgA production in the intestine compared to the control diets. Moreover, BLE intake significantly enhanced the intestinal total IgA level in the LPS-challenged control diet group as well as the standard group.

#### **IgA-producing activity of <1,000 Da glycopeptides and 5,000-10,000 Da glycoproteins from BLE**

**Fig. 5** shows that peptides with a MW less than 1,000 Da and that around 5,000-10,000 Da induced greater IgA-production than that of the other peptide sizes. BLE was fractionated into five peaks using DEAE-Sephadex A-50 ion exchange chromatography (**Fig. 6A**). The first 2 peaks (F-1 and F-2), which were not retained by the column, had no effect on IgA production (**Fig. 6B**). The second 3 retained peaks (F-3, F-4, F-5), which were eluted with 200 mM sodium chloride, enhanced IgA production (**Fig. 6A**). The F-3 fraction was applied to a Bio-Gel P-10 column and eluted with distilled water at 2-4 °C. Absorbance at 220 nm or 490 nm, after color development using the phenol-sulfuric acid reaction, was determined for alternate fractions. Glycopeptides and glycoproteins can be accurately determined by the colorimetric phenol-sulfuric acid method at 490 nm. Fractionation of F-3 yielded five fractions (**Fig. 7A**), the high-molecular-weight fractions (F-6, F-7) that emerged at the void volume and the low-molecular-weight fractions that emerged at the bed volume (F-

8, F-9, F-10), representing a mixture of small molecules (**Fig. 7A**). Fractions F-6, 7, 9 and 10 of the absorption peaks at 220 nm and 490 nm significantly enhanced IgA production (**Fig. 7B**). These results suggest that the IgA-producing molecules from BLE are glycopeptides (<1,000 Da) and non-polysaccharide glycoproteins (around 5,000-10,000 Da), because the carbohydrate composition of the 70% ethanol-insoluble precipitates from *B. leucomelas* reduced IgA-producing activity (data not shown).

## Discussion

Previously, extracts from various mushrooms have been reported to have anti-tumor cell proliferation activity [Koyama et al., 2002; Yue et al., 2012; Zhu et al., 2008]. Specifically, Lavi et al. showed that an extract of the edible mushroom *Pleurotus pulmonarius* inhibited colitis-associated colon carcinogenesis induced in mice, through the modulation of cell proliferation, induction of apoptosis, and inhibition of inflammation [Lavi et al., 2012]. However, there was previously no evidence of an IgA response after ingestion of mushroom extract. This is the first report to describe a mushroom extract with IgA-inducing activity. In this study, the oral administration of BLE enhanced intestinal IgA production in LPS-challenged mice. We propose that the oral ingestion of BLE stimulates certain natural cellular immune systems, while enhancing adaptive immune responses in LPS-challenged mice. We also propose that the IgA-producing molecules from BLE are glycopeptides and non-polysaccharide glycoproteins. The increase in IgA may have the potential to improve intestinal mucosal immunity. The IgA response is promoted by the regulatory activities of helper T-cell type 2 cytokines, integrins, and chemokines secreted within the tissues of the mucosal immune system, linking the gut and other mucosal sites, such as the mammary glands,

through the traffic of effector cells [Koyasu & Moro 2012; Mora & Von Andrian 2008; Schippers et al., 2012]. Exploiting this property may also prove useful in the design and production of new physiologically functional foods using BLE. Further studies are required to reveal in detail the IgA-producing molecules of BLE. Recent studies have shown that mushrooms contain a wide range of soluble polysaccharides capable of activating macrophages, a key event for effective innate and adaptive immunity [Chandrasekaran et al., 2011; Sun et al., 2012; Thompson et al., 2010]. It is important to determine the significance of such changes induced by a dietary intake of BLE on the overall defense capacity of the intestine and how this could lead to increased protection against the risk of infections, such as upper intestinal illness, in susceptible populations.

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## Figure legends

**Fig. 1.** Mitogenic activity of BLE in mouse spleen cells treated with or without 50 ng/mL LPS (A) *in vitro*. Mitogenic activity was examined in the presence of 10, 50, and 100 µg/mL extract. Effect of BLE (10, 50, and 100 µg/mL) on the production of IgA in mouse spleen cell cultures (B). The IgA level was determined by ELISA. Values represent means, and error bars indicate the standard deviations. \*\*\* $P < 0.001$ , \*\* $P < 0.01$  and \* $P < 0.05$  versus cells cultured in the absence of extract. All assays were conducted in a minimum of triplicate using three separate culture wells per experiment. Similar results were obtained from at least three different mice.

**Fig. 2.** Schedule for the oral administration of the test diets. After preliminary breeding for 1 week, mice were fed standard diet with or without 0.16% BLE. The mice were orally administered PBS or PBS containing 10 µg LPS weekly at days 7, 14 and 21. The body weight of mice was monitored during the experiment. Five weeks later, all mice were sacrificed and spleen and blood samples extracted for analysis of antibody production. Grouping: 1. Control+PBS (CP) ( $n=4$ ), 2. Control+LPS (CL) ( $n=4$ ), 3. BLE+LPS (BL) ( $n=4$ ). Group 2 and 3: Oral administration of LPS 10 µg/mouse at days 7, 14, and 21. BW: Body weight.

**Fig. 3.** Serum LPS-specific IgA levels of mice fed diets containing 0.16% BLE for 35 days and treated with 10 µg LPS/mouse at days 7, 14, and 21. The antibody levels were determined by indirect ELISA. The data are presented as the mean  $\pm$  SD ( $n = 3$ ). Items indicated with different letters (i.e., *a*, *b*, *c*) are significantly different ( $P < 0.01$ ). All assays were conducted in a minimum of triplicate using

**three separate culture wells per experiment. Similar results were obtained from at least three different mice.**

**Fig. 4.** Intestinal total IgA (A) or LPS-specific IgA (B), LPS-specific IgG (C) and LPS-specific IgM (D) levels of mice fed diets containing 0.16% BLE for 35 days and treated with LPS on days 7, 14, and 21. Antibody levels were determined by indirect ELISA. The data are presented as the mean  $\pm$  SD ( $n = 3$ ). Items indicated with different letters (i.e., *a*, *b*, *c*) are significantly different ( $P < 0.01$ ). All assays were conducted in a minimum of triplicate using three separate culture wells per experiment. Similar results were obtained from at least three different mice.

**Fig. 5.** Effect of each ultrafiltered BLE fraction on mouse spleen cell IgA production. \*\*\* $P < 0.001$  (compared to the control, 0  $\mu\text{g/mL}$ ). All assays were conducted in a minimum of triplicate using three separate culture wells per experiment. Similar results were obtained from at least three different mice.

**Fig. 6.** Fractionation of BLE using DEAE-Sephadex A-50 column chromatography (A) and IgA production assay of the fractionated substance (B). The data are presented as the mean  $\pm$  SD ( $n = 3$ ). \*\*\* $P < 0.001$ , \*\* $P < 0.01$  (compared to the control, 0  $\mu\text{g/mL}$ ). All assays were conducted in a minimum of triplicate using three separate culture wells per experiment. Similar results were obtained from at least three different mice. **280 nm (black diamond).**

**Fig. 7.** Gel filtration of F-3 fraction (Fig. 5A) on a Bio gel P-10 column (A). The eluate

was collected in 5.0 mL fractions and assayed for IgA production (ng/mL) in mouse spleen cells (B). The data are presented as the mean  $\pm$  SD ( $n = 3$ ). \*\*\* $P < 0.001$ , \*\* $P < 0.01$  (compared to the control, 0  $\mu\text{g/mL}$ ). All assays were conducted in a minimum of triplicate using three separate culture wells per experiment. Similar results were obtained from at least three different mice. Vo, void volume. **220 nm (white diamond); 490 nm (black diamond).**

**Supplemental Fig. 1. Serum LPS-specific IgG (A), IgM (B), and IgE (C) levels of mice fed diets containing 0.16% BLE for 35 days and treated with 10  $\mu\text{g}$  LPS/mouse at days 7, 14, and 21. The antibody levels were determined by indirect ELISA. The data are presented as the mean  $\pm$  SD ( $n = 3$ ). Items indicated with different letters (i.e., *a*, *b*, *c*) are significantly different ( $P < 0.01$ ). All assays were conducted in a minimum of triplicate using three separate culture wells per experiment. Similar results were obtained from at least three different mice.**

**Supplemental Fig. 2. Intestinal LPS-specific IgG (A) and LPS-specific IgM (B) levels of mice fed diets containing 0.16% BLE for 35 days and treated with LPS on days 7, 14, and 21. Antibody levels were determined by indirect ELISA. The data are presented as the mean  $\pm$  SD ( $n = 3$ ). Items indicated with different letters (i.e., *a*, *b*) are significantly different ( $P < 0.01$ ). All assays were conducted in a minimum of triplicate using three separate culture wells per experiment. Similar results were obtained from at least three different mice.**

## Supplemental Table 1

**Table 1. Mushroom samples and IgA-producing activity**

Scientific name	Japanese name	IgA-producing activity <sup>1)</sup>	Source
<i>Hypsizygus marmoreus</i>	Bunashimeji	None	JA-Kamiina
<i>Pleurotus eryngii</i>	Eringi	++	JA-Kamiina
<i>Pleurotus ostreatus</i>	Hiratake	None	JA-Kamiina
<i>Pholiota nameko</i>	Nameko	None	JA-Kamiina
<i>Tlammulina velutipes</i>	Enokitake	None	JA-Kamiina
<i>Hericium erinaceus</i>	Yamabushitake	None	JA-Kamiina
<i>Lentinus edodes</i>	Shiitake	None	Shinano-ransyouen
<i>Bunapi shimeji</i>	Bunapi	None	Hokuto Co.Ltd.
<i>Agaricus blazei Murrill</i>	Agarikusutake	None	wild
<i>Rozites caperatus</i>	Syougenji	+	wild
<i>Tricholoma protentosum</i>	Shimofuri-shimeji	None	wild
<i>Ramaria botrytis</i>	Houkitake	None	wild
<i>Pleurotus ostreatus</i>	Hiratake	None	wild
<i>Boletopsis leucomelas</i>	Kurokawa	+++	wild
<i>Sarcodon imbricatus</i>	Koutake	++	wild
<i>Sarcodon aspratus</i>	Shishitake	++	wild
<i>Entoloma sarcopum</i>	Urabenihoteishimeji	None	wild
<i>Suillus laricinus</i>	Shironumeri-iguchi	None	wild

<sup>1)</sup> IgA-producing activity; '+++>50%, '++>10%, '+>5%, 'none' > not detected.

## Supplemental Table 2

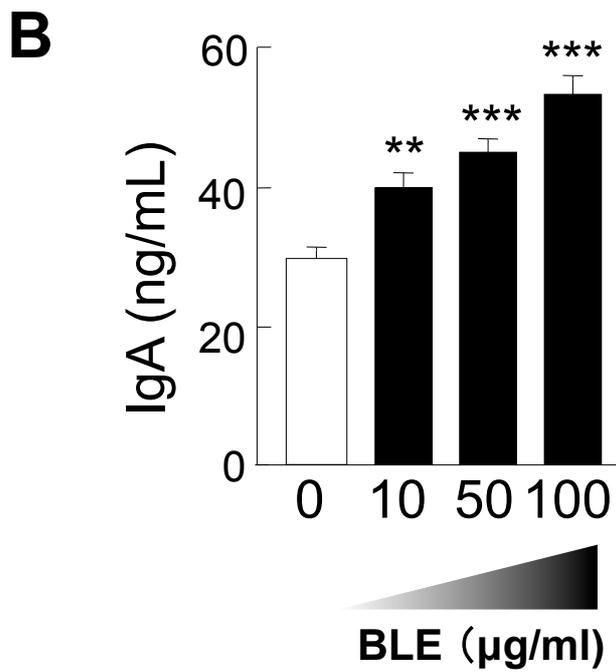
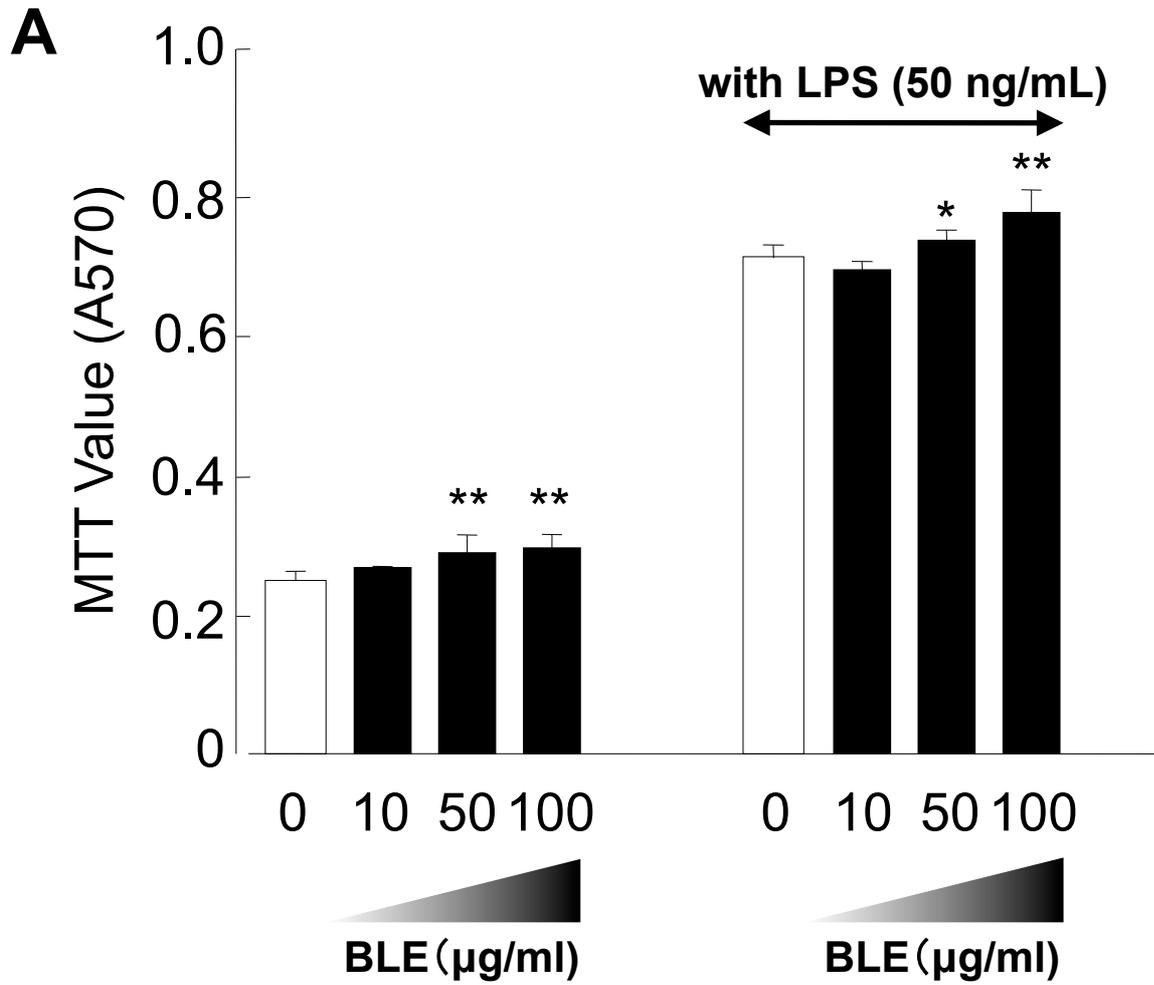
**Table 2. Composition of the test diets (%)**

	Control diet (%)	BLE diet (%)
BLE	None	0.16
OVA	25.00	24.97
Sucrose	27.11	27.08
Solka Floc	2.25	2.24
RP vitamini mix*	1.50	1.49
RP mineral mix #10**	3.75	3.74
Choline chloride	0.15	0.14
Lard	3.75	3.74
Corn oil	3.75	3.74
Dextrin	32.74	32.70
Total	100.00	100.00

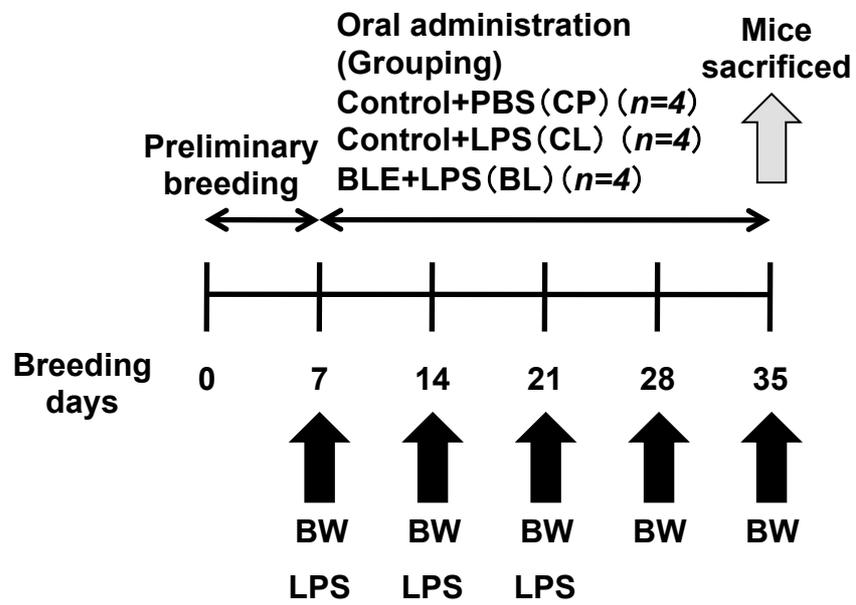
\*RP vitamin mix: thiamin hydrochloride, 20 ppm; riboflavin, 20 ppm; nicotinic acid, 90 ppm; pyridoxine hydrochloride, 20 ppm; d-calcium pantothenate, 60 ppm; folic acid, 4.0 ppm; biotin, 0.4 ppm; i-inositol, 200 ppm; vitamin B12, 20µg/kg; menadione dimethylpyrimidinol bisulfite, 20 ppm; vitamin A acetate, 22 IU/g; vitamin D3, 2.2 IU/g; dl-alpha tocopheryl acetate, 50 IU/kg.

\*\*RP mineral mix #10: calcium, 0.60%; phosphorus, 0.40%; potassium, 0.40%; magnesium, 0.065%; sodium, 0.20%; chlorine, 0.20%; fluorine, 5.0 ppm; iron, 60 ppm; zinc, 20 ppm; manganese, 65 ppm; copper, 15 ppm; cobalt, 3.2 ppm; iodine, 0.6 ppm; chromium, 3.0 ppm; molybdenum, 0.8 ppm; selenium, 0.2 ppm.

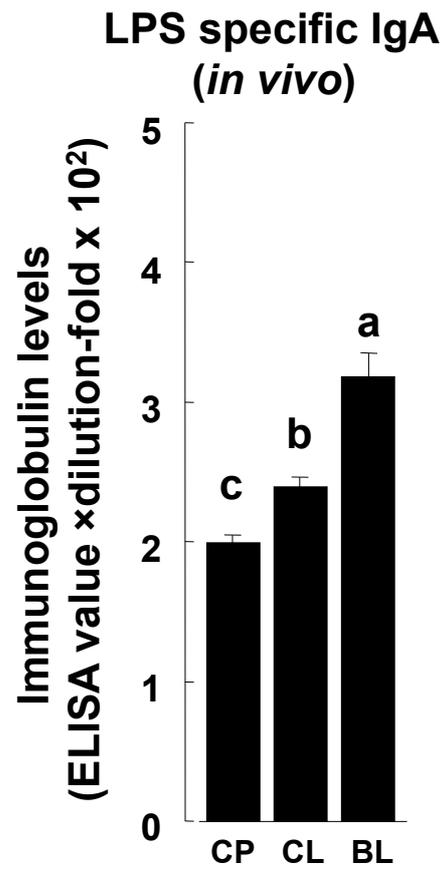
**Figure 1(revise)**



**Figure 2(revise)**



**Figure 3(revise)**



**Figure 4(revise)**

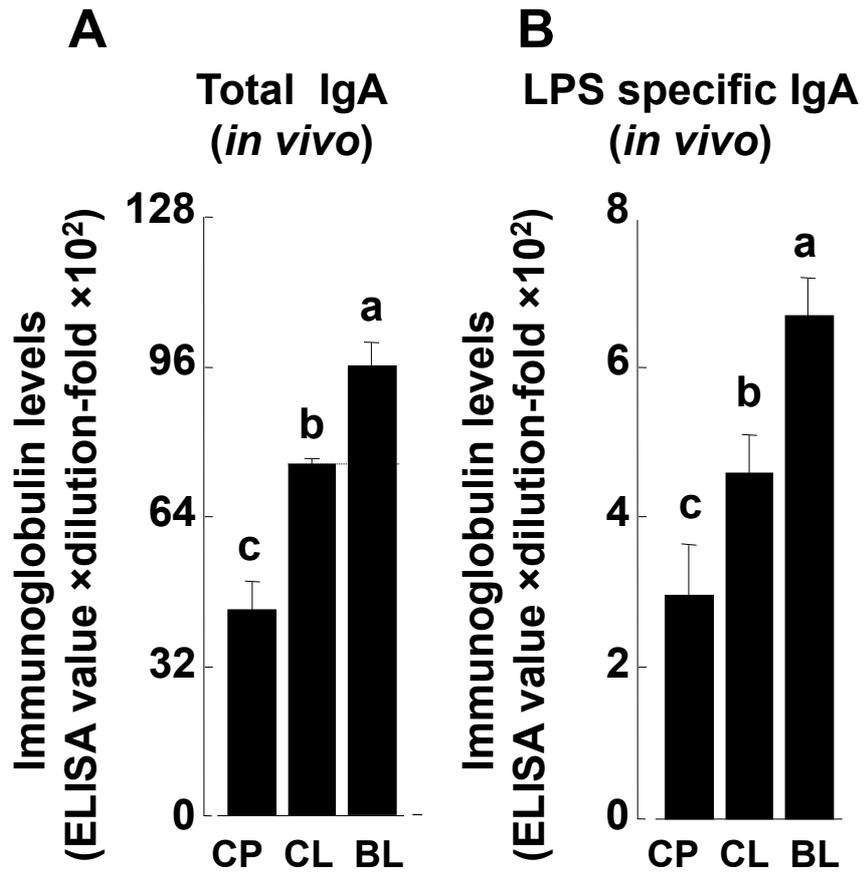


Figure 5(revise)

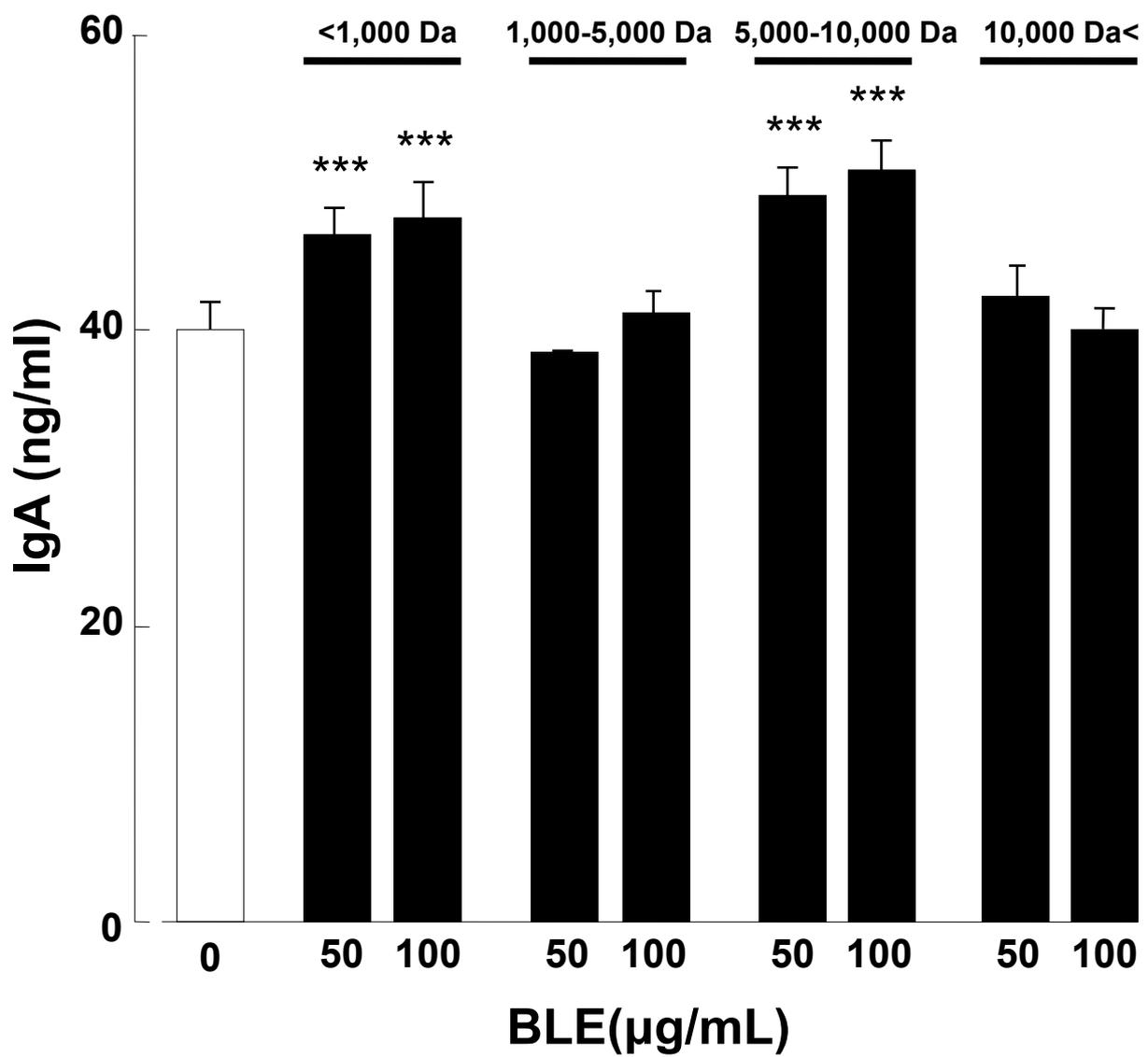
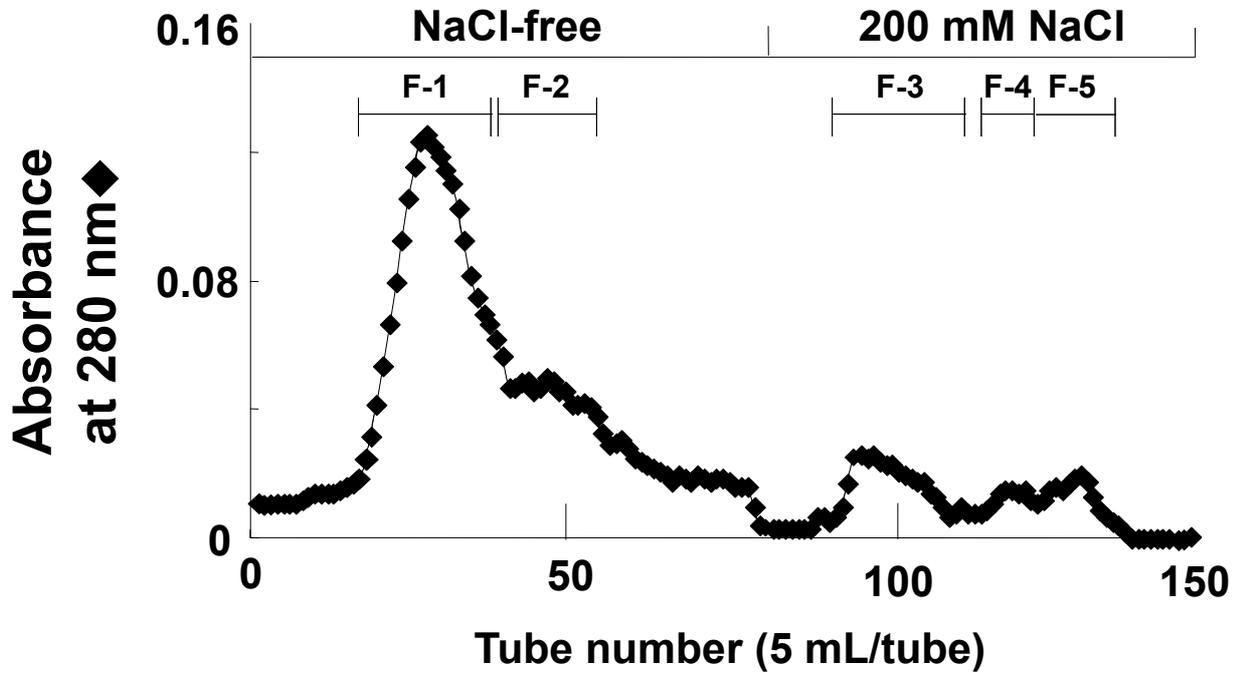


Figure 6(revise)

**A**



**B**

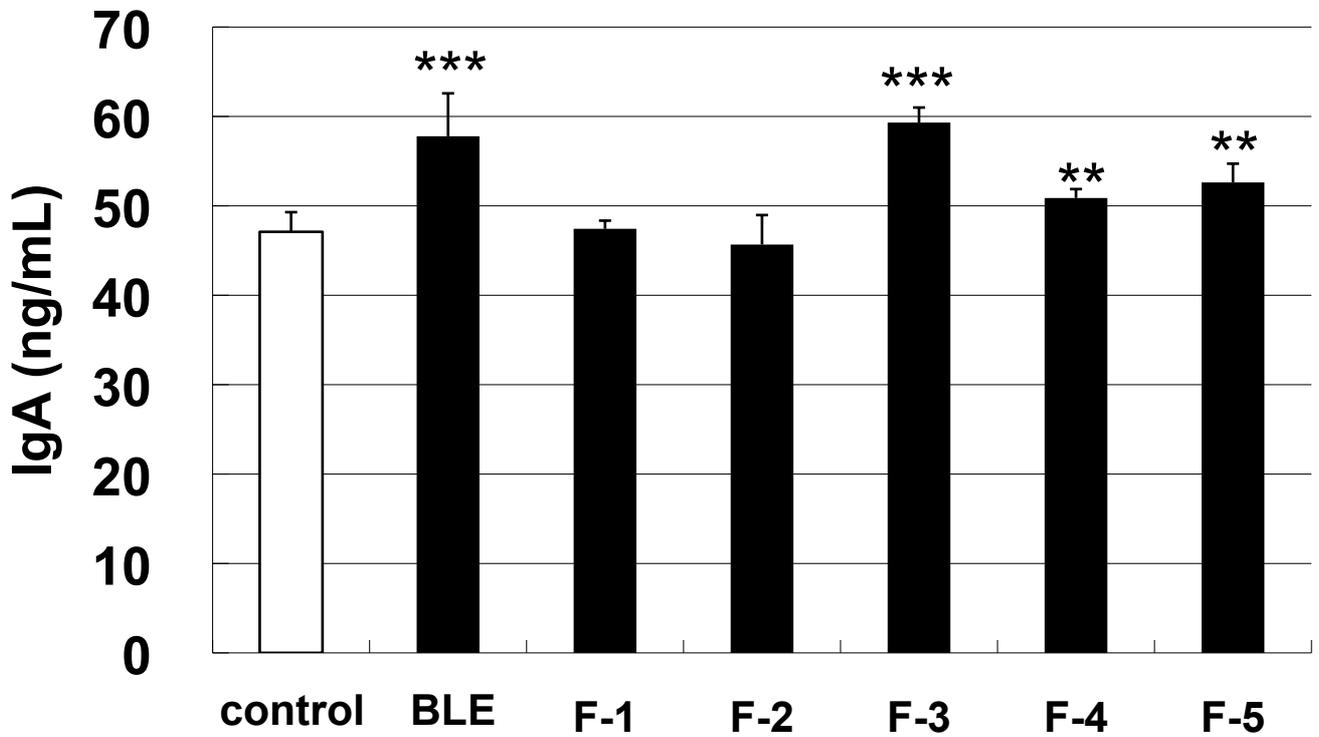
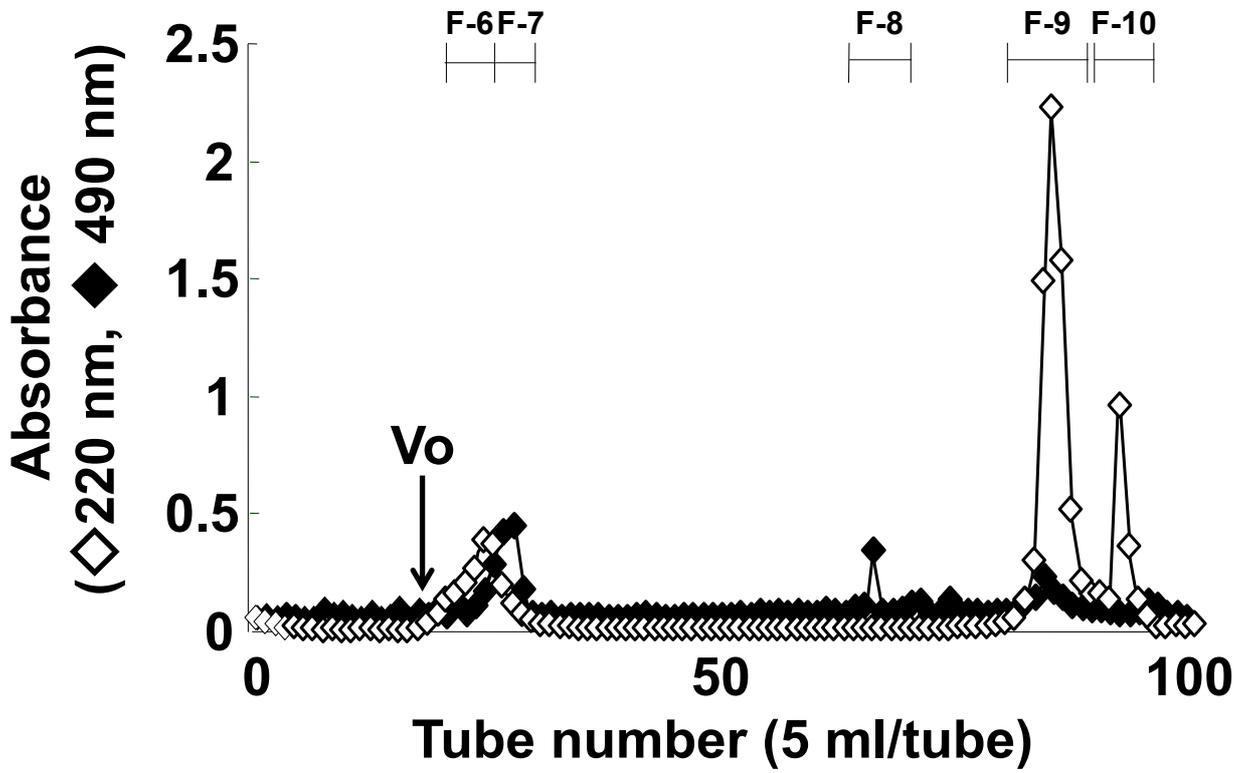
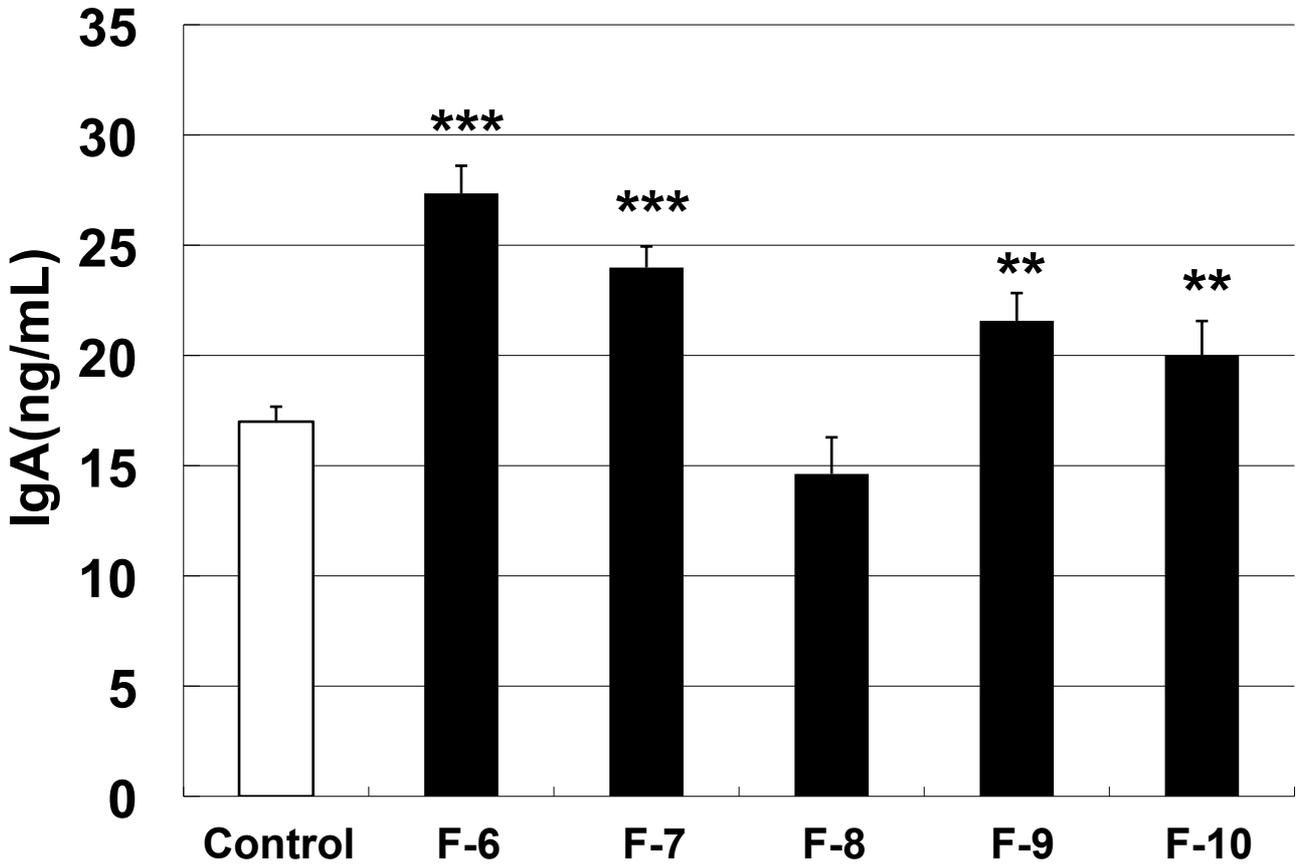


Figure 7(revise)

**A**

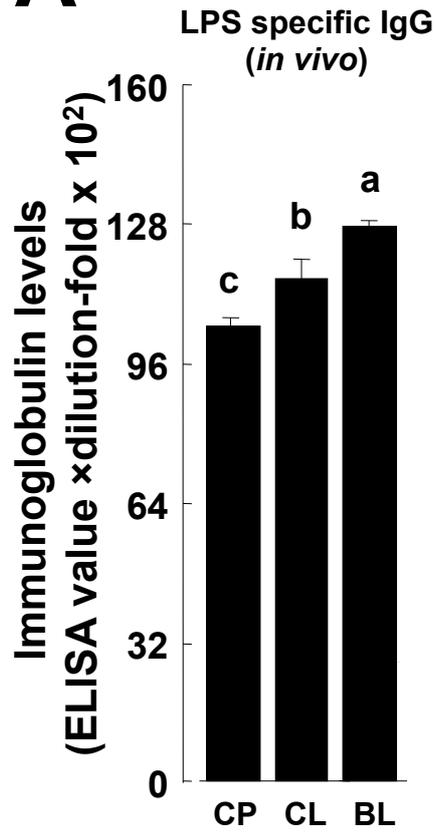


**B**

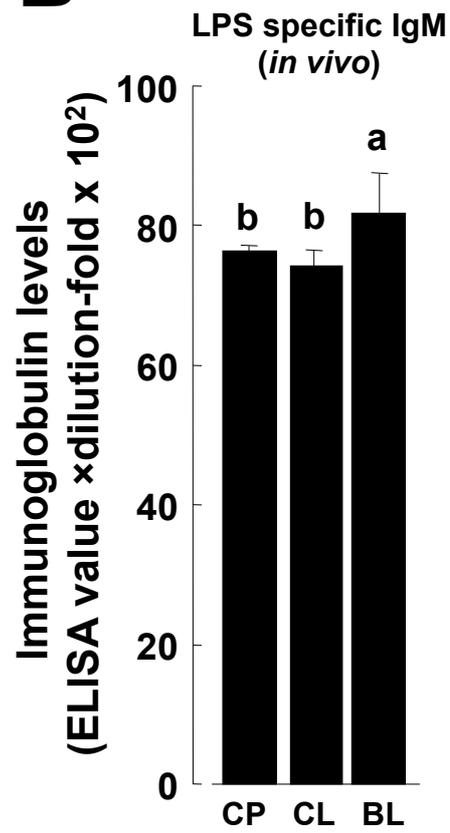


# Supplemental Figure 1 (revise)

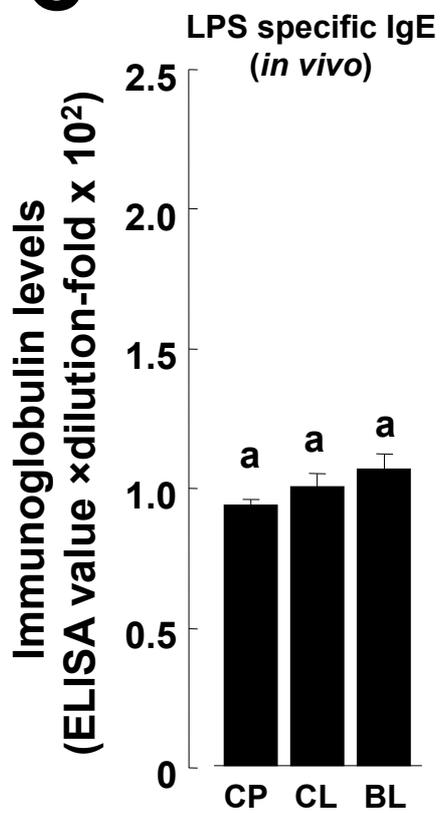
## A



## B



## C



## Supplemental Figure 2(revise)

