Soybean protein fraction digested with neutral protease preparation, "Peptidase R", produced by *Rhizopus oryzae,* stimulates innate cellular immune system in mouse

Shintaro Egusa[a,b], Hajime Otani[a,*]

[a]*Interdisciplinary Graduate School of Science & Technology, Shinshu University, Minamiminowa-mura 8304, Kamiina-gun, Nagano 399-4598, Japan.*
[b]*MARUSAN-AI Co., Ltd., 1, Arasita, Aza, Nikki-cho, Okazaki-shi, Aichi 444-2193, Japan.*

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*Corresponding author. Tel & Fax, +81-265-77-1430;
E-mail: otani84@gipmc.shinshu-u.ac.jp (H. Otani)

Abstract

A soybean protein fraction was prepared from defatted soybean seed flour and digested with 29 kinds of commercially available protease originating from preparations of animals, plants, and microorganisms. Some digests, in particular, Ro-digest prepared using a *Rhizopus oryzae* neutral protease preparation (Peptidase R), displayed strong mitogenic activity toward C3H/HeN mouse spleen cells. The number of spleen $CD11b^+$, $CD49b^+$, interleukin (IL)-12^+CD11b^+, and interferon (IFN)-γ^+CD49b^+ cells significantly increased when cultured with Ro-digest. Similarly, the number of spleen IFN-γ^+CD4^+ cells significantly increased in the presence of Ro-digest while that of spleen IL-4^+CD4^+ cells was largely unchanged. Additionally, 5-week-old male C3H/HeN mice were given diets consisting of ovalbumin (OVA) alone (control diet) or a mixture of OVA and Ro-digest (Ro-digest-added diet) as a protein source for 5 weeks, and the immune properties of the mice were investigated. The number of IL-12^+CD11b^+ cells was greater in spleens from mice given the Ro-digest-added diet than in those given the control diet. The cytotoxic activity of spleen cells toward the human erythroleukemia cell line, K562, was significantly higher in mice given the Ro-digest-added diet than in those given the control diet. Furthermore, in a microarray analysis of mRNAs extracted from mice Peyer's patch cells, gene expression related to innate immune responses was increased in mice given the Ro-digest-added diet. These results indicate that the Ro-digest might stimulate cellular immune systems, in particular, an innate immunity in mice.

Keywords: Soybean protein digest; Immunomodulation; Innate immunity; Microarray analysis; Mouse

1. Introduction

Soybeans are known as a source of high-quality protein because of their well-balanced amino acid composition. Additionally, soybeans have recently been demonstrated to possess a variety of physiological functions including anti-hypercholesterolemic and antitumor activities [1-3]. Some of these functions have been reported to be due to soybean proteins and their digests. Tamaru et al. [4] found that a low molecular weight peptide fraction from soybean protein isolate digested with Protin SD-AY10 displayed triglyceride-lowering activity in rats fed a cholesterol-enriched diet. Kim et al. [5] reported that the peptide corresponding to residues 83 to 89 of soybean glycinin showed cytotoxic activity toward P388D1, a mouse monocyte macrophage cell line. In addition, Jeong et al. [6] found that lunasin (a small subunit of 2S albumin) inhibited the carcinogenesis of mouse fibroblast NIH 3T3 cells induced by chemical carcinogens such as DMBA and MNU. These reports suggest that soybean proteins and their digests might be utilized as functional food additives with hypocholesterolemic and antitumor activities.

The immune system plays an important role in host defense against microorganisms, and is divided into two groups: innate and adaptive immunities. The former forms the first line of defense against attacks by pathogens, and is mediated by macrophages, dendritic cells (DC), and natural killer (NK) cells. The latter is involved in the elimination of pathogens in the late phase of infection, as well as the generation of immunological memory, and is initiated by T cell activation following T cell receptor (TCR) engagement with antigen-presenting cells (APC). Recently, some food proteins have been demonstrated to modulate immune systems. Artym et al. [7] found that oral administration of lactoferrin improved the humoral immune response in immunodeficient mice. Hata et al. [8] isolated a peptide with mitogenic activity toward mouse spleen cells from αs1-casein digested with trypsin, and identified the peptide casein phosphopeptide (CPP), which corresponds to residues 59 to 79 of αs1-casein. Otani et al. [9, 10] found that the oral administration of a commercially available CPP preparation enhanced mucosal IgA responses in mice and piglets. In addition, Otani and Wakatsuki [11] observed that the oral administration of CPP preparation reduced allergic symptoms through the enhancement of intestinal IgA levels and the reduction of serum IgE levels in NC/Jic Jcl mice. Moreover, Ohnuki et al. [12] reported that the oral ingestion of cow's milk IgG stimulated some of the innate cellular immune systems and suppressed humoral adaptive immune responses in mice. These reports suggest that food components may be utilized as anti-infectious and anti-allergic food additives.

However, there are few reports on the immunomodulatory activities of soybean proteins and their digests, and their activities are restricted to modulation of phagocytic cells. Yoshikawa et al. [13] demonstrated that the oral administration of a peptide corresponding to residues 296 to 301 of the glycinin A1a subunit stimulated phagocytosis in mice. Tsuruki et al. [14] isolated a phagocytosis-stimulating peptide from β-conglycinin digested with trypsin and named it soymetide-13.

The authors hypothesized that there were various kinds of immunomodulatory peptides in soybean protein digests. Hence, the authors investigated the effect of acid-precipitated soybean protein fractions digested with 29 kinds of commercially available protease preparations on mouse immune systems and found that a digest (Ro-digest), prepared with *R. oryzae*-produced "Peptidase R", stimulated cellular immune systems in both *in vitro* and *in vivo* experiments. In this work, the authors demonstrate the immunomodulatory properties of Ro-digest in mice.

2. Materials and methods

2.1. Materials

Defatted soybean seed flour was obtained from J-Oil Mills, Inc. (Tokyo, Japan). A total of 29 kinds of commercially available protease preparations were obtained from the following manufacturers: Protease MG, Protease AG, Protease NG, Protease SG, Protease P3G, Peptidase R, Protin A, Protin P, Neurase F3G, Prorezer FGF, Umamizyme G, Thermoase, Bromelain, and Papin W-40 were from Amano Enzyme, Inc. (Nagoya, Japan); Sumizyme LP, Sumizyme MP, Sumizyme FP, Sumizyme LPL, Sumizyme RP, Sumizyme CP, Sumizyme AP, Sumizyme LP, Sumizyme MMR, Sumizyme FLAP, and Sumizyme TP were from Shin Nihon Chemicals, Inc. (Anjyo, Japan); Alcalase 2.4 L, Protamex, and Neutrase 0.8 L were from Novo Nordisk A/S (Chiba, Japan); and pepsin and pancreatin were from Sigma Chemical (St. Louis, MO). Defined fetal bovine serum (FBS) was obtained from Equitech-Bio, Inc. (Kerrville, TX). RPMI-1640 medium was purchased from Nissui Pharmaceutical (Tokyo, Japan). 3-(4,5-Dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) was obtained from Sigma Chemical (St Louis, MO). Biolegend (San Diego, CA) was the supplier of phycoerythrin (PE)-labeled anti-mouse interleukin (IL)-4 monoclonal antibodies (mAbs, clone 11B11), PE-labeled anti-mouse IL-12 IL-23/p40 mAb (clone C15.6), PE-labeled anti-mouse interferon (IFN)-γ mAb (clone XMG1.2), PE-labeled anti-mouse CD49b mAb (clone DX5), biotin-labeled anti-mouse CD4 mAb (clone RM4-5), biotin-labeled anti-mouse CD11b

mAb (clone M1/70), and phycoerythrin/cyanine 5 (PE/Cy5)-labeled streptavidin. Brefeldin A (BFA), ionomycin, and phorbol 12-myristate 13-acetate (PMA) were obtained from Wako Pure Chemical Industries (Osaka, Japan). IntraPrep Permeabilization Reagent was bought from Beckman Coulter, Inc. (Tokyo, Japan). A defined protein-free purified diet (PM15765) was obtained from Purina Mills (St. Louis, MO). Ovalbumin (OVA) was acquired from Wako Pure Chemical Industries. Guava Viacount reagent was purchased from Guava Technologies (Hayward, CA).

2.2. Mice

Pathogen-free male C3H/HeN mice were purchased from Japan SLC (Shizuoka, Japan) and housed at 25 ± 2 °C with a 12 h-light/dark cycle. Mice at 6 weeks of age and 18 to 20 g body weight were used in accordance with the Guidelines for Regulation of Animal Experimentation at Shinshu University, and according to Law No. 105 and Notification No. 6 of the Japanese government.

2.3. Preparation of soybean protein digests

Defatted soybean seed flour (100g) was mixed with 20 volumes of 0.03 M Tris-HCl buffer (pH 8.0) and stirred for 2 h at room temperature. The supernatant was collected by centrifugation (9,000 × *g*, 30 min), adjusted to pH 4.5, and centrifuged at 9,000 × *g* for 30 min. The obtained precipitate was dispersed in water, dialyzed against tap water for 1 day, lyophilized, and used as an acid-precipitated protein fraction (28.8g).

The acid-precipitated protein fraction (1 g) was resuspended in 15 ml distilled water and boiled for 2 min. The boiled protein solution was adjusted to the optimal pH of each protease, distilled water was added for a total volume of 20 ml, and then this solution was used as the substrate solution. The substrate solution was incubated with protease (protein/enzyme = 50:1, w/w) for 2 h at 45 °C. The reaction was stopped by boiling the solution for 10 min and adjusting the pH to 4.5. The supernatant was collected by centrifugation at 3,000 rpm for 30 min at 4 °C, lyophilized, and used as digest.

2.4. Cells and cell cultures

Six-week-old male C3H/HeN mice were sacrificed by vertebral dislocation and

their spleens were removed aseptically. A single-cell suspension was prepared according to a previously described procedure [15]. Cell cultures were set up in quadruplicate in a 96-well flat-bottomed plate (BD Biosciences, San Jose, CA) for the MTT assay, and in duplicate in a 24-well flat-bottomed plate (Sarstedt, Inc., Newton, NC) for cell function analysis. The final concentrations in the RPMI-1640 medium were as follows: spleen cells, 5×10^6 viable cells/ml; digest, 0 to 500 μg/ml; FBS, 5%; penicillin, 100 units/ml; and streptomycin, 100 μg/ml. The cell culture was incubated at 37 °C in a humidified 5% CO_2-95% air atmosphere for 48 h or 72 h.

The human erythroleukemia cell line, K562 (TKG 0210), was obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. K562 was grown in RPMI-1640 medium supplemented with 10% FBS, and the cell density was maintained at between 2×10^5 and 2×10^6 cells/ml.

2.5. Feeding procedure

Four-week-old male C3H/HeN mice were assigned to test regimes and given commercial pellets (MF, Oriental Yeast Co., Tokyo, Japan) for 1 week. They were then given PM15765 supplemented with 25% OVA (control diet, $n = 5$) or a mixture of 12.5% OVA and 12.5% soybean protein digest prepared from the acid-precipitated protein fraction digested with Peptidase R (Ro-digest-added diet, $n = 5$) for 5 weeks. The detailed composition of each diet is shown in Table 1. The diets were continuously available in columnar form from stainless-steel feeders. Water was provided *ad libitum* from drinking bottles. The mice were maintained at 25 ± 2 °C under a 12 h-light/dark cycle. After 5 weeks, the mice were fasted overnight and used for analysis.

Table 1

2.6. Cell function analysis

Cell proliferation was estimated by the MTT assay [16] and using a Guava Personal Cell Function Analyzer (Guava Technologies). Spleen cells were incubated for 15 min at 4 °C with biotin-labeled anti-mouse mAbs specific to CD4 (clone RM4-5) and CD11b (clone M1/70), or PE-labeled anti-mouse mAb specific to CD49b (clone DX5), and were visualized by incubation with PE/Cy5-labeled streptavidin for 15 min at 4 °C. The cells were analyzed by the Guava Personal Cell Function Analyzer. In the case of cells having intercellular cytokines, cells were incubated with BFA 40 μg/ml, ionomycin 4 μg/ml, and PMA 40 ng/ml for 4 h, and then washed and fixed in IntraPrep

Regent 1 for 15 min at room temperature. The cells were then washed and incubated with IntraPrep Regent 2 for 5 min at room temperature. Finally, the cells were reacted with PE-labeled anti-mouse mAb specific to IL-4, IL-12, or IFN-γ, and analyzed by the Guava Personal Cell Function Analyzer.

To assay cytotoxic activity, 1-ml spleen cell suspension (1×10^6 cells/ml) was seeded in each well of a 24-well microtiter plate. The erythroleukemia cell line (K562) was added to the well to give spleen cell/K562 ratios of 1, 20, 100, 500, and 1,000. The plate was incubated for 24 h at 37 °C in a humidified 5% CO_2-95% air atmosphere, and 380 μl Guava Viacount reagent was added to 20 μl of each cell suspension. K562 viability was measured by the Guava Personal Cell Function Analyzer with Viacount software.

2.7. Microarray

The genome-wide gene expression of Peyer's patch cells was examined using the Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA) that contains 45101 probe sets for approximately 34,000 mouse genes. Briefly, total RNA was extracted from Peyer's patch cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA). GeneChip analysis was performed according to the GeneChip Eukaryotic Target Preparation & Hybridization Manual (Affymetrix). Data analysis was performed with GeneChip Operating software 1.4 (Affymetrix). Expression data were selected when there was a 1.5-fold difference between the control diet group and the Ro-digest-added diet group.

2.8. Statistical analysis

All data are expressed as means ± standard deviation ($n = 4$). The significance of the difference was tested using the Student's *t* test.

3. Results and discussion

3.1. Effects of protease digests on mouse spleen resting cells

Figure 1 shows the typical effects of the digests prepared from an acid-precipitated soybean protein fraction hydrolyzed by proteases originating from animals, plants, molds, and bacteria. Many protein digests prepared using proteases originating from molds and bacteria (*Aspergillus oryzae*: Sumizyme LP, Sumizyme FLAP, Sumizyme

Fig. 1

LPL, and Protease M amano G, *R. oryzae*: Peptidase R, *Bacillus amyloliqufaciens*: Neutrase 0.8 L, and *B. sterrothermophilus*: Protease S amano G) displayed mitogenic activities, while a few digests originating from animals, molds, and bacteria (Porcine pancreas: pancreatin, *Asp. niger*: Sumizyme AP, and *B. subtilis*: Protin A) were cytotoxic. Of these digests, Ro-digest, prepared using Peptidase R, exhibited the strongest mitogenic activity. Thus, Ro-digest was used in the following experiment. The Ro-digest yield obtained from a 1-g acid-precipitated protein fraction was 0.25 g.

3.2. Number of immunocompetent cells in mouse spleen cells cultured with Ro-digest

The number of immunocompetent cells in spleen cells cultured with Ro-digest is shown in Fig. 2. The number of $CD11b^+$, $CD49b^+$, IL-12^+CD11b^+, and IFN-γ^+CD49b^+ cells significantly increased in the presence of Ro-digest. Similarly, the number of IFN-γ^+CD4^+ cells was significantly greater in cells cultured with Ro-digest, while that of IL-4^+CD4^+ cells was largely unchanged.

Fig. 2

CD11b is a typical cell surface antigen of macrophages and DC [17]. IL-12 is one of the major cytokines produced by these cells [18]. A monoclonal antibody specific to CD49b (DX5) is a typical antibody toward pan-NK cells [19]. IFN-γ is one of the major cytokines produced by NK cells and type 1-helper T (Th1) cells [20]. Macrophages and DC play an essential role in host defense and form the first line of defense as components of innate immune response. NK cells are also important accessory cells, which, in addition to macrophages and DC, play a critical role in host defense as well as display cytotoxic effects on tumor cells [21]. The proliferation and activation of NK cells are induced by IL-12, and the activated NK cells produce IFN-γ [22, 23]. These facts suggest that the increase in the number of IFN-γ^+CD49b^+ cells is due to the increase in the number of IL-12^+CD11b^+ cells.

IFN-γ^+CD4^+ and IL-4^+CD4^+ cells are Th1 and type 2-helper T (Th2) cells, respectively. IL-12 produced from macrophages stimulates IFN-γ production by T cells, as well as NK cells, and enhances the differentiation of naive T cells into Th1 cells [19, 24]. In addition, there are two distinct types of DC precursors: myeloid monocytes (pre-DC1) and plasmacytoid DC precursors (pre-DC2). The myeloid DC1, derived from monocytes, produce a large amount of IL-12 and preferentially induce Th1 development [25]. These facts indicate that the Ro-digest stimulates IL-12 produced by macrophages and/or DC, suggesting that IL-12 activates NK cells and differentiates Th0 cells into Th1 cells.

3.3. Properties of spleen cells in mice given Ro-digest-added diet

Five-week-old male C3H/HeN mice were given the control or Ro-digest-added diet for 5 weeks. No significant difference was observed in the body weights of mice given the different diets (data not shown). Hence, there was little nutritional difference between the control and Ro-digest-added diets.

As shown in Fig. 3, the number of spleen $CD11b^+$ and $IL\text{-}12^+CD11b^+$ cells were significantly higher in mice given the Ro-digest-added diet than in those given the control diet. As shown in Fig. 4, the viability of K562, a human erythroleukemia cell line, in the presence of spleen cells from mice given the Ro-digest-added diet was significantly lower than that from mice given the control diet. The viability decreased with the increase in the spleen cell/K562 ratio. These results indicate that spleen cells from mice given the Ro-digest-added diet possess a higher cytotoxic activity toward K562 than those from mice given the control diet, suggesting that the increase of $IL\text{-}12^+CD11b^+$ cells in mice given the Ro-digest-added diet would stimulate spleen NK cells and activate their cytotoxicity.

Fig. 3

Fig. 4

3.4. Gene expression relating to innate immune responses in Peyer's patch cells of mice given Ro-digest-added diet

Figure 5 shows the increased transcriptomes of genes relating to innate immune responses in Peyer's patch cells of mice given the Ro-digest-added diet compared with those given the control diet. Transcriptomes exhibiting *Ela3*, *Aqp8*, *Ela1*, *Dmbt1*, *Igh-4*, *Slpi*, and *Mx1* increased more than 1.5-fold in mice given the Ro-digest-added diet than in those given the control diet. In particular, the transcriptome of *Ela3* was approximately 5-fold higher in mice given the Ro-digest-added diet.

Fig. 5

The proteins produced by transcription of these genes are associated with host defense. The proteins originating from *Igh-4* and *Aqp8* up-regulate phagocytosis and the migration of leukemia, respectively [26, 27]. Elastases originating from *Ela3* and *Ela1* attach to microorganisms and foreign substances [28]. Proteins originating from *Dmbt1*, *Slpi*, and *Mx1* are known as anti-bacterial and anti-viral components [29-31]. The inner surface of the intestinal tract possesses a large area of mucosal membranes, and they are continuously exposed to various substances in the intestinal lumen [32]. The gut-associated lymphoid tissues exist on the intestinal mucosal site and play an important role in the immune system. Peyer's patches are considered to be lymphoid tissues, where mucosal immune responses such as local IgA production and systemic immunological responses are induced [32]. These facts suggest that Ro-digest enhances host defense via phagocytosis and anti-microbial activity and plays a valuable role in the protection of intestinal mucosal sites against attacks by pathogens.

As described above, Ro-digest was screened for immunomodulatory activity from acid-precipitated protein fractions digested with 29 kinds of commercially available protease preparations. The Ro-digest significantly increased the number of spleen IL-12^{+}CD11b^{+}, IFN-γ^{+}CD49b^{+}, and IFN-γ^{+}CD4^{+} cells in mouse spleen cell cultures. The oral administration of Ro-digest increased the number of spleen IL-12^{+}CD11b^{+} cells and stimulated the cytotoxic activity of spleen cells toward the human erythroleukemia cell line (K562) compared to non-administration. Moreover, DNA microarray analysis showed that gene expression relating to the innate immune responses in Peyer's patch cells increased in mice given the Ro-digest-added diet.

In general, adjuvants are known as substances that stimulate antibody responses [33]. On the other hand, immunomodulators are known as substances that modulate not only antibody production but also immune systems, including innate immunity [34]. Several bacterial components such as DNA, lipopolysaccharides, and liposomes are known to be typical immunomodulators [35]. Recently, some food components have been demonstrated to modulate immune systems and are noted as oral immunomodulators because of their safety [34]. For example, the oral administration of CPP preparation enhanced mucosal IgA responses in piglets [10], and a piglet fed with CPP preparation has been sold in Japan. The oral administration of lactoferrin enhanced IFN-γ production in mice [36], and some foods and feeds have been sold for the purpose of preventing infectious diseases in animals. In this work, the authors found that Ro-digest prepared from soybean protein hydrolyzed by Peptidase R (*R. oryzae*) stimulated cellular immune systems, in particular, the innate immunity in mice. This finding suggests that Ro-digest may be an effective immunomodulator for preventing infectious diseases. In order to use Ro-digest as an immunomodulator, the characterization of the immunostimulating peptide is now in progress.

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Figure legends

Fig. 1. Typical effects of protease digests on mouse spleen resting cells. Spleen cells were cultured with the digest for 72 h. The data is represented as mean ± SD (n = 4). The value is significantly different from that without the digest ($^*P < 0.05$).

Fig. 2. Number of immunocompetent cells in mouse spleens cells cultured with Ro-digest. In the case of $CD11b^+$ and $CD49b^+$ cells, the spleen cells were cultured for 72 h while in the case of other cells, the spleen cells were cultured for 48 h. The data is represented as mean ± SD (n = 4). The value is significantly different from that without the digest ($^*P < 0.05$).

Fig. 3. Number of spleen $CD11b^+$ and IL-12^+CD11b^+ cells in mice given the control or Ro-digest-added diet for 5 weeks. The data is represented as mean ± SD (n = 4). The value is significantly different from that of the control diet ($^*P < 0.05$).

Fig. 4. The viability of the erythroleukemia cell line, K562, in the presence of spleen cells of mice given the control (●) or Ro-digest-added diet (○) for 5 weeks. The data is represented as mean ± SD (n = 4). The value is significantly different from that of the control diet ($^*P < 0.05$).

Fig. 5. Gene expression relating to innate immune responses in the Peyer's patch cells of mice given the Ro-digest-added diet. Relative mRNA expression was determined using the GeneChip Operating software 1.4.

Table 1. Composition of diets

	Control diet (25% OVA alone)	Ro-digest-added diet (A mixture of 12.5% OVA and 12.5% digest)
	%	
Digest	0.000	12.500
Ovalbumin	25.000	12.500
Dextrin	32.738	32.738
Sucrose	27.112	27.112
RP mineral mix #10[†]	3.750	3.750
Corn oil	3.750	3.750
Lard	3.750	3.750
Powdered cellulose	2.250	2.250
RP vitamin mix[‡]	1.500	1.500
Choline chloride	0.150	0.150
Total	100.000	100.000

[†]RP mineral mix #10: calcium, 0.6%; phosphorus, 0.4%; potassium, 0.4%; magnesium, 0.07%; sodium, 0.21%; chlorine, 0.24%; fluorine, 5.0 ppm; iron, 60 ppm; zinc, 21 ppm; manganese, 65 ppm; copper, 15.0 ppm; cobalt, 3.2 ppm; iodine, 0.57 ppm; chromium, 3.0 ppm; molybdenum, 0.82 ppm; selenium, 0.23 ppm. [‡]RP vitamin mix: vitamin K, 10.4 ppm; thiamin hydrochloride, 20.6 ppm; riboflavin, 20.0 ppm; niacin, 90 ppm; pantothenic acid, 55 ppm; choline chloride, 1400 ppm; folic acid, 4.0 ppm; pyridoxine, 16.5 ppm; biotin, 0.4 ppm; vitamin B12, 20 mcg/kg; vitamin A, 22.1 IU/g; vitamin D3, 2.2 IU/g; vitamin E, 50 IU/kg; ascorbic acid, 0.0 ppm.

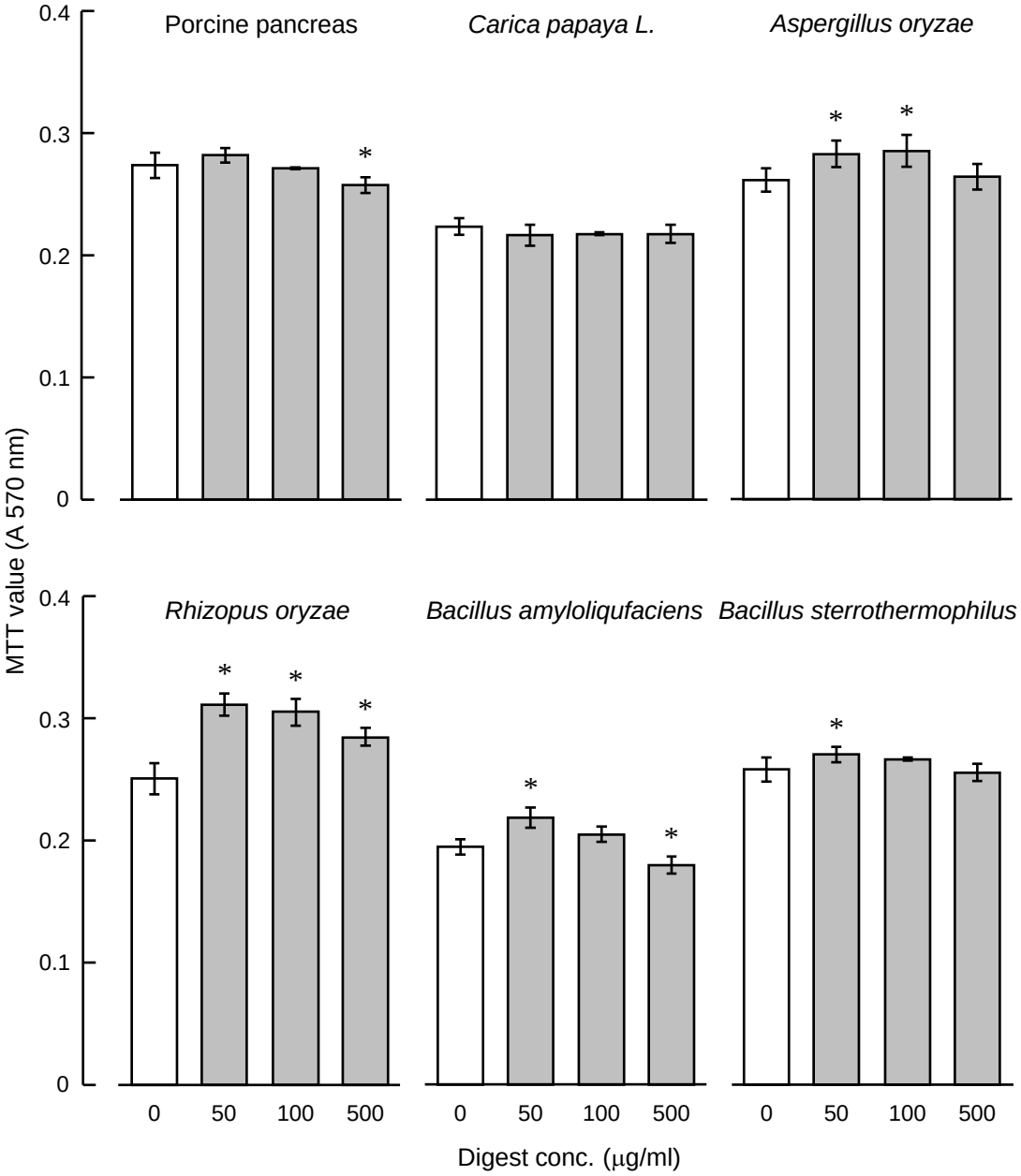

Fig. 1 (Egusa & Otani)

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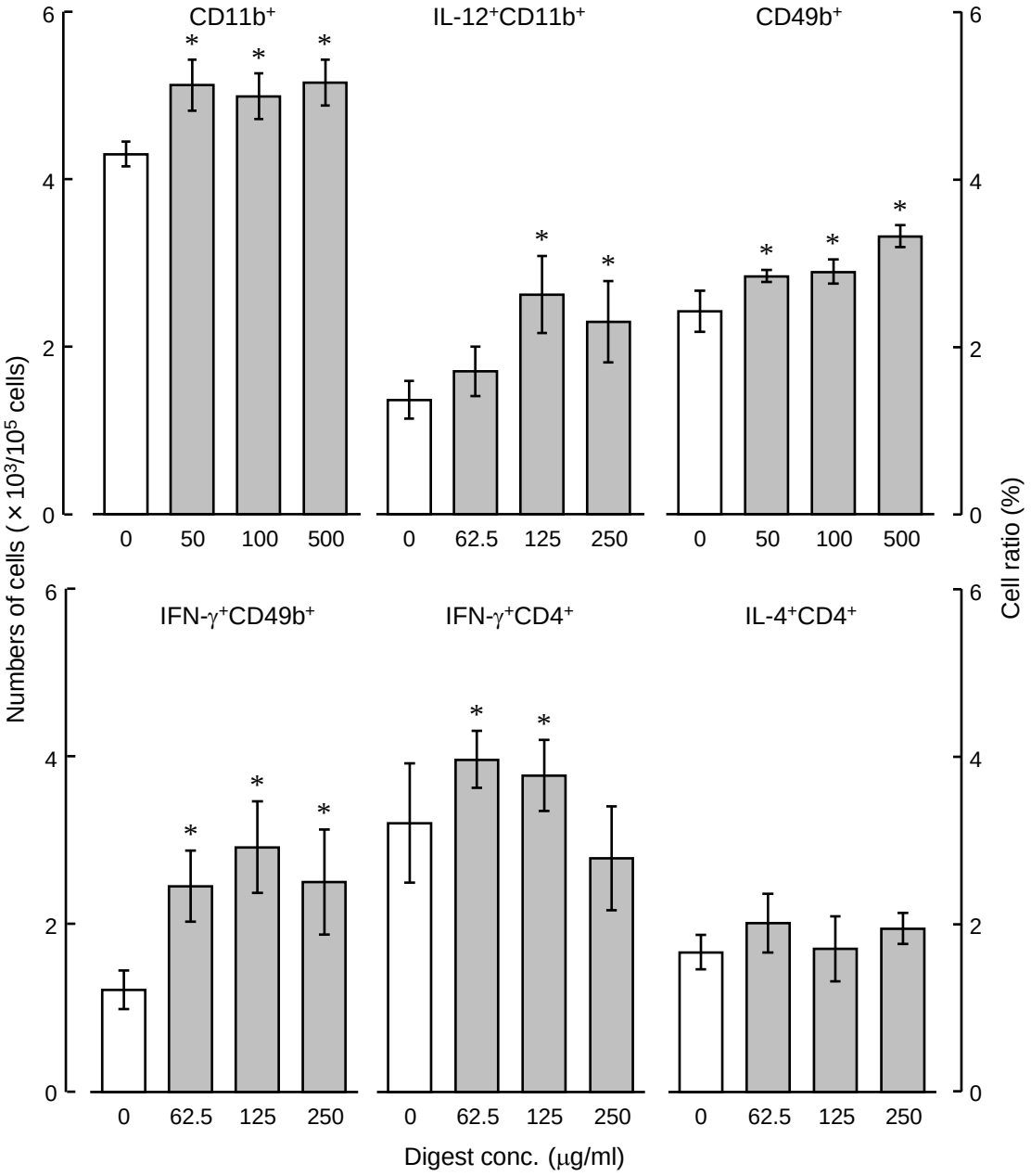

Fig. 2 (Egusa & Otani)

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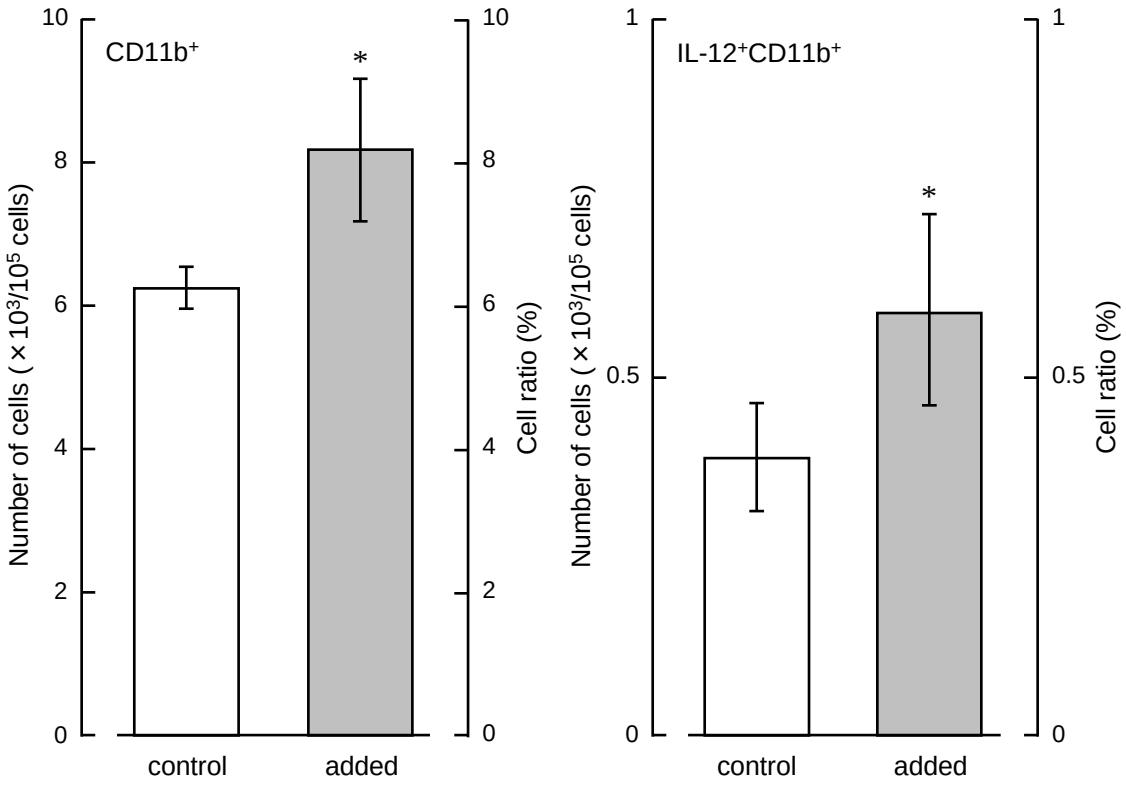

Fig. 3 (Egusa & Otani)

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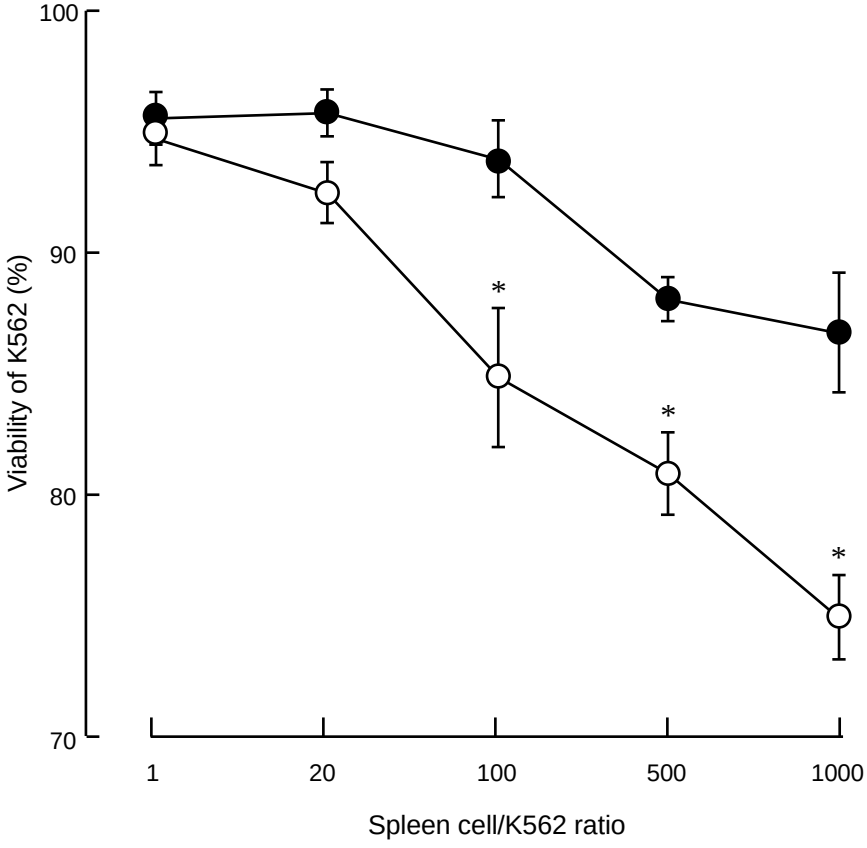

Fig. 4 (Egusa & Otani)

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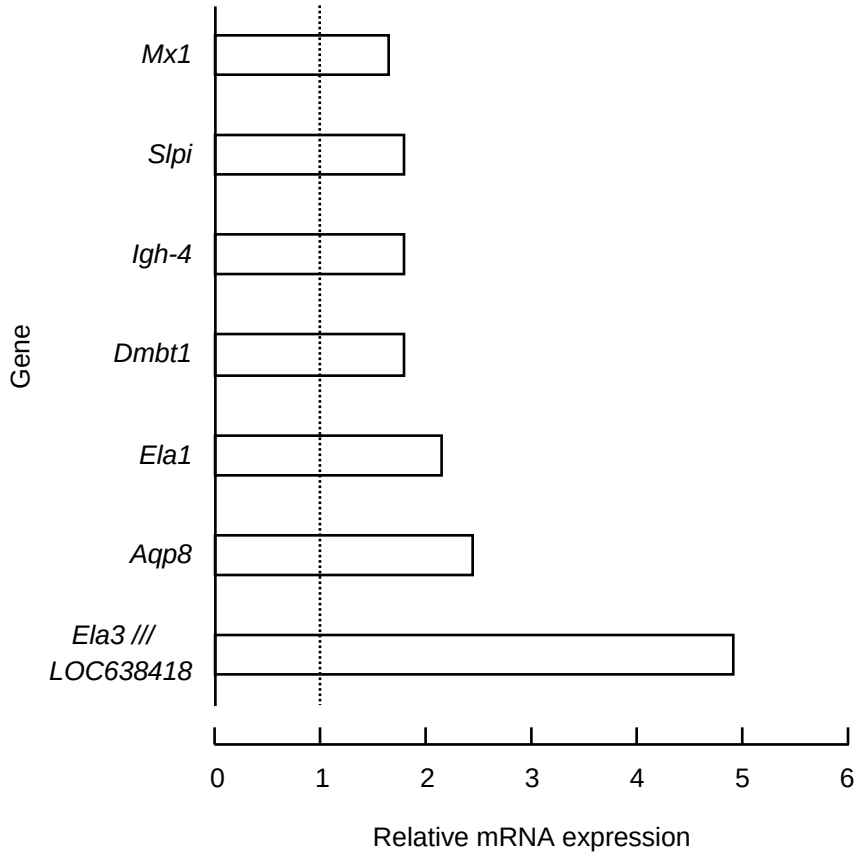

Fig. 5 (Egusa & Otani)

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