Characteristics of B cell growth substance produced by *Bacillus licheniformis* E1

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Characteristics of B cell growth substance produced by *Bacillus licheniformis* E1

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ABSTRACT

Characteristics of B cell growth substance produced by *Bacillus licheniformis* E1

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It was reported that B cell-specific growth substance (BGS) was found in Doenjang, the Korean-style fermented soybean paste (Kfsp) but not in non-fermented soybean paste, thus it was considered that bacteria involved in fermentation of Kfsp to be the cause to produce the KfspBGS. In this study, the bacterium which produced BGS was isolated from Kfsp, and was identified as *Bacillus licheniformis* E1. The BGS produced by *B. licheniformis* E1 (bBGS) was originated from the slime layer of the bacterium, and appeared to be a glycoprotein that shares similar physical and biological characteristics to those of BGS isolated from Kfsp (KfspBGS).

Unlike LPS, bBGS was not affected by polymixin B, an inhibitor of LPS,

or by TLR4, and has resulted in the growth of B cells.

When BALB/C mouse was treated with bBGS, increases of B cell population were resulted in the bone marrow and in the spleen. In the bone marrow, the increase was marked at 24 h after treatment with bBGS, and, in the spleen at 48h.

Through the analysis of B cell population increased by bBGS using antibodies to the B cell lineage-restricted surface molecules, it was found that immature B cells (IgM^+ and $AA4.1^+$) and mature B cells (IgM^+ and IgD^+) were increased in the bone marrow. Whereas in the spleen, mature B cells decreased, and IgG^+ B cells increased at 24 h after bBGS treatment.

When bBGS was injected with OVA antigen into peritoneal cavity of BALB/C mice, the high concentration of OVA-specific antibody was found in their sera. The increase of OVA-specific antibody titer induced by bBGS was found to be similar to that induced by aluminum hydroxide.

It is therefore expected that mass production of bBGS by *B. licheniformis* E1 could be used for studies on B cells in immunology, and help to cure the patients who are medically in need of reinforcement of B cells proliferation. It is also expected that bBGS may contribute to new adjuvant development in vaccine manufacture.

Key words: B cell–specific growth substance (BGS), Korean-style fermented soybean paste (Kfsp), *Bacillus licheniformis* E1

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I. INTRODUCTION

Immunomodulators are substances that may affect the host immune response. They control the immune responses by increasing or suppressing the growth, differentiation, and also the activities of immune cells[1, 2]. Protein, glycoprotein, peptide, lipopolysaccharide (LPS) and lipid derivative[3] are the well known immunomodulators. Some of the substances are derived from animals and plants, but they are mainly produced in microorganisms.

Polysaccharide A (PS A), β-(1-3)-glucans, mannan, krestin (PSK), polysaccharopeptide (PSP), and hyaluronic acid (HA) are some of the currently known immunomodulators that are derived from microbes[3]. Such polymers unusually increase the activities of T cells or antigen-presenting cells (APCs)[3]. PS A from *Bacteriodes fragilis* increases the production of interlukin-2 (IL-2) from helper T cell[4]. β-(1-3)-glucans, a glucose polymer

from the yeast, increase the antimicrobial activity of mononuclear cells and neutrophils, and to increase the functional activity of macrophages[5, 6]. Mannan, derived from *Candida albicans,* increases the proliferation of type 2 helper T cells and also increases the production of IL-4[7]. PSK and PSP are glycoproteins formed in mushrooms that induce the production of interferon-γ (IFN-γ) and IL-2 by increasing T cell activity[8]. HA is a major carbohydrate component of the extracellular matrix of mammalian tissue, formed from streptococci and induces T cell activity[9].

Some substances function as T cell-independent antigen in host immune system that differentiate B cell to be short-lived effector cell having no immunologic memory by stimulating B cell without help from T cell, and also function to induce the production of low-affinity IgM and IgG antibodies[10, 11]. LPS from Gram-negative bacteria, immunostimulating factor (ISTF) of *Actinobacillus actinomycetemcomitans,* macrophage-activating lipopeptide-2 (MALP-2) of *Mycoplasma fermentans*, and outer surface lipoprotein A (OspA) which is a lipoprotein from *Borrelia burgdorferi* are the known examples. LPS is a typical bacterial B cell mitogen that induces differentiation of B cell and also promotes expression of IgM on the B cell surface[12]. ISTF not only proliferates B cells and APCs, but also induces the production of IL-6 and tumor necrosis factor-α (TNF-α)[13]. MALP-2 increases the proliferation and differentiation of B cell through Toll-like receptor 2 (TLR2) in T cell independent manner[14]. And, OspA is known to proliferate B cell through

CD40/CD40L interaction[10].

Korean-style fermented soybean paste (Kfsp) is a traditional food that is consumed as a protein source in Korea. It is produced through the fermentation of soybeans by natural microbial flora for 1 year. It is also known to contain biologically active substances. Cytotoxic effect against carcinoma[15], antithrombotic peptides[16], angiotensin I converting enzyme inhibitory peptides[17, 18], antioxidants, a phenolic compound, and hydrophilic brown pigment[19, 20] have all been identified in Kfsp.

In our previous studies on the functionality of Kfsp, we have reported that B cell-specific growth substance (BGS) was found in Kfsp. The intraperitoneal treatment of BALB/C mice with KfspBGS significantly enhanced the number of B cells expressing surface immunoglobulins (IgM and IgG). Subsequently, an increased level of immunoglobulins in the sera was also observed. *In vitro*, KfspBGS was found to upregulate the production of IL-1 and IL-6 by macrophages and B cells but not the production of IL-2 by T cells. KfspBGS was found only in the fermented soybean paste, and not in the non-fermented soybean paste, thus it was considered that bacteria involved in fermentation of Kfsp to be the cause to produce the KfspBGS[21]. Therefore, in this study, we attempted to isolate the bacteria from Kfsp that produce BGS similar to KfspBGS, and investigated the physicochemical, and biological characteristics of the BGS obtained from the isolate.

II. MATERIALS AND METHODS

1. Animals

BALB/C, C3H/HeJ and C3H/HeN female mice between 6 and 7 weeks of age were purchased from Orientbio (Seoul, Korea) and Central Lab. Animal (Seoul, Korea), respectively. The animals were maintained and used in accordance with the guidelines prepared by the Yonsei University College of Medicine (Seoul, Korea), which has been fully accredited by Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC), for the care and use of laboratory animals.

2. Reagents

Aluminum hydroxide (Al), Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FICA), LPS, Ovalbumin (OVA), peroxidase substrate and polymixin B (PMB) were purchased from the Sigma-Aldrich (Steinheim, Germany), and a EZ-link Sulfo-NHS-LC-biotinylation kit used to biotinylated OVA was purchased from the Pierce (Rockford, IL, USA).

3. Antibodies

The antibodies used for cell staining and FACS analysis were as follows: phycoerythrin (PE) anti-mouse CD45R/B220, fluorescein isothiocyanate (FITC) anti-mouse CD3, FITC anti-mouse CD25 (IL-2R), FITC anti-mouse

CD117 (c-*kit*), FITC anti-mouse IgM (BD Pharmingen, SanDiego, CA, USA), FITC anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA), PE anti-mouse IgD and PE anti-mouse C1qRp (AA4.1) (eBioscience, Burlingame, CA, USA).

The antibodies used for ELISA were as follows: horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, purified anti-mouse IgG, HRP-conjugated anti-mouse IgG (BD Pharmingen), affiniPure rabbit anti-mouse IgM and IgG Abs (ImmunoResearch Laboratories, West Grove, PA, USA), purified mouse IgM and IgG, HRP-conjugated goat anti-mouse IgM and IgG (Southern Biotechnology Associates, Birmingham, Alabama, USA) and HRP-conjugated anti-biotin (Vector Laboratories).

4. Isolation of bacteria from Kfsp

Kfsp was obtained from Sunnchang Traditional Foods Co. (Sunchang, Korea). Samples were harvested from the top, middle, and bottom of the jar containing the Kfsp, and then pooled. One gram of the pooled sample was suspended with 10 ml of saline. One milliliter of the suspension was spun down at room temperature (RT) at 10,000×g for 10 min. The supernatant was discarded, and the sediment was resuspended in 1 ml of saline. The suspension was serially diluted with saline, inoculated on brain heart infusion (BHI: Difco, Becton Dickinson, Sparks, MA, USA) agar plates supplemented with 5% NaCl, and cultured at 37℃ for 2 to 5 d under aerobic or anaerobic

conditions. The isolation of the bacteria was based on the morphology of the colony and Gram staining.

5. Screening of bacteria producing KfspBGS

The bacterial isolates obtained from Kfsp were inoculated in 20 ml of BHI broth in 50 ml plastic tube and cultured by shaking at 37℃ under aerobic and anaerobic conditions for 2 and 5 d respectively. The cultured fluids were boiled, and spun down at 4℃ at 10,000×g for 1 h. The supernatant was concentrated at 1 ml by using DIAFLO Centricon Plus-80 (Amicon, Bedford, MA, USA). The productivity of the KfspBGS obtained from each of the bacterial isolates was measured by ELISA methods with anti-KfspBGS Ab[21].

6. Isolation of mouse T and B cells

The lymphocytes were obtained from the spleen of mice. A single cell suspension was prepared by gently teasing the spleen between two glass slides, and the red blood cells were lysed using an ACK lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA).

For the isolation of T cells, splenocytes were incubated in MACS buffer (PBS containing 0.5% bovine serum albumin and 2 mM EDTA) with biotin-antibody cocktail (biotin-conjugated monoclonal antibodies against CD45R, CD49b, CD11b, and Ter-119; Miltenyi Biotec, Auburn, CA, USA) for 10 min at 4℃, washed, and incubated with anti-biotin MicroBeads

(Miltenyi Biotec) for 15 min at 4℃, according to procedures provided by the manufacturer. Cells were applied onto the magnetic column (Miltenyi Biotec) and the effluent was collected, and washed in RPMI 1640 medium supplemented with 2 mM L-glutamine, 2.2 mM sodium bicarbonate, 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO BRL, Grand Island, NY, USA).

For the isolation of B cells, biotin-conjugated monoclonal antibodies against CD43, CD4 and Ter-119 (Miltenyi Biotec) were used.

7. Lymphocyte proliferation assay for substance produced by bacteria

The activity of substance produced by bacteria was measured by the proliferation of lymphocytes from BALB/C mice. The fractionated T or B cells in a complete medium supplemented with RPMI 1640 and 10% heat-inactivated fetal calf serum (2.5×10^5 cells/well) were plated in 96-well round bottomed microtiter plates (Corning, NY, USA). Fifty microliters of the bacterial culture fluids or the purified bBGS were added to each well, and the plates were cultured for 48 h. The cell proliferation was measured using 6 h ^{3}H-tymidine (^{3}H-TdR, New England Nuclear, Boston, MA, USA) incorporation.

8. Identification of bacteria

The bacterial isolate that shows a high level of B cell growth activity was identified by *Bergey's Manual Systematic Bacteriology*[22] based on general

characteristics, and by sequencing of 16S ribosomal DNA. For the biochemical testing, the bacterial isolate was cultured in a nutrient broth at 30℃ for 24 h. The cultured bacterial suspension was inoculated in API 50 CHB kit (bioMerieux, Marcy 1'Etoile, France), then incubated at 30℃ for 24 or 48 h, and identified by using API 50 CHB database v. 3.0. The gene sequencing of 16S ribosomal DNA of the bacterial isolate was performed by PCR technique. To amplify a partial 16S rDNA fragment of the isolate, the universal primers (27F: 5'-AGAGTTTGATCATGGCTCAG-3' and 1492R: 5'-GGATACCTTGTTACGACTT-3') were used. The amplified PCR product was ligated into a T vector (Invitrogen, Seoul, Korea), and DNA sequencing was performed using an ABI 377 Genetic Analyzer (PE Applied Biosystems, Fostercity, CA, USA). The 16S rDNA sequence was then aligned with reference sequences obtained from the GenBank databases (NCBI, Bethesda, MD, USA), using the Blast searches (http://www.ncbi.nlm.nih.gov).

9. Purification of BGS from *Bacillus licheniformis* E1

Purification of concentrate from *B. licheniformis* E1 was performed as described[21]. Briefly, *B. licheniformis* E1 was cultured in BHI broth on a shaker at 37℃ for 48 h. The cultured supernatant was spun down at 10,000×g at 4℃ for 1 h. The harvested supernatant was boiled at 100℃ for 30 min and spun down again in the same way as the above. The supernatant was concentrated using a DIAFLO ultrafiltration membrane (Filter code: YM 100,

Millipore, Bedford, MA, USA). Five milliliters of the concentrate were applied to a column (25×330 mm) of DEAE Sepharose Fast Flow (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) which was previously equilibrated with 0.05 M phosphate buffer (pH 7.4), and the column was eluted with a linear gradient of 0.0-1.0 M NaCl in the phosphate buffer at a flow rate of 2 ml/min. Five milliliters of the fractions were collected, and monitored at 280 nm, and their activities were measured by BALB/C mouse B cell proliferation assay. Those elutes showing mouse B cell proliferation activity were pooled and concentrated using the same ultrafiltration membrane. And then 1 ml of the concentrate was subjected to a column (16×900 mm) of Sephacryl S-500 (GE Healthcare Bio-Sciences AB) which was previously equilibrated with the phosphate butter and eluted with the same buffer at a flow rate of 1 ml/min. Three milliliters of each fraction were collected and monitored at 280 nm. Those elutes showing mouse B cell proliferation activity were pooled and concentrated by the same ultrafiltration membrane. The concentrate was again subjected to a Superose TM 6HR column (GE Healthcare Bio-Sciences AB), and the fractions were monitored at 214 nm. The fractions at the peak were collected, pooled, concentrated, and freeze-dried. The concentrate produced by the *B. licheniformis* E1 is hereafter referred to as bacterial B cell-specific growth substance (bBGS).

10. Molecular weight analysis

The molecular weight of bBGS was estimated by the gel permeation chromatography (GPC) system with a column of ultrahydrogel 250 (7.8x300 mm), ultrahydrogel 500 (7.8x300 mm), and ultrahydrogel 1,000 (7.8x300 mm), using a conventional HPLC system with a refractive index detector. The columns were eluted with distilled water at a flow rate of 1 ml/min. Calibration of the columns was performed using several kinds of dextrans (molecular weights of 10-2,000 kDa; Sigma-Aldrich)

11. Carbohydrate analysis

The total carbohydrate content was quantified by the method of Dubois et al.[23]. The bBGS (75 μl) was mixed with 5% phenol reagent (75 μl), and 375 μl of H_2SO_4. The mixed solution was allowed to stand in an ice bath for 5 min and then reacted at 80℃ for 30 min, and the total carbohydrate content was measured by a spectrophotometer at 492 nm. Galactose (G-6404, Sigma-Aldrich) was used as a standard. Sugar composition of the bBGS was analyzed by a modified method of Park and Yun[24]. Briefly, the bBGS was hydrolyzed with 2 M trifluoroacetic acid (TFA) for the neutral sugar and 6 N HCl for the amino sugar at 100℃. A Dionex CarboPac PA column (4.5x250 mm) was used, and the hydrolysates were analyzed using Bio-LC DX-600 (Dionex, Sunnyvale, CA, USA) with an electrochemical detector (Dionex ED50). The column was eluted with 16 mM NaOH at a flow rate of 1 ml/min.

12. Protein analysis

The total protein content of bBGS was determined by the method of Ohnishi and Barr[25]. The bBGS (50 μl) was added to 550 μl of Biuret reagent (Sigma-Aldrich) and allowed to stand at RT for 10 min. This solution was mixed with 25 μl of Folin and Ciocalteu's phenol reagents (Sigma-Aldrich) and allowed to stand at RT for 30 min. The protein content of bBGS was estimated by spectrophotometer at 752 nm, and bovine serum albumin (BSA) was used as a standard protein. The amino acid composition of bBGS was carried out by the Pico-Tag method[26]. After hydrolysis with 6N HCl at 110℃ for 24 h, derivatization of the hydrolyzed bBGS was accomplished using the derivatizing solution [ethanol/distilled water/triethylamine/phenylisothiocyanate (PITC), 7/1/1/1, v/v] for 15 min. PITC-derivatized amino acids were applied to a Pico-Tag column (3.9×300 mm, Waters, Milford, MA, USA) equilibrated with 140 mM sodium acetate and equipped with a Waters HPLC system. The column was eluted with a linear gradient of 0-60% acetonitrile in 140 mM sodium acetate at a flow rate of 1 ml/min at 46℃.

13. FT-IR spectrum analysis

The bBGS was hydrolyzed with 2 M HCl for 5 h at 100℃. The infrared spectrum of the hydrolyzate was measured using a Fourier transform infrared (FT-IR) spectrophotometer (Perkin-Elmer, Boston, MA, USA) with Miracle™ attenuated total reflectance (ATR).

14. Sensitization

In order to induce bBGS-specific immune response, BALB/C mice were injected once intraperitoneally (i.p.) (day 0) with the bBGS (25 μg/mouse). Subsequently, mice were sacrificed 1, 2 and 3 d after the i.p. sensitization and cell suspensions from spleens and bone marrows from each group of mice were used to FACS analysis.

For OVA sensitization, BALB/C mice were injected with an initial intradermal (i.d.) sensitization of OVA (100 μg/mouse) mixed with one of the following adjuvants: FCA adjuvant (100 μl/mouse), Al (20 μg/mouse) or bBGS (25 μg/mouse). Subsequently, mice were intradermally boosted with OVA (100 μg/mouse) mixed with FICA (100 μl/mouse), Al (20 μg/mouse) or bBGS (25 μg/mouse) 1, 2 and 3 weeks after the initial sensitization. One week after last sensitization, blood was harvested through vena cava of mouse, and sera were stored at -20℃ until ELISA.

15. Isolation of bone marrow cells

Bone marrow cells from BALB/C mice were flushed out of femurs with ice-cooled complete medium by using a 10-ml syringe with a 21-gauge needle. Red blood cells were depleted using ACK lysis buffer, and bone marrow cells were washed with RPMI 1640 medium.

16. Thymidine incorporation assay

A total of 1×10^6 cells/ml was cultured in 200 μl volumes in 96-well round bottom plates. Cells were cultured for 48 h and pulsed with 1 μCi per well of ^{3}H-TdR for the last 8 h before ^{3}H-thymidine incorporation was determined.

17. FACS analysis

For a cytometric analysis of bone marrow cells, splenocytes, and MACS-purified T or B cell suspensions, the cells were incubated with antibodies labeled with FITC and/or PE in FACS buffer (PBS, 1% FCS, 0.05% sodium-azide) for 30 min on ice and washed twice with FACS buffer. Data were acquired on a FACSCalibur (Becton Dickinson, San Jose, USA) and analyzed using CellQuest software (Becton Dickinson).

18. ELISA methods

The concentrate (100 μl) obtained from each of the bacteria isolated from Kfsp was mixed with 200 μl of carbonate-bicarbonate coating buffer, and the mixture (100 μl/well) was coated on 96-well ELISA plate (Nunc, Copen-hagen, Denmark) at 4℃ for 24 h. The plates were washed three times with PBS containing 0.05% Tween 20 (PBST), and blocked with 150 μl/well of PBS containing 10% BSA for 2 h at RT. The primary antibody used was anti-KfspBRM prepared from rabbits immunized with KfspBRM in the previous study[21], and the secondary antibody was HRP-conjugated goat

anti-rabbit IgG. The plates were then incubated at RT for 1 h, washed five times with PBST, and reacted with peroxidase substrate at 37℃ for 30 min. The reaction was stopped by adding 2N H_2SO_4, and read an optical density (OD) of 450 nm using an ELISA plate reader.

Total IgM and IgG levels were measured by capture-ELISA. Plates were coated with 100 μl/well of affiniPure rabbit anti-mouse IgM (1 μg/ml) or IgG (2 μg/ml) Ab for 24 h and blocked with 150 μl of PBS containing 10% BSA for 2 h at RT. The plates were then washed three times with PBST, and the sera of BALB/C mice diluted by PBS containing 10% BSA (100 μl/well) were added and incubated for 1 h. The plates were then washed five times and 100 μl of 1/1,500 HRP-conjugated goat anti-mouse IgM or IgG Ab was added to the well. The plates were then incubated at RT for 1 h, washed five times with PBST, and reacted with peroxidase substrate at 37℃ for 30 min. The reaction was stopped by adding 2N H_2SO_4, and read an OD of 450 nm using an ELISA plate reader. Standard curves for IgM or IgG Ab were plotted using purified mouse IgM or IgG.

The OVA-specific IgG levels were measured by capture-ELISA. Plates were coated with 100 μl/well of a purified anti-mouse IgG mAb (2 μg/ml) for 24 h and blocked with 150 μl of PBS containing 10% BSA for 2 h at RT. The plates were then washed three times with PBST, and the sera of BALB/C mice diluted with PBS containing 10% BSA (100 μl/well) were added and incubated for 1 h. One hundred microliters per well of the biotinylated OVA

(10 μg/ml) were added and incubated at RT for 1 h. The plates were then washed five times and 100 μl of 1/1,500 HRP-conjugated goat anti-biotin mAb was added to the well. The plates were then incubated at RT for 1 h, washed five times with PBST, and reacted with peroxidase substrate at 37℃ for 30 min. The reaction was stopped by adding 2N H_2SO_4, and read an OD of 450 nm using an ELISA plate reader.

19. Statistical analysis

All data were expressed as mean ± S.D. of three independent experiments (*n*=3). The differences were analyzed using the Student's *t*-test and results were considered significant with a *P* value <0.05.

III. RESULTS

1. Isolation of bacteria from Kfsp

In order to isolate bacteria growing in the fermentation process of soybean paste, samples harvested from several areas of a jar containing Kfsp were inoculated on BHI agar plate supplemented with 5% NaCl (as Kfsp usually contains 5% or more of NaCl) and incubated at 37℃ for 2 to 5 d under either aerobic or anaerobic conditions. The cultured bacteria were preliminarily identified on the basis of their colony and cellular morphology. As shown in Table 1, a total of 31 kinds of bacteria were isolated from Kfsp fermented for 1 year. Among the isolates, there were 25 kinds of aerobic bacteria and 6 kinds of anaerobic bacteria. Aerobic isolates included 20 kinds of Gram-positive ($Gram^+$) bacteria (12 rod and 8 coccus forms) and 5 kinds of Gram-negative ($Gram^-$) ones (3 rod and 2 coccus forms), while anaerobic isolates appeared to include 4 kinds of $Gram^+$ bacteria (3 rod and 1 coccus forms) and 2 kinds of $Gram^-$ cocci.

Table 1. Isolation of bacteria from Kfsp[a] fermented for 1 year

Group	Number of isolates[b] obtained from Kfsp			
	Gram-positive		Gram-negative	
	Rod	Coccus	Rod	Coccus
Aerobes	12	8	3	2
Anaerobes	3	1		2

[a] Samples were harvested from the top, middle and bottom of the jar contained Kfsp.
[b] Isolation of bacteria was based on the morphology of colony and Gram's staining.

2. Selection of bacteria that produce substance reactive with anti-KfspBGS

Among 31 isolates, 24 kinds of Gram-positive bacteria were cultured in BHI broth under aerobic or anaerobic conditions, and their culture fluids were boiled at 100℃ for 30 min. The serological responses of the concentrates from the bacterial culture fluids to anti-KfspBGS[21] that were obtained from a rabbit immunized with KfspBGS were examined using the ELISA method. The concentrates from the bacterial culture fluids obtained from each of the 9 isolates (A2, B1, B4, C1, C3, C5, D1, E1 and E2) of aerobic Gram-positive bacilli strongly reacted with anti-KfspBGS. Their reactions were more than 0.5 of OD. In contrast, those from the rest of the isolates (13 strains) showed low levels of OD (below 0.1) in the reaction with the anti-KfspBGS (Table 2).

Table 2. Serological response of the concentrated culture fluids from each of the isolates to anti-KfspBGS

Isolate[a]	OD[b]	Isolate[a]	OD[b]	Isolate[a]	OD[b]
A1	0.08 ± 0.03	C2	0.02 ± 0.03	D4	0.06 ± 0.04
A2	0.56 ± 0.05	C3	0.72 ± 0.08	D5	0.05 ± 0.03
A3	0.07 ± 0.04	C4	0.08 ± 0.04	D6	0.05 ± 0.02
B1	0.67 ± 0.07	C5	0.92 ± 0.11	E1	0.97 ± 0.07
B2	0.08 ± 0.03	C6	0.07 ± 0.04	E2	1.23 ± 0.09
B3	0.05 ± 0.02	D1	0.83 ± 0.08	E3	0.06 ± 0.02
B4	0.92 ± 0.15	D2	0.04 ± 0.03	E4	0.08 ± 0.05
C1	0.74 ± 0.09	D3	0.07 ± 0.04	E5	0.06 ± 0.02

[a] Gram-positive bacteria isolated from Kfsp.

[b] Optical density (the mean ± SD of three independent experiments).

3. B cell growth activity of the bacterial culture concentrates reactive with anti-KfspBGS

As shown in Table 2, the concentrated culture fluids obtained from each of the 9 isolates strongly reacted with anti-KfspBGS. Therefore, to find out whether the concentrates would share the same biological function with KfspBGS, the effect of the concentrated culture fluid on the proliferation of B and T cells was examined by 6 h-^{3}H-TdR incorporation assay. As shown in Fig. 1, high levels of ^{3}H-TdR incorporation by B cells were shown in the groups treated with the concentrated culture fluids from each of the 5 isolates: A2 (9.92 ± 1.58 kcpm), B4 (8.62 ± 0.67 kcpm), C5 (6.5 ± 0.68 kcpm), E1 (16.7 ± 2.08 kcpm), and E2 (12.22 ± 2.08 kcpm). In contrast, those treated with the concentrated culture fluids from each of the rest of the 4 isolates (B1, C1, C3 and D1) were below 1.0 kcpm, and were similar to that of B cells treated with the medium as control. On the other hand, none of the enhanced proliferation of T cells was found in groups treated with the concentrated culture fluids from the 9 isolates, and their ^{3}H-TdR incorporation was below 1.0 kcpm, which is similar to that of T cells treated with the medium. Even if the concentrated culture fluids from each of the 9 isolates showed the serologic responses to anti-KfspBGS in the ELISA test as shown in Table 2, their responses to induce the proliferation of B cells were found only in those from each of the 5 isolates, suggesting that the 5 isolates produce substances specific for B cells, but not for T cells.

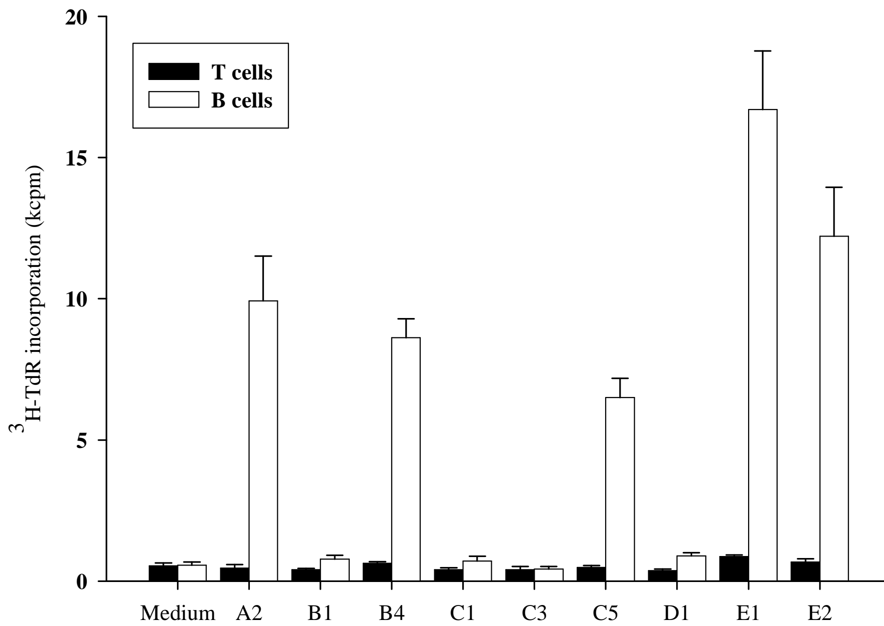


Fig. 1. The proliferative responses of T or B cells treated with the concentrates from the bacterial culture fluids that react to anti-KfspBGS. T and B cells fractionated from spleen of BALB/C mice were distributed into 96-well round bottomed microtiter plates. Added to the wells was 50 μl of the 5-fold diluted concentrates from each of the bacterial culture fluids, and incubated for 48 h. The cell proliferation was measured using the 6 h ^{3}H-TdR incorporation. Each value represents the mean±SD of three independent experiments (*n*=3).

4. Identification of the isolate producing substances specific for B cells

For the identification, we selected the isolate E1 that induced the highest level of ^{3}H-TdR incorporation by B cells. For macro- and microscopic examinations, the isolate E1 was grown in aerobic conditions and found to be Gram-positive spore-forming rod. In the biochemical test using API 50 CHB, the isolate E1 utilized 99.9% identical to the carbohydrates used by *B. licheniformis* (Table 3). When 16S ribosomal DNA gene sequence of the isolate E1 was compared with that of GenBank using BlastN program, the isolate E1 appeared to be closely connected with *B. licheniformis*, as shown in the phylogenetic tree (Fig. 2). These results suggest that the isolate is *B. licheniformis* (hereafter referred as *B. licheniformis* E1).

Table 3. Utilization of carbohydrates by the isolate E1 in API 50 CHB kit[a]

Substrate	Result	Substrate	Result
Glycerol	+	Salicine	+
Erythritol	-	Cellobiose	+
D-Arabinose	-	Maltose	+
L-Arabinose	+	Lactose	+
Ribose	+	Melibiose	-
D-Xylose	+	Saccharose	+
L-Xylose	-	Trehalose	+
Adonitol	-	Inuline	-
β-Methyl-D-Xyloside	-	Melezitose	-
Galactose	+	D-Raffinose	-
D-Glucose	+	Amidon	+
D-Fructose	+	Glycogene	+
D-Mannose	+	Xylitol	-
L-Sorbose	-	β-Gentiobiose	+
Rhamnose	-	D-Turanose	+
Dulcitol	-	D-Lyxose	-
Inositol	-	D-Tagatose	+
Mannitol	+	D-Fucose	-
Sorbitol	+	L-Fucose	-
α-Methyl-D-mannoside	-	D-Arabitol	-
α-Methyl-D-glucoside	+	L-Arabitol	-
N-Acetyl-glucosamine	+	Gluconate	+
Amygdaline	+	2-keto-gluconate	-
Arbutine	+	5-keto-gluconate	-
Esculine	+		

Isolate	Result[b]	Identification %	Comments
E1	*Bacillus licheniformis*	99.9	Very Good Identification

[a]API 50 CHB kit inoculated with the isolate E1 was incubated at 30℃ for 24 or 48 h.
[b]The experimental result was identified using API 50 CHB database V3.0.

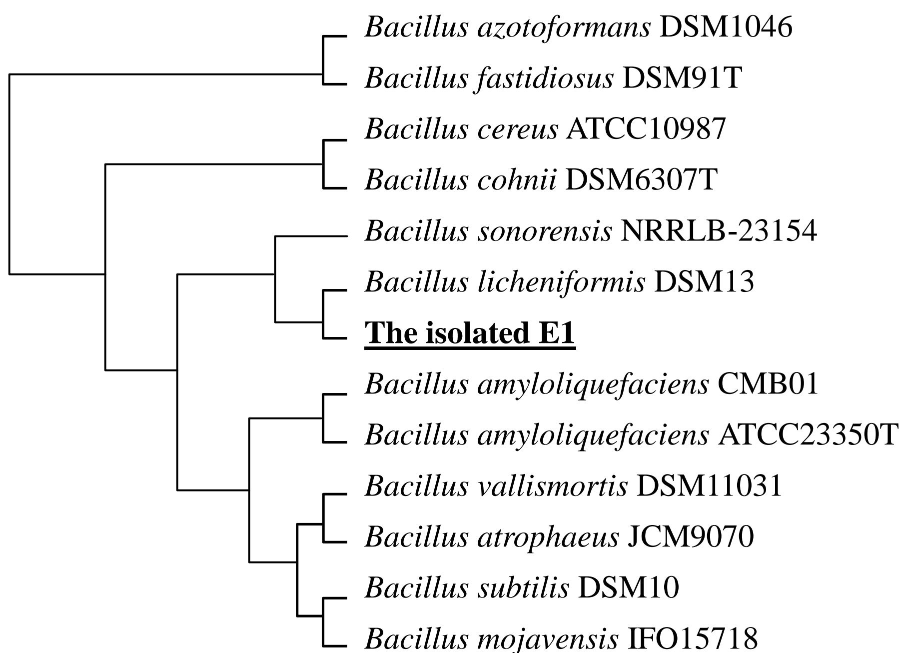


Fig. 2. The phylogenetic tree of the isolate E1 based on homology of 16S rDNA gene sequence. A 16S ribosomal DNA gene sequencing of the isolate was performed by PCR. The universal primers used for amplification of a partial 16S rDNA fragment of the isolate were 27F: 5'-AGAGTTTGATCATGGCTCAG-3' and 1492R: 5'-GGATACCTTG TTACGACTT-3'. The PCR product was ligated into a T vector, and sequenced using an ABI 377 Genetic Analyzer. The 16S rDNA sequence was then aligned with reference sequences obtained from the GenBank databases, using the Blast searches.

5. Purification of bBGS from *B. licheniformis* E1 and determination of its molecular weight

For a mass production of the bBGS, *B. licheniformis* E1 was cultured in BHI broth at shaker for 48 h. Since the bBGS is heat-stable, the supernatant harvested from the bacterial culture was boiled in order to denature contaminated proteins. Purification of the bBGS from the supernatant was performed by the same method as that used to purify the KfspBGS in the previous study[21]. Measurement of molecular weight of the purified bBGS was performed by GPC system, and various molecular weights of dextrans were used for calibration of the column. As shown in Fig. 3, the bBGS appeared to be highly polymerized, having a molecular weight of 1,594 kDa.

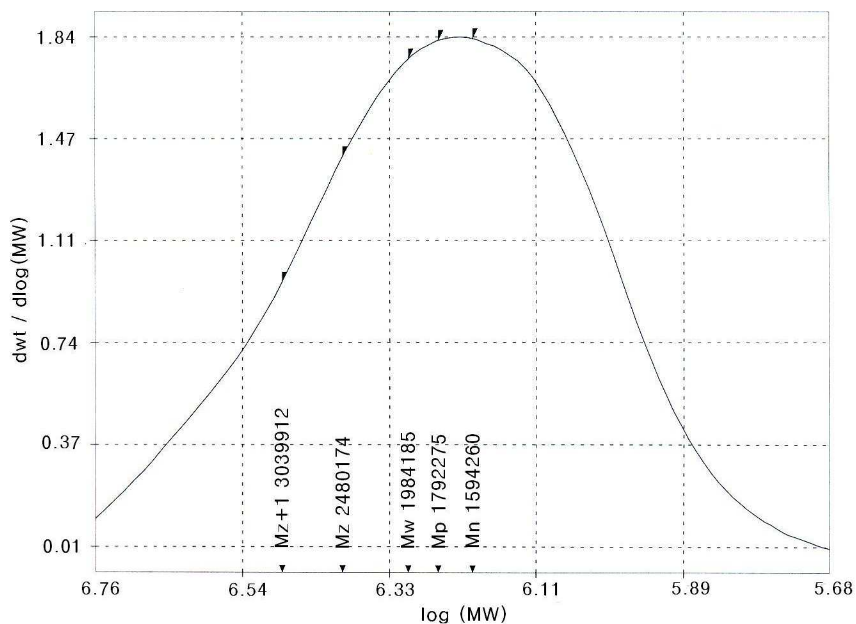


Fig. 3. Determination of molecular weight of bBGS. The molecular weight of the bBGS was estimated by GPC system with a column of ultrahydrogel 250, ultrahydrogel 500 and ultrahydrogel 1000. The running sample was eluted with distilled water. Calibration of the columns was performed using dextrans (10 – 2,000 kDa) as the reference molecular weights.

6. Chemical composition of bBGS

Carbohydrates and proteins of bBGS were analyzed, and their contents were calculated in terms of percentages. The bBGS contained 33% (w/w) of reduced sugar, and its sugars included glucosamine (35.1%), galactose (34.8%), galactosamine (22.6%) and glucose (7.5%) (Table 4). On the other hand, the protein content of the bBGS was shown to be 4.6% (w/w), and its major amino acids included serine (17.2%), proline (11.7%), tryptophan (11.5%) and alanine (10.1%) (Table 5).

Table 4. Sugar composition of bBGS

Sugar	Pmol	mole%
Galactosamine	255	22.6
Glucosamine	396	35.1
Galactose	392	34.8
Glucose	84	7.5
Total	1127	100.0

Table 5. Amino acid composition of bBGS

Amino acid	pmol	mole%	Amino acid	pmol	mole%
Cys	388	4.6	Tyr	406	4.9
Asp[a]	625	7.5	Val	168	2.0
Glu[b]	487	5.8	Met	209	2.5
Ser	1431	17.2	Cys2[c]	50	0.6
Gly	502	6.0	Ile	178	2.1
His	199	2.4	Leu	209	2.5
Arg	89	1.1	Phe	83	1.0
Thr	438	5.3	Trp	955	11.5
Ala	838	10.1	Lys	100	1.2
Pro	979	11.7	Total	8336	100.0

[a] Asp; amount of aspartate and asparagines. [b] Glu; amount of glutamate and glutamine.

[c] Cys2; disulfide linkaged cysteine.

7. FT-IR spectrum of bBGS

To further obtain molecular information, the bBGS was analyzed using FT-IR. As shown in Fig. 4, the FT-IR spectrum of the bBGS showed a absorption peak at 3,280 cm^{-1}, representing a typical OH stretching from a bound sugar group. The peak at 2,925 cm^{-1} is characteristic absorption by the methyl group, and that at 1,737 cm^{-1} is a typical spectrum of uronic acid. The absorption peaks at 1,581 cm^{-1} and 1,414 cm^{-1} indicate amide and carboxyl groups, respectively. These data indicate that the bBGS includes protein. Therefore, the bBGS might be a glycoprotein.

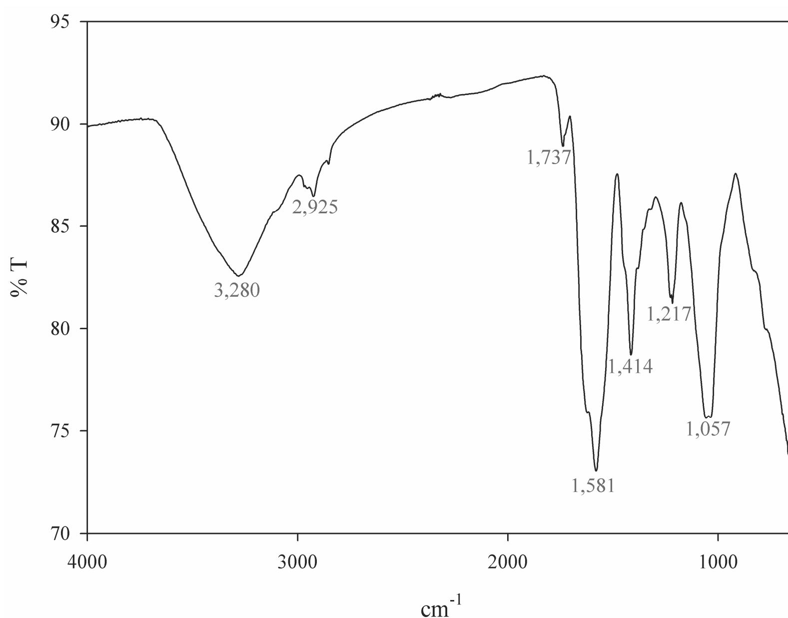


Fig. 4. Infrared absorption spectrum of bBGS. OH group of sugar; 3,280 cm^{-1}, methyl group; 2,925 cm^{-1}, uronic acid; 1,737 cm^{-1}, amide group; 1,581 cm^{-1}, carboxyl group; 1,414cm^{-1}, ester group; 1,217 cm^{-1}.

8. B cell growth activity of the excreta and the slime layer of *B. licheniformis* E1

To investigate whether the bBGS derived from the excreta or from the slime layer of *B. licheniformis* E1, the *B. licheniformis* E1 was cultured in 3 L of BHI broth for 48 h, spun down, and divided into two groups, group A and group B: Group A was a mass of bacteria (5.4 g of wet weight) and group B was a culture fluid. Group A was suspended in 100 ml of saline, heated at 100℃ for 30 min to release the water soluble slime layer from the bacterium, and then spun down. Group B was also heated at 100℃ for 30 min and spun down. The supernatant harvested from each of the groups was concentrated to 50 ml by using DIAFLO ultrafiltration membrane (Filter code: YM 100). Fig. 5 shows B cell growth activities of the concentrates from both group A and B, estimated by ^{3}H-TdR uptake. B cells isolated from spleen of BALB/C mice treated with the diluted concentrates obtained from group B showed the marked proliferative responses, and their ^{3}H-TdR incorporations at 1/40, 1/160 and 1/640 diluted concentrates were 16.46 ± 0.72, 12.32 ± 0.64 and 8.24 ± 0.62 kcpm, respectively. In contrast, the proliferation of B cells treated with the diluted concentrates from group A appeared to be low, compared to those of B cells treated with group B, and their ^{3}H-TdR incorporation at 1/40, 1/160 and 1/640 diluted concentrates were 5.3 ± 0.52, 3.45 ± 0.51 and 2.05 ± 0.39 kcpm, respectively, but were significantly higher compared to that of B cells treated with medium as control. These data indicates that both groups induced the

proliferation of B cells, suggesting that the bBGS might have originated from the slime layer of *B. licheniformis* E1.

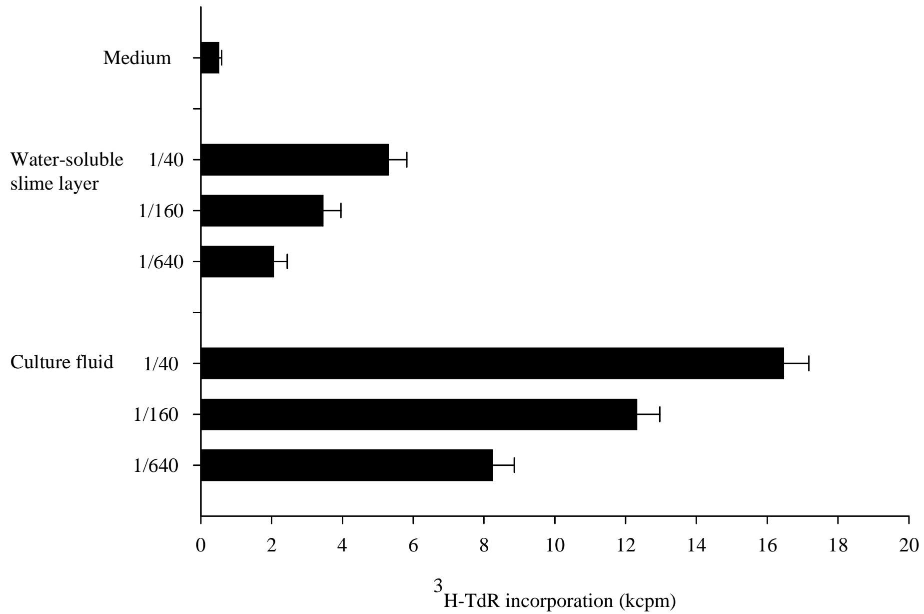


Fig. 5. B cell growth activity of the slime layer and culture fluid obtained from *B. licheniformis* E1. B cells isolated from spleen of BALB/C mice were distributed into 96-well round bottomed microtiter plates. The wells were then added with 50 μl of either the diluted concentrates from the culture fluid or the diluted concentrates from the slime layer, and incubated for 48 h. The cell proliferation was measured using the 6 h ^{3}H-TdR incorporation. Each value represents the mean±SD of three independent experiments (*n*=3).

9. The specific binding of anti-bBGS to the slime layer of *B. licheniformis* E1

To further clarify that the origin of bBGS was the slime layer of the bacteria, *B. licheniformis* E1 cultured in a BHI broth for 24 h was reacted with rabbit anti-bBGS or control rabbit serum, stained with FITC-conjugated goat anti-rabbit immunoglobulin, and analyzed by FACSCalibur flow cytometry. As shown in Fig. 6, *B. licheniformis* E1 showed specific immunoreactivity with anti-bBGS (62.05%, Fig. 6b), but not with normal rabbit serum (1.98%, Fig. 6a). These results indicate that anti-bBGS bind to the slime layer of *B. licheniformis* E1, suggesting that the origin of bBGS is the slime layer of *B. licheniformis* E1.

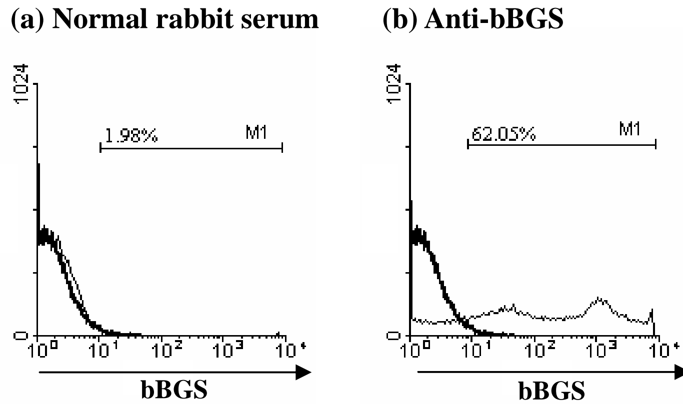


Fig. 6. The specific immunoreactivity of *B. licheniformis* E1 to anti-bBGS. *B. licheniformis* E1 cultured for 24 h was reacted with rabbit anti-bBGS or control rabbit serum, and stained with FITC-conjugated goat anti-rabbit immunoglobulin. The stained bacterium was analyzed by FACSCalibur flow cytometry (a and b). Similar results were obtained with three independent experiments.

10. The effect of bBGS on the proliferation of mouse spleen lymphocytes

Since LPS are well-known as the B cell mitogens[27, 28], bBGS's effects on the proliferation of BALB/C mice lymphocyte were compared to LPS. Lymphocytes isolated from BALB/C mice spleen were treated with various doses of bBGS or LPS, and incubated for 48 h and then measured by ^{3}H-TdR incorporation assay. As shown in Fig. 7, bBGS and LPS induced the lymphocyte proliferation. And the lymphocyte proliferation activities of bBGS and LPS are both shown to be dose-dependent. The optimized concentration doses of bBGS and LPS for mice lymphocyte proliferation were 10 μg/ml and 5 μg/ml, respectively.

As to find out which of the spleen lymphocyte proliferated in responses to bBGS and LPS, the proliferation responses of T and B cell were measured by ^{3}H-TdR incorporation assay. The T and B cell were treated with optimized concentration of bBGS or LPS, and then incubated for 48 h. As expected, both bBGS and LPS induced B cell proliferation, whereas neither bBGS nor LPS affected T cell proliferation. The B cell proliferation measurements for bBGS and LPS were 18.66±1.80 and 20.15±2.19 kcpm, respectively, and the statistical difference between the two was p=0.2004, which was not noticeable (Fig. 8).

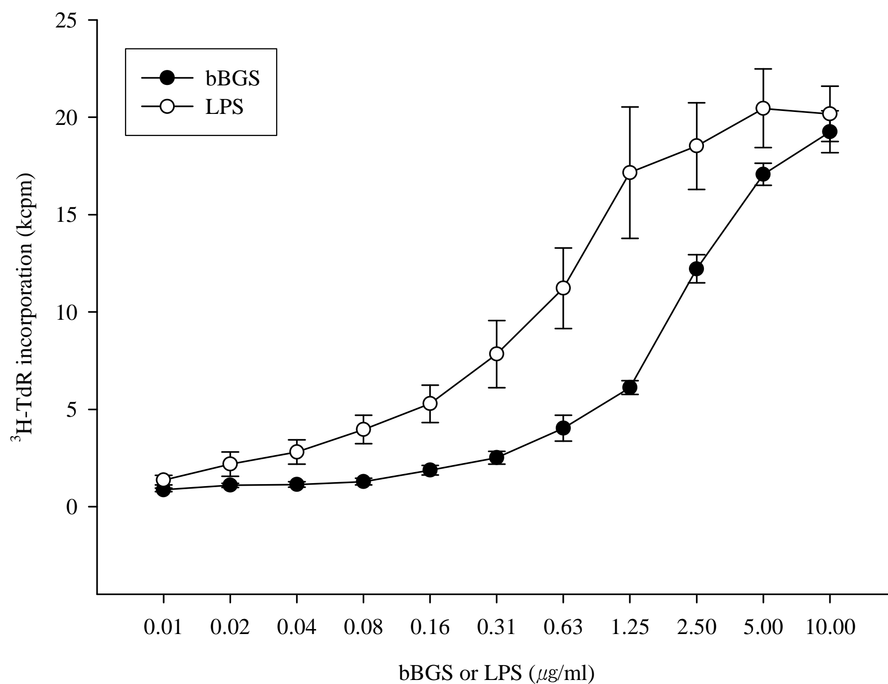


Fig. 7. Effect of bBGS on the proliferation of splenocytes. Splenocytes (2×10^5 cells/200 μl) from BALB/C mice were incubated with various concentrations (horizontal axis) of bBGS or LPS for 48 h and then 6 h ^{3}H-TdR incorporation assay was performed. Each value represents mean±SD of three independent experiments (*n*=3).

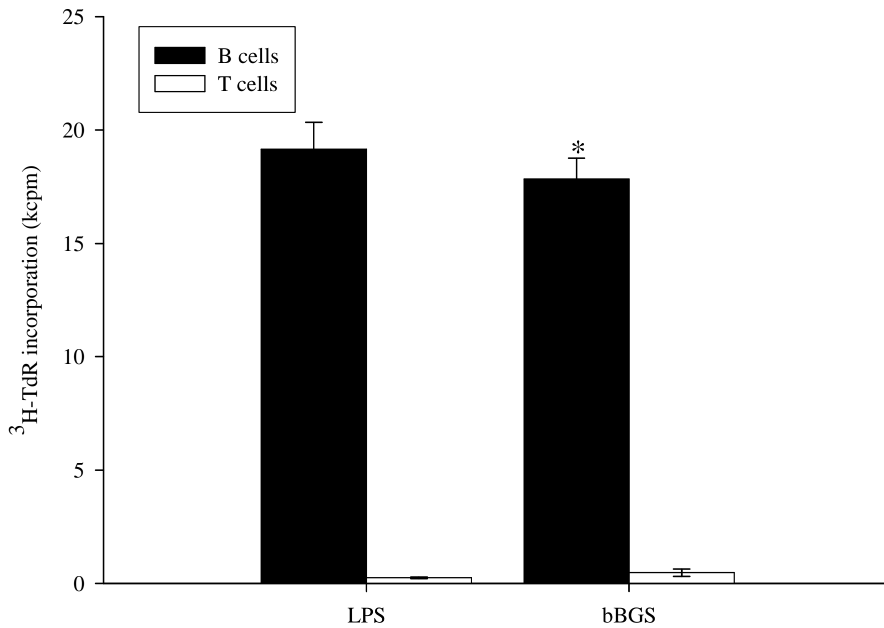


Fig. 8. bBGS induces proliferation of B cells *in vitro*. Spleen B or T cells were cultured for 48 h with bBGS (10 μg/ml) or LPS (5 μg/ml). The cell proliferation was measured using the 6 h ^{3}H-TdR incorporation. Each value represents the mean±SD of three independent experiments (*n*=3). *, Statistical significance from "LPS" (*p*=0.2004).

11. The effect of PMB on the B cell proliferative activity of bBGS

It is the case that both bBGS and LPS affect the increase of B cell proliferation, but bBGS and LPS are different in regards to their origin and structure. To find out whether their acting ingredients to B cell would also be different, the response of bBGS to PMB, an inhibitor of LPS[29], was tested. The spleen B cells isolated from BALB/C mice were treated with bBGS and LPS alone or bBGS+PMB and LPS+PMB, and then incubated for 48 h, and the B cell proliferation was measured by using ^{3}H-TdR incorporation assay. B cell proliferation measurement of the sample treated with the bBGS+PMB was 13.46±2.24 kcpm which showed slight decrease when compared to 17.33±0.12 kcpm, the measurement of that of the bBGS alone treated sample. The shown decrease in statistical measurement between the two bBGS samples is p=0.1179, and the difference is statistically not noticeable (Fig. 9).

LPS responded differently on PMB, compared to those of bBGS. B cell proliferation measurement of the sample treated with the LPS+PMB resulted in 4.95±0.069 kcpm, and that is considerably lower than 23.86±1.52 kcpm which is the measurement for the sample treated with LPS alone. The difference resulted to be p=0.0172, and it is statistically noticeable (Fig. 9).

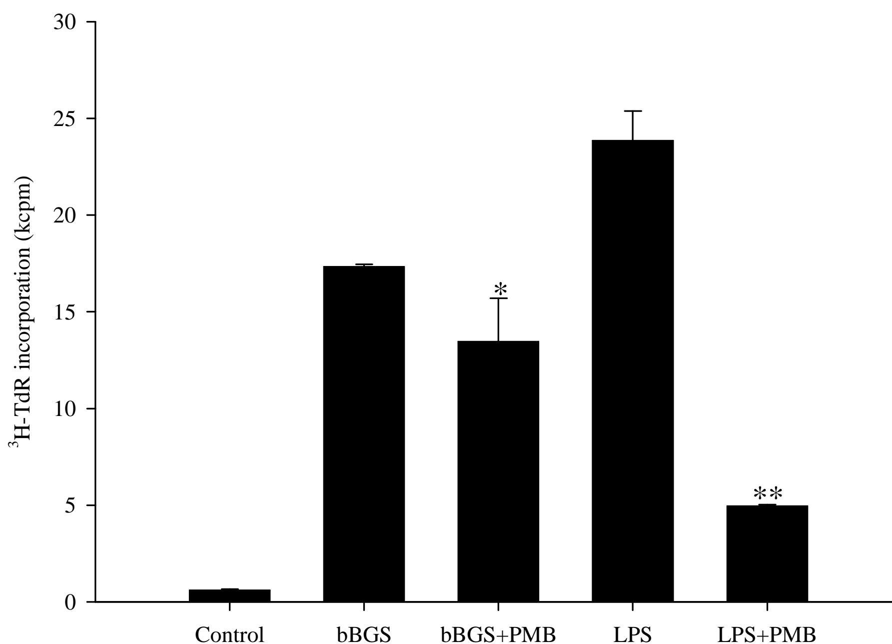


Fig. 9. PMB inhibits the B cell proliferative activity of LPS, but not that of bBGS. The spleen B cells isolated from BALB/C mice were treated with bBGS (10 μg/ml) and LPS (5 μg/ml) alone or bBGS+PMB (5 U/ml) and LPS+PMB. B cell proliferation was measured by ^{3}H-TdR incorporation assay. Each value represents mean±SD of three independent experiments (*n*=3). *, Statistical significance from "bBGS" (*p*=0.1179). **, Statistical significance from "LPS" (*p*=0.0172).

12. bBGS induces the proliferation of B cells isolated from C3H/HeJ mouse lacking Toll-like receptor 4

Inducing B cell response to LPS can be achieved by binding LPS with cell surface Toll-like receptor 4 (TLR4)[30-32]. To find out the difference in terms of the B cell proliferation activity between LPS and bBGS might be originated from the TLR4 binding, we tested how LPS and bBGS affect the splenic B cell proliferation of each C3H/HeJ mouse lacking TLR4[33] and C3H/HeN mouse with intact TLR4. The splenic B cell from C3H/HeJ mice or C3H/HeN mice was treated with bBGS or with LPS, respectively, and incubated for 48 h. As shown in Fig. 10, when splenic B cells from C3H/HeJ mice treated with LPS showed noticeable decrease in the measurement of 11.99±1.39 kcpm when compared to 22.74±2.12 kcpm which is the measurement for C3H/HeN mice. And, it leads to p=0.0021 of statistical significance. However, for those cases treated with bBGS, the splenic B cell proliferation for C3H/HeJ mice and for C3H/HeN mice were each measured to be 18.39±4.22 and 21.30±0.82 kcpm, respectively. The two measurements show only slight yet not noticeable difference, leading to statistical insignificance of p=0.1609.

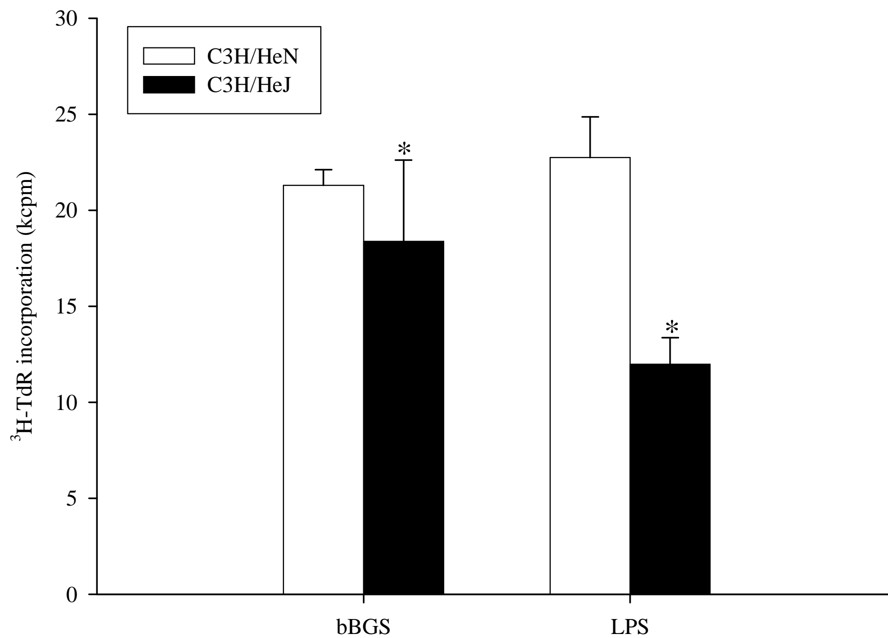


Fig. 10. The B cell proliferative activity of bBGS is not affected by TLR4. Splenic B cells from C3H/HeN and C3H/HeJ mice were stimulated with bBGS (10 μg/ml) or LPS (5 μg/ml) for 48 h. The degree of lymphocyte proliferation was measured by ^{3}H-TdR incorporation assay. Each value represents mean±SD of three independent experiments (*n*=3). *, Statistical significance from "C3H/HeN": *p*=0.1609 (bBGS) and *p*=0.0021 (LPS).

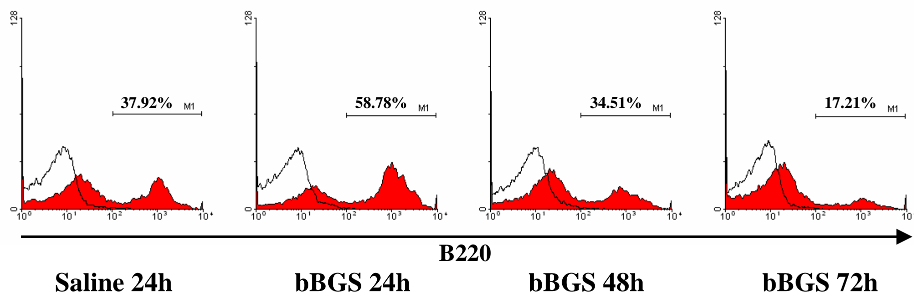
13. Change of B cell population in the bone marrow and spleen of bBGS-treated BALB/C mouse

To test the effects of bBGS on B cell proliferation *in vivo*, BALB/C mice were treated with 25 μg of bBGS per mouse once into the peritoneal cavity and then divided into three sub-groups to be sacrificed after 24, 48, and 72 h. The control group treated with saline was sacrificed after 24 h. Cells harvested from their bone marrow and spleen were stained with CD45R/B22O-PE and then analyzed using FACSCalibur flow cytometry (Fig. 11).

The bone marrow B cell of the bBGS-treated group that was sacrificed after 24 h resulted to be 58.78%, showing marked increase in the B cell population compared to that of the saline-treated control group, 37.92%. The B cell population in the bBGS-treated group, however, appeared to go down to 34.51 and 17.21% when sacrificed after 48 and 72 h, respectively (Fig. 11a).

The splenic B cell population increased in the bBGS-treated group that was sacrificed after 24 h, to 44.23%. And, that of the saline-treated control group which was also sacrificed after 24 h was 39.84%. However, the splenic B cell population in the bBGS-treated group after 48 and 72 h increase to 54.14 and 52.24%, respectively when compared to that of the saline-treated control group (Fig. 11b).

(a) Bone marrow



(b) Spleen

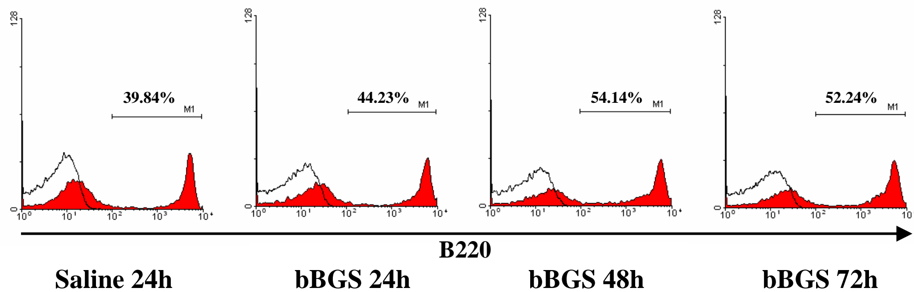


Fig. 11. bBGS increases the B cell population *in vivo*. BALB/C mice were treated intraperitoneally with bBGS (25 μg/mouse) or saline. As lapse of time, mice from each group were sacrificed and B cells were isolated from their bone marrows or spleens. FACS analysis was performed for the staining of CD45R/B220-PE. Similar results were obtained in three independent experiments.

14. Target stage of bBGS in B cell lineage

To find out target stage of bBGS within B cell development, BALB/C mice were treated with 25 μg of bBGS per mouse once into the peritoneal cavity and then sacrificed either after 24 or 48 h. For the control, another group of BALB/C mice were treated with saline, and sacrificed after 24 h. Bone marrow cells and splenocytes were isolated from each group, and MACS bead was used to separate B cells from them. The separated B cells were then stained with FITC-conjugated anti-CD117, -CD25, -IgM, -IgG, and PE-conjugated anti-AA4.1 and -IgD, respectively, on B cell lineage-restricted surface molecules, and then analyzed by FACSCalibur flow cytometry as shown in Fig. 12 and in Fig. 13.

Transitions in the bone marrow-derived B cell lineage are shown in Figure 12. Pro B cells ($CD117^{+}$), a type of B cell progenitor, were increased to 6.89 and 8.01% in the bBGS-treated groups when sacrificed after 24 and 48 h, respectively. The measurements are higher when compared to 3.81% of the saline-treated control group. The cell population for pre B cell ($CD25^{+}$), another type of B cell progenitor, resulted to be 21.71 and 16.34% in the bBGS-treated groups after 24 and 48 h, respectively. The percentages showed no noticeable difference when compared to the cell population of 20.82% in the saline-treated control group (Fig. 12a).

Immature B cells (IgM^{+} and $AA4.1^{+}$) in the bBGS-treated groups came out to be 29.4 and 27.2% after 24 and 48 h, respectively, and those percentages

are slightly higher than 21.2% of the saline-treated control group. Increases in cell population were also shown for mature B cells (IgM^+ and IgD^+), resulting 27.2 and 22.7% in the bBGS-treated groups after 24 and 48 h, respectively, whereas it was 18.4% in the saline-treated control group (Fig. 12b).

IgG^+ B cells showed different percentage changes in cell population, unlike the results of immature or mature B cells. After 24 h, the cell population of IgG^+ B cells in the bBGS-treated group came out to be 36.5%, which showed no noticeable difference when compared to 34.1% of the saline-treated control group. However, the percentage in the bBGS-treated group went down to 26.2% after 48 h (Fig. 12c).

Since the spleen is the secondary lymphoid organ, our studies on B cell lineage for mice spleen when treated with bBGS were limited only to immature B cells and mature B cells, and the results are shown in Fig. 13.

Splenic immature B cells showed 15.2% of the cell population in the bBGS-treated group after 24 h, which was similar to 13.4% of the saline-treated control group. In comparison to the control group, the percentage in the bBGS-treated group was increased to 19.9%, after 48 h. Interestingly, splenic mature B cells showed lower cell populations in the bBGS-treated groups than in the saline-treated control group. The splenic mature B cells showed the population of 46.2% in the saline-treated control group, whereas it showed comparably lower populations of 33.6 and 36.9% in the two bBGS-treated groups, one with 24 h lapse and the latter one with 48 h

lapse, respectively (Fig. 13a).

The population of splenic IgG^+ B cells in the saline-treated control group came out to be 38.6%. After 24 h, the population in the bBGS-treated group was increased to 50.7%. However, the percentage was dropped to 25.3% in the bBGS-treated group, after 48 h. (Fig. 13b).

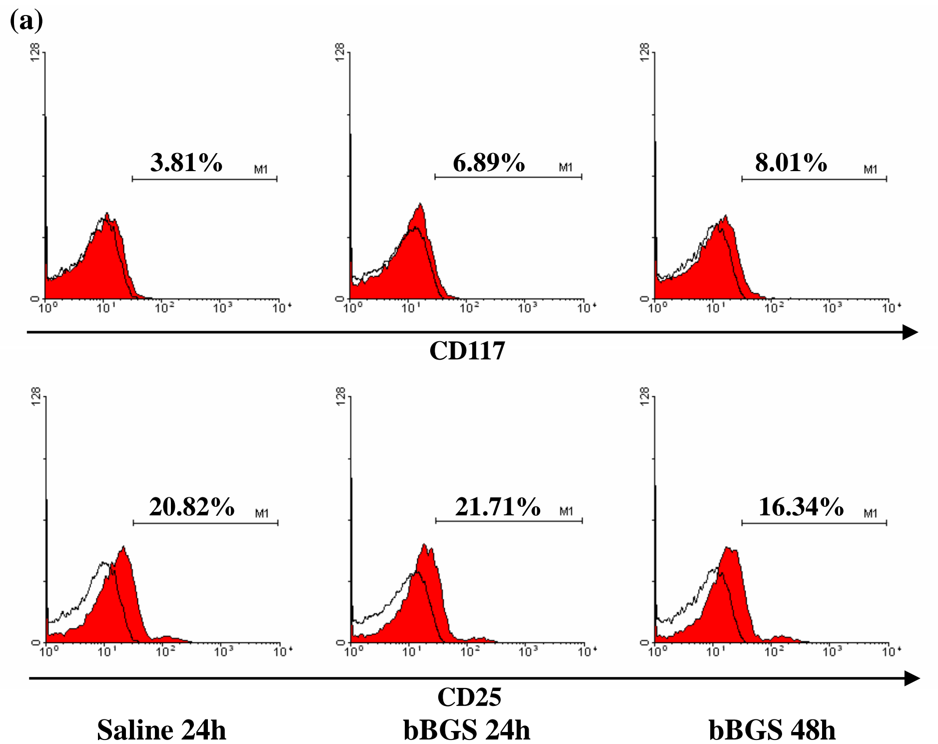
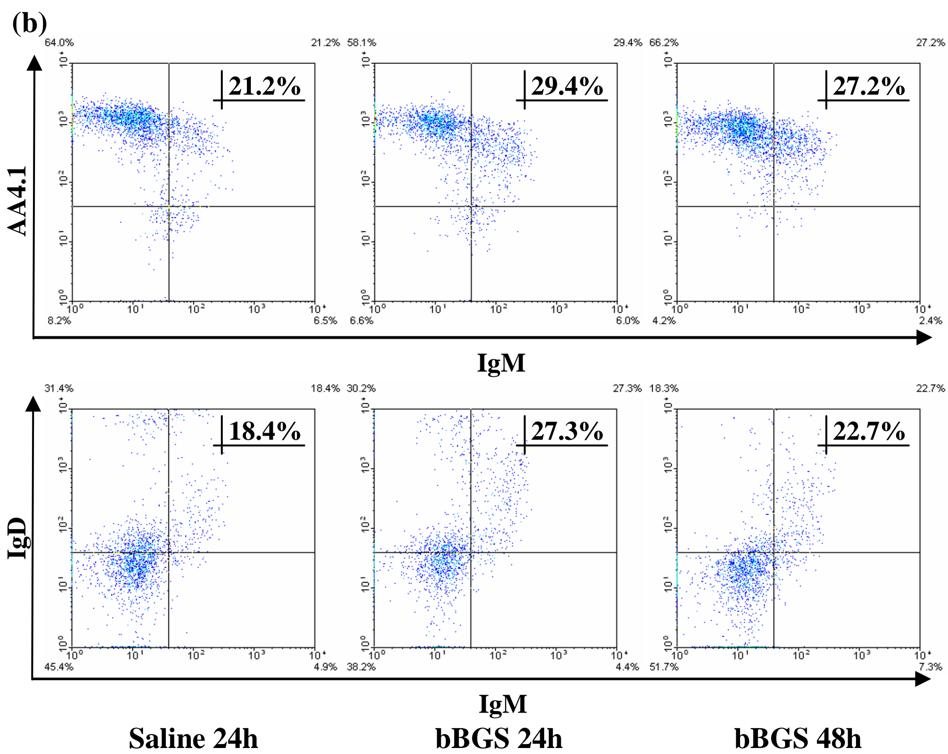


Fig. 12-1. Effect of bBGS on early stage of B lineage cell *in vivo*. BALB/C mice were treated intraperitoneally with bBGS (25 μg/mouse) or saline. As lapse of time, mice from each group were sacrificed and isolated from their bone marrow. (a) FACS analysis was performed using antibodies (CD117-FITC, CD25-FITC). Similar results were obtained in three independent experiments.

(b)



(c)

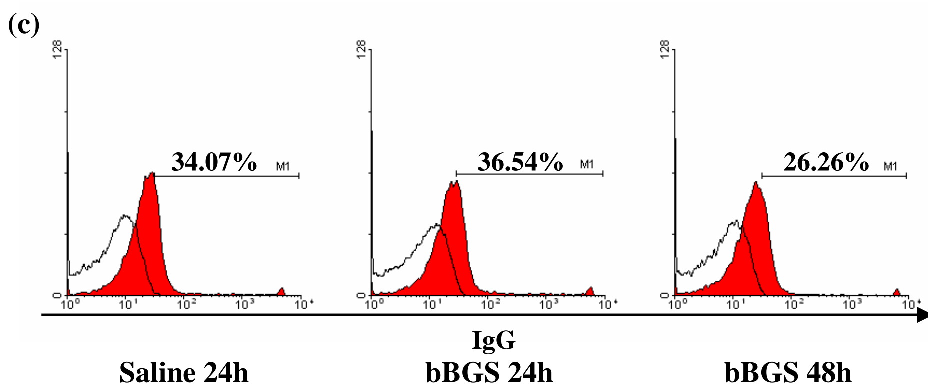
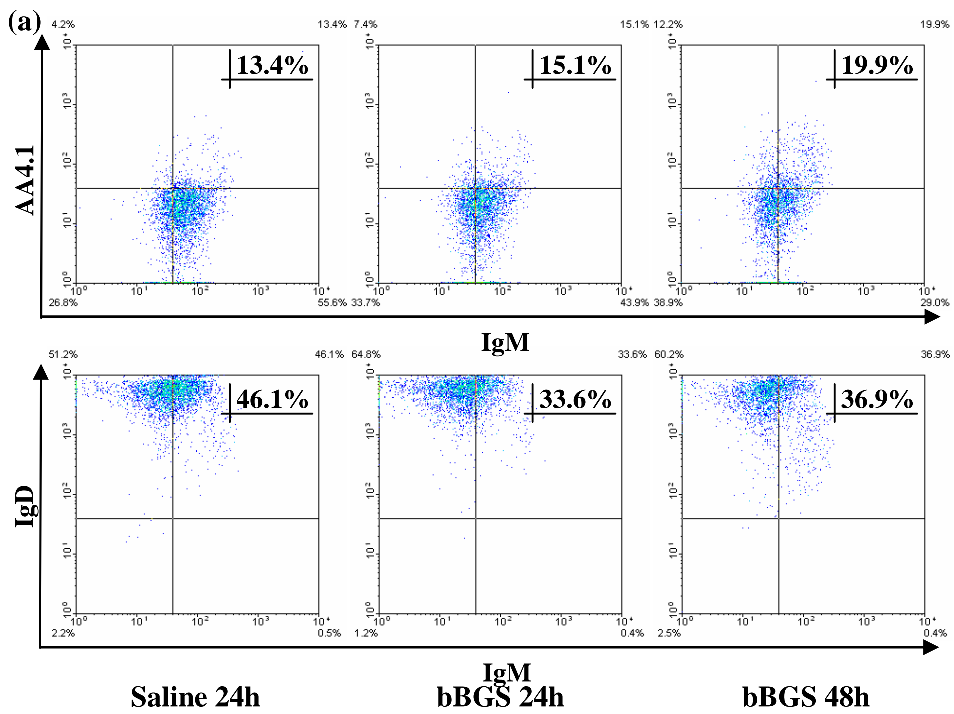


Fig. 12-2. Effect of bBGS on early stage of B lineage cell *in vivo*. BALB/C mice were treated intraperitoneally with bBGS (25 μg/mouse) or saline. As lapse of time, mice from each group were sacrificed and isolated from their bone marrow. (b) FACS analysis was performed using antibodies (IgM-FITC/AA4.1-PE, IgM-FITC/IgD-PE). (c) FACS analysis was performed using antibodies (IgG-FITC). Similar results were obtained in three independent experiments.


(a)
AA4.1
13.4%
15.1%
19.9%
IgM
IgD
46.1%
33.6%
36.9%
IgM
Saline 24h
bBGS 24h
bBGS 48h

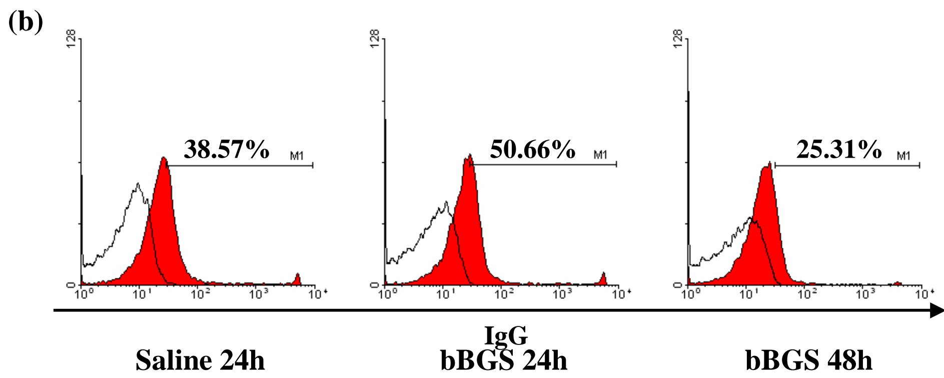

(b)
38.57%
50.66%
25.31%
IgG
Saline 24h
bBGS 24h
bBGS 48h

Fig. 13. Effect of bBGS on splenic B cell differentiation *in vivo*. BALB/C mice were treated intraperitoneally with bBGS (25 μg/mouse) or saline. As lapse of time, mice from each group were sacrificed and isolated from their spleen. FACS analysis was performed using antibodies (IgM-FITC/AA4.1-PE, IgM-FITC/IgD-PE, or IgG-PE). Similar results were obtained in three independent experiments.

15. bBGS increases the concentration of IgM and IgG *in vivo*

To find out the effect of bBGS on antibody-producing in B cells, BALB/C mice were treated with bBGS (25 μg/mouse), once into the peritoneal cavity. The bBGS-treated-mice were then sacrificed after 72 h. Serum was separated from the blood, and the concentrations of IgM and IgG in the serum were measured. The concentrations of IgM and IgG in the saline-treated control group were 0.25±0.15 and 14.26±2.18 mg/ml, respectively, and those of the bBGS-treated group were 7.38±1.60 and 33.63±7.92 mg/ml, respectively. The results showed the concentrations in the bBGS-treated group to be noticeably higher than those in the saline-treated control group (Fig. 14).

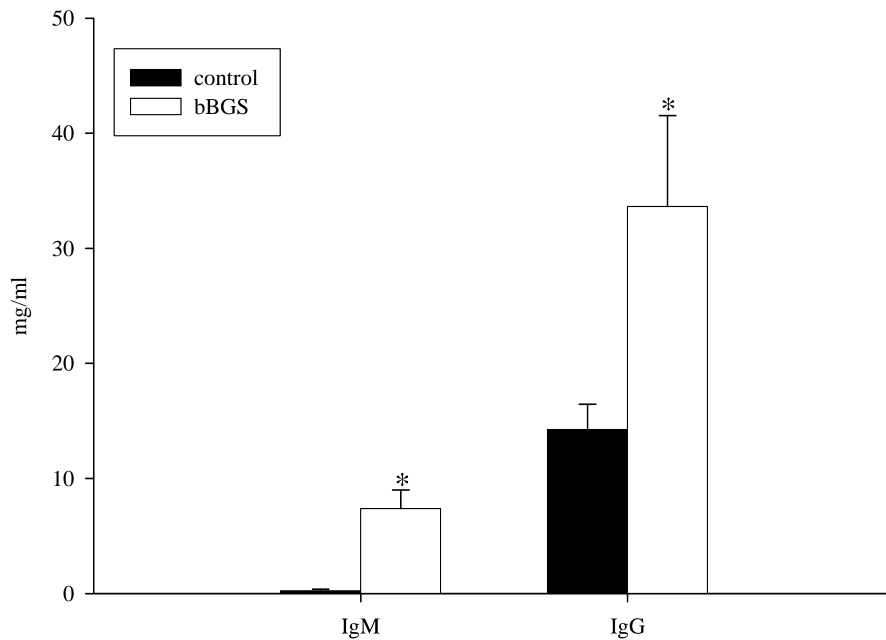


Fig. 14. bBGS increases the levels of serum IgM and IgG. The BALB/C mice were injected with bBGS (25 μg/mouse) once into the peritoneal cavity. Three days later, 3 mice of each group were sacrificed, and their bloods were harvested. The levels of immunoglobulins in their sera were measured by ELISA. Each value represents mean±SD of three independent experiments (n=3). *, Statistical significance from "control": p=0.0016 (IgM) and p=0.0083 (IgG).

16. Adjuvant effect of bBGS

To find out whether bBGS may elicit adjuvant effects in terms of the antigen-specific antibody production, testing of other adjuvants was performed. Groups of BALB/C mice were intradermally sensitized with either OVA alone, or OVA+bBGS, or OVA+Al, or OVA+FA, once a week, for 4 weeks. One week after last sensitization, OVA-specific IgG within their blood serum was measured. The OVA-specific IgG antibody production was greater within the groups that were sensitized with the mixture of OVA and one of the adjuvants than the control group that was sensitized with OVA alone. As shown in Fig. 15, bBGS show almost the same effect as Al but lower than FA, when used as adjuvant.

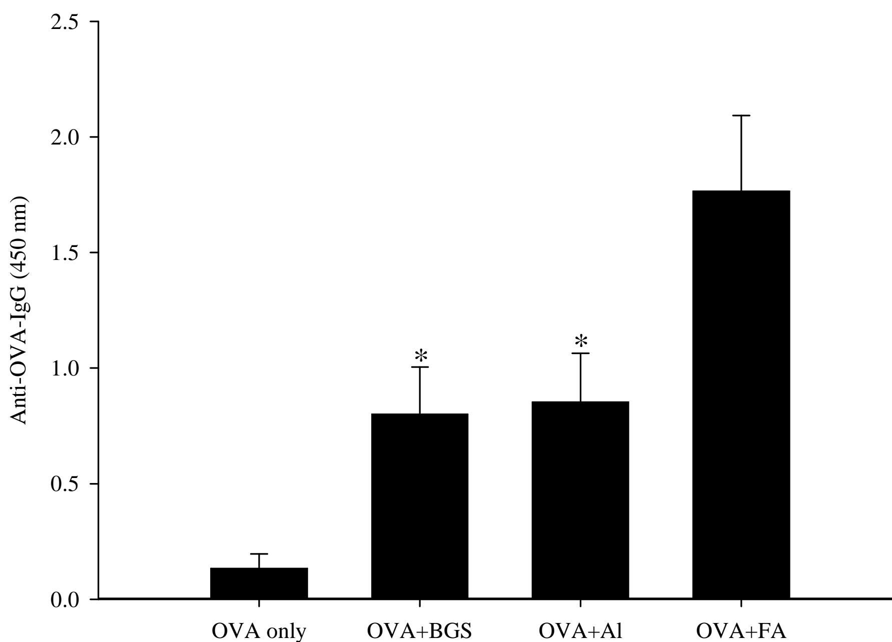


Fig. 15. bBGS affected the OVA-specific antibody production as an adjuvant. The BALB/C mice were intradermally sensitized with a OVA (100 μg/mouse) or a mixture of OVA and adjuvants (bBGS, Al and FA) once a week for 4 weeks. One week after last sensitization, mice from each group were sacrificed and sera were obtained. The levels of OVA specific IgG in the sera were measured using ELISA. Each value represents mean±SD of three independent experiments (n=3). *, Statistical significance from "OVA only": p=0.0044 (OVA+bBGS) and p=0.0035 (OVA+Al).

IV. DISCUSSION

It has been reported that microorganisms produce a great variety of immunomodulator, and most of them appeared to increase the growth, differentiation or activation of T cells but not B cells[3]. Only a few immunomodulators such as LPS and polysaccharide from streptococci have been known to be specific to B cells[1, 27, 28].

We recently have isolated a BGS from Kfsp, and it was ascertained that KfspBGS was only found in the fermented soybean paste but not in the non-fermented one[21]. Evidently, we attempted to isolate the bacteria that produce KfspBGS in Kfsp. We first isolated a total of 31 kinds of bacteria including aerobes and anaerobes in Kfsp. And, because the cell envelope of Gram-negative bacteria contains LPS which functions as B cell mitogen, we then selected 24 kinds of Gram-positive bacteria in order to isolate the bacteria-producing KfspBGS found in Kfsp. Screening for KfspBGS-producing bacteria in 24 kinds of Gram-positive bacteria was performed by ELISA method, using the concentrated culture fluids and anti-KfspBGS that were obtained from KfspBGS-immunized rabbits[21]. The concentrates in the culture fluids from 9 kinds of Gram-positive bacteria were shown to strongly react to anti-KfspBGS. The proliferative response was detected only in B cells and not in T cells, in 5 of 9 kinds of the culture concentrates. The results implicated that KfspBGS was produced in 5 kinds of Gram-positive bacteria that were isolated from

Kfsp. Among 5 isolates, a noticeable increase of B cell proliferation was resulted in the concentrated culture fluid of E1 isolate. Hence, the E1 isolate was selected to be used in the study to find out the characteristics of bBGS. The E1 isolate was identified to be *B. licheniformis* after by the morphological, growth and biochemical tests, and by gene sequencing of 16S-rDNA.

The molecular weight of bBGS was 1,594 kDa which was only slightly different from the weight of KfspBGS of 2,000 kDa. Chemical composition of bBGS was 33% of reduced sugar, and 7.5% of protein. The analysis of the remaining 59.5% has not yet been determined, and therefore further studies are to be followed in the future to complete the remaining chemical composition of bBGS. Components in the sugar were glucosamine, galactose, galctosamine, and glucose. And, the major protein components were consisted of serine, proline, tryptophan, and alanine residues. By using FT-IR, it was revealed that bBGS showed a typical peak of OH stretching from the bound sugar group. There were several peaks that indicate the amide and the carboxyl sugar group as well as the acid sugar group. Since bBGS was found to contain not only the neutral, acid, and amino sugar, but also the proteins, it could be suggested that bBGS may be a glycoprotein.

In the study for the origin of bBGS, both the concentrated culture fluid and the concentrated water-soluble slime layer of *B. licheniformis* E1 induced the proliferation of B cells. The B cell proliferation activity of bBGS was higher in the concentrated culture fluid than the concentrated water-soluble slime

layer, and this might be the release of the slime layer to the culture fluid. The slime layer of bacteria is thus shown to be the origin of bBGS. And, the slime layer being the origin of bBGS can also be supported by high specific immunoreactivity when *B. licheniformis* E1 was treated with rabbit anti-KfspBGS.

Even though bBGS and LPS share no common in their origin and structure, they both induce the growth of B cells. Thus, it was questioned whether their acting ingredients to cause the growth of B cells might be similar to each other. We studied the effect of PMB, an LPS inhibitor, on the functional activity of bBGS, and the response of bBGS to TLR4 that is a receptor of LPS. When PMB+LPS was added, the growth of B cells isolated from BALB/C mice spleen was indeed inhibited. However, regardless of PMB treatment, bBGS still induced the B cell growth. When B cells from C3H/HeJ mice lacking TLR4 were used, LPS could not induce the cell growth in spleen whereas bBGS successfully induced the cell growth. Consequently, unlike LPS, the study showed that bBGS activities are not inhibited by PMB nor be affected by using TLR4 as a receptor, suggesting that their acting ingradients are different.

In the study for the effect of bBGS on the growth of B cells, *in vivo*, the B cell population was shown to be increased both in the bone marrow cells and in the spleen cells from BALB/C mice that were treated with bBGS.

In examining bBGS target in B cell development, B cells were harvested

and separated from the bone marrows and the spleens of the bBGS-treated BALB/C mice. Those B cells were studied by using anti-B cell lineage-restricted surface molecules antibodies. When looked at the B cell population in the bone marrow, only IgG^+ B cells showed no change in cell population compared to the increased cell population in other cells such as less-proportioned pro-B cells, and large-proportioned immature B cells and mature B cells. But as for the B cell population in the spleen, IgG^+ B cells showed high increase when compared to only a little bit of increase in immature B cells and to a decrease in mature B cells. Gathered from the results, it can be derived that when treated with bBGS, the B cell population gets to be increased due to the increase of immature B cells and mature B cells. The outcomes are shown to coincide with results from the precedently conducted researches that B cell progenitor which is then developed to be immature B cells in bone marrow, migrates into spleen and become mature B cells[34, 35], and that a portion of short-lived immature B cells in spleen is differentiated and gets to be long-lived mature B cells[36-38].

Along B cell development, V-D-J rearrangements lead to formation of the clonotypic BCR and surface expression of IgM molecules[39]. Surface lgM (sIgM) of B cells starts class switch recombination (CSR) for $C\mu$ gene, heavy chain constant region, to $C\gamma$, $C\alpha$ or $C\varepsilon$ gene, then by recombination, each gets to be secondary heavy chain isotypes such as IgG, IgA or IgE[40, 41]. In particular, IgG begins to be manifested on the cell surface of memory B cell

stage, and it is known to be the most abundant form of Ig isotype amongst immunoglobulins[42-44]. In this study, the results showed that IgG^+ B cells in bBGS-treated BALB/C mice have not been increased in bone marrow cells but have been largely increased in spleen cells, and therefore could be led to an assumption that bBGS takes part in differentiation of mature B cells into IgG^+ B cells. Such possibility is also supported by the result that the concentrations of IgM and IgG were noticeably increased in the blood serum from the bBGS-treated BALB/C mice.

Adjuvants are usually defined as compounds that can increase and/or modulate the intrinsic lower immunogenicity of an antigen. Adjuvants are therefore required to assist the vaccines to induce potent and persistent immune responses, with additional benefits that like lesser antigen and fewer injections are needed[45]. It was found that bBGS induced the growth of B cells, *in vivo*, and that it increased the IgM and IgG production, and therefore led to assume that bBGS functions as an adjuvant. For the study of adjuvant effects of bBGS, BALB/C mice were sensitized with various mixtures of OVA and bBGS. In the case where bBGS was used as an adjuvant, highly concentrated anti-OVA antibodies were found to be produced in the mice. The adjuvant effect of bBGS was shown to be less than that of Freund's adjuvant which is a highly effective adjuvant using dominantly in animal studies[46]. However, the adjuvant effect of bBGS was almost similar to that of aluminum hydroxide which is used in making vaccine for human[47].

The overall safety concerns including possible immediate and long-term side effects of bBGS as an adjuvant have not yet been studied. However, due to the studied results that bBGS showed an adjuvant effect in producing antigen-specific antibody, bBGS is considered to become a new possible immuno-stimulator or an adjuvant.

In conclusion, we isolated BGS-producing bacterium from Kfsp, and identified it to *B. licheniformis* E1. It was then confirmed that the physical and biological characteristics of bBGS produced by *B. licheniformis* E1 are similar to those of KfspBGS found in Kfsp, and that bBGS is a glycoprotein originated from the slime layer of the bacteria. In addition, bBGS was revealed to increase the proliferation of immature and mature B cells from BALB/C mice. The concentrations of IgM and IgG were noticeably increased in the serum from the bBGS-treated BALB/C mice. The results in the studies show that bBGS shows an adjuvant effect to increase the producing of antigen-specific antibody, *in vivo*.

V. CONCLUSION

It was reported that BGS was found in Kfsp but not in non-fermented soybean paste, thus it was considered that bacteria involved in fermentation of Kfsp to be the cause to produce the KfspBGS. In this study, the bacterium which produced BGS was isolated from Kfsp, and was identified as *B. licheniformis* E1. The bBGS was originated from the slime layer of the bacterium, and appeared to be a glycoprotein that shares similar physical and biological characteristics to those of KfspBGS.

bBGS was not affected by polymixin B or by TLR4, and has resulted in the growth of B cells.

When BALB/C mouse was treated with bBGS, increases of B cell population were resulted in the bone marrow and in the spleen. In the bone marrow, the increase was marked at 24 h after treatment with bBGS, and, in the spleen at 48h.

Through the analysis of B cell population increased by bBGS, it was found that immature B cells and mature B cells were increased in the bone marrow. Whereas in the spleen, mature B cells decreased, and IgG^+ B cells increased at 24 h after bBGS treatment.

When bBGS was injected with OVA antigen into peritoneal cavity of BALB/C mice, the high concentration of OVA-specific antibody was found in their sera. The increase of OVA-specific antibody titer induced by bBGS was found to be similar to that induced by aluminum hydroxide.

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ABSTRACT (IN KOREAN)

Bacillus licheniformis E1에서 분리한 B 세포 특이 성장물질의 특성

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김 주 영

최근 식품의 기능성에 대한 연구가 활발해지면서 한국의 전통발효된장(Korean-style fermented soybean paste; Kfsp)에서 삶은 콩에서는 존재하지 않는, 특이적으로 B 세포의 성장을 증가시키는 물질(BGS)이 존재한다는 사실이 보고되었다. 이에 본 연구에서는 Kfsp가 미생물에 의한 발효식품이라는 점을 감안하여, Kfsp로부터 BGS를 생산하는 미생물의 분리를 시도하였고, *Bacillus licheniformis* E1이라 명명하였다. *Bacillus licheniformis* E1에서 생산되는 bBGS는 세균의 slime layer에서 기원하는 당단백 물질로서, Kfsp에서 추출한 BGS와 유사한

특성을 나타내었다. B 세포의 증식을 유발함에서 bBGS는 LPS와는 다르게, LPS의 억제제인 PMB와 LPS 수용체인 TLR4에 영향을 받지 않았다.

bBGS를 BALB/C 마우스 생체 내로 투여하였을 때, 골수 및 비장에서 B 세포 집단이 증가하였다. 특히, 골수의 B 세포 집단은 bBGS 투여 후 24시간에서, 비장의 B 세포 집단은 48시간에서 증가하였다.

bBGS 투여 후 나타나는 B 세포 분화단계를 조사한 실험에서, BALB/C 마우스의 골수에서 immature B 세포와 mature B 세포가 증가되었다. 한편, 비장에서는 bBGS 투여 후 mature B 세포가 감소하였으나 IgG^{+} B 세포는 두드러진 증가를 나타내었다. 또한, bBGS를 OVA와 함께 BALB/C 마우스의 복강 내로 투여하였을 때, 혈청 내에서 높은 수준의 OVA 항원에 특이적인 항체가 생성되었다.

Bacillus licheniformis E1의 배양을 통한 bBGS의 대량생산은 면역학 분야에서의 B 세포에 관한 연구에 기여할 것이며, 의학적으로 B 세포 증식이 필요한 환자를 대상으로 한 면역강화요법에 도움이 될 것으로 기대된다. 또한, bBGS는

백신제조에 있어서 새로운 adjuvant로서 기여할 것으로 예상된다.

핵심되는 말: B 세포 특이 성장물질(BGS), 한국 전통발효된장(Kfsp),
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