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# Isolation of Bacteria Producing a B-Cell-Specific Biological Response Modifier Found in Korean Fermented Soybean Paste

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**Abstract** In a previous study, a biological response modifier (BRM) specifically enhancing the function of B-cells was isolated from Korean fermented soybean paste (Kfsp), but not from non-fermented soybeans. In this study, we attempted to isolate the bacteria producing the BRM from Kfsp (KfspBRM) by ELISA using anti-KfspBRM and by B-cell proliferation. Five bacteria whose culture supernatants showed the BRM activities were isolated, and one of them was identified as *Bacillus licheniformis* E1. The bacterial BRM (bBRM) originated from a slime layer of *B. licheniformis* E1 had a molecular weight of 1,594 kDa, and contained 33% (w/w) of reduced sugar and 4.6% (w/w) of protein content. The bBRM appeared to be a glycoprotein that is physically, structurally, and functionally similar to the KfspBRM, suggesting that the isolates including *B. licheniformis* E1 may produce the KfspBRM in the fermentation process of soybean paste. The mass production of the BRM by the bacterium may help to study B-cells in immunology, and the enrichment of the BRM in Kfsp may help patients in future who are medically in need of potentiation of B-cell proliferation and antibody production.

**Key words:** *Bacillus licheniformis*, biological response modifier (BRM), B-cell-specific BRM

Several classes of compounds including proteins, peptides, lipopolysaccharides, glycoproteins, and lipid derivatives are recently being known as BRMs that upregulate or downregulate the host immune system [32]. Certain polysaccharides, produced by microorganisms that were introduced by researchers, act as potent BRMs. Most of the polymers show specific activity for both T-cells and antigen-presenting cells (APCs) such as monocytes and macrophages, but not for B-cells [32]. One Kfsp, Doenjang, is a traditional food produced through the fermentation of soybeans by bacteria and fungi in nature, and has been consumed for centuries as a protein source and flavoring [19]. In the investigation of the functionality of foods, Kfsp has been shown to contain various biologically active substances, including antitumor [1, 16], antithrombosis [26], antioxidants, and hydrophilic brown pigment [3, 4]. Recently, we isolated BRM from Kfsp and found that the BRM induces the proliferation of B-cells, but not T-cells, and that it enhances the antibody production of B-cells. The BRM was detected only in fermented soybean paste and not in non-fermented soybean paste [19], suggesting that microorganisms involved in the fermentation process of soybean paste might produce the BRM.

In this study, we attempted to isolate the KfspBRM-producing bacteria from the Kfsp, and investigated the characteristics of the BRM produced by the isolate.

## Materials and Methods

### Animals

BALB/c female mice between 6 and 7 weeks of age and female rabbits (2.5 kg/rabbit) were purchased from the Korean Advanced Institute of Science and Technology (Daejeon, Korea) and Samtaco Animal Farm (Osan, 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 the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC), for the care and use of laboratory animals.

### Isolation of Bacteria from Kfsp

Kfsp was obtained from Sunnchang Traditional Foods Co. (Sunchang, Korea). Ten g of samples were harvested from

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the top, middle, and bottom of the jar containing the Kfsp, and then pooled. One g of the pooled sample was suspended in 10 ml of saline. One ml of the suspension was harvested and spun down at room temperature (RT) and 10,000 ×*g* for 10 min using a microfuge (Type; A-14, Jouan, Milan, Italy). The supernatant was discarded, and the sediment was suspended in 1 ml of saline. The suspension was diluted at various concentrations with saline, inoculated on brain heart infusion (BHI: Difco, Becton Dickinson and Company, Sparks, MA, U.S.A.) agar plates supplemented with 5% of NaCl, and cultured at 37°C 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.

**Screening of KfspBRM-Producing Bacteria**

The isolates obtained from Kfsp were inoculated in 20 ml of BHI broth in 50-ml plastic tubes and cultured by shaking at 37°C under aerobic and anaerobic conditions. Their incubation periods were 2 d for aerobes and 5 d for anaerobes. The cultured fluids were boiled and then spun down at 4°C at 10,000 ×*g* for 1 h using a Sorvall centrifuge (Type; ss-34, Thermo Electron Corporation, Asheville, NC, U.S.A.). Ten ml of the supernatant was concentrated to 1 ml by using DIAFLO Centricon Plus-80 (Amicon, Inc., Bedford, MA, U.S.A.). The concentrate (100 µl) obtained from each of the isolates was mixed with 200 µl of carbonate-bicarbonate coating buffer, and the mixture (100 µl/well) was coated on a 96-well ELISA plate (Nunc, Copen-hagen, Denmark) at 4°C for 24 h. The plates were washed three times with phosphate-buffered saline containing 0.05% Tween 20 (PBST) and blocked with 150 µl/well of PBS containing 10% bovine serum albumin (Sigma-Aldrich, Steinheim, Germany) for 2 h at RT. The primary antibody used was anti-KfspBRM prepared in the previous study from rabbits immunized with KfspBRM [19], and the secondary antibody was peroxidase-conjugated goat anti-rabbit immunoglobulin (PharMingen, San Diego, CA, U.S.A.). The reaction products were read with an ELISA plate reader at 450 nm.

**Preparation of T-Cells and B-Cells**

T-cells and B-cells were obtained from the lymphocytes of mouse spleen. A single cell suspension of lymphocytes was prepared by gently teasing the spleen between two glass slides. Then, the contaminated red blood cells were lysed using an ammonium-chloride-potassium lysing buffer [17]. The cells ($2\times10^7$ cells/ml) were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 2.2 mM sodium bicarbonate, 100 U/ml penicillin, and 100 µg/ml streptomycin (GIBCO BRL, Grand Island, NY, U.S.A.) (hereafter, referred to as the complete medium). To discard adherent cells, the cells were cultured in 75 $cm^2$ plastic culture flasks (Corning Inc., NY, U.S.A.) for 3 h at 37°C in a humidified 5% $CO_2$ incubator (hereafter referred to as the incubator). Nonadherent cells were harvested by gently shaking the culture flasks. T-cells and B-cells from nonadherent cells were isolated by negative selection with Dynabeads mouse pan B (B 220) and pan T (Thy1.2) (Dynal AS, Oslo, Norway), respectively, according to the manufacturer's direction.

**Assay for B-Cell-Specific BRM**

The activity of BRM produced by bacteria was measured by the proliferation of B-cells. The fractionated B-cells in a complete medium ($2.5\times10^5$ cells/well) were plated in 96-well round bottomed microtiter plates (Corning). Fifty ml of the bacterial culture fluids or the purified bBRM was added to each well, and the plates were cultured in an incubator for 48 h. The cell proliferation was measured using the technique of 6 h $^3$H-tymidine ($^3$H-TdR, New England Nuclear, Boston, MA, U.S.A.) incorporation.

**Identification of Bacteria**

The isolate that showed a high level of BRM activity was identified by *Bergey's Manual of Systematic Bacteriology* [28], based on general characteristics, and by sequencing the 16S DNA gene. For biochemical testing, the isolate was cultured in a nutrient broth at 30°C for 24 h. After cultivation, the cultured suspension was inoculated in an API 50 CHB kit (bioMerieux Co., Marcy 1'Etoile, France), incubated at 30°C for 24 or 48 h, and identified by using the API 50 CHB database v. 3.0. The gene sequencing of the 16S rDNA of the isolate was performed by PCR technique. To amplify a partial 16S rDNA fragment of the isolate, universal primers (27F:5'-AGAGTTTGATCATGGCTCAG-3' and 1492R:5'-GGATACCTTGTTACGACTT-3') were used. The amplified PCR product was ligated into a T vector (Invitrogen Co., Seoul, Korea), and DNA sequencing was performed using an ABI 377 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, U.S.A.). The 16S rDNA sequence was then aligned with reference sequences obtained from the GenBank databases (NCBI, Bethesda, MD), using the Blast searches (http://www.ncbi.nlm.nih.gov).

**Purification of Bacterial BRM**

Purification of BRM from the bacterial culture fluid was performed by the method used for isolation of KfspBRM in the previous study [19]. Briefly, the isolate E1 producing BRM was cultured in BHI broth on a shaker at 37°C for 2 d. The cultured fluid was spun down at 10,000 ×*g* at 4°C for 1 h. The harvested supernatant was boiled at 100°C for 30 min and spun down again in the same way as above. The supernatant was concentrated using a DIAFLO ultrafiltration membrane (Filter code: YM 100, Millipore Corporation, Bedford, MA, U.S.A.). Five ml of the concentrate was applied to a column (25×330 mm) of DEAE Sepharose Fast Flow (Pharmacia, Biotech, AB Uppsala, Sweden)

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 ml of the fractions was collected, and monitored at 280 nm, and their BRM activities measured by mouse B-cell proliferation assay. Those elutes showing bBRM activity were pooled and concentrated using the same ultrafiltration membrane. One ml of the concentrate was subjected to a column (16×900 mm) of Sephacryl S-500 (Amersham Biosciences, Uppsala, Sweden) previously equilibrated with the phosphate buffer and eluted with the same buffer at a flow rate of 1 ml/min. Three ml of each fraction was collected and monitored at 280 nm. Those eluates rich in bBRM were pooled and concentrated by the same ultrafiltration membrane. The concentrate was again subjected to a Superose TM 6HR column (Amersham Biosciences), and the fractions were monitored at 214 nm. The fractions at the peak of bBRM activity were collected, pooled, concentrated, and freeze-dried (the BRM produced by the isolate E1 is hereafter referred to as bBRM).

**Molecular Weight Analysis**

The molecular weight of bBRM was estimated by the gel permeation chromatography (GPC) system with a column of ultrahydrogel 250 (7.8×300 mm), ultrahydrogel 500 (7.8×300 mm), and ultrahydrogel 1000 (7.8×300 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).

**Carbohydrate Analysis**

The total carbohydrate content was quantified by the method of Dubois *et al.* [7]. The bBRM (75 µl) was mixed with 5% phenol reagent (75 µl), and 375 µl of $H_2SO_4$ was also added to the solution. The mixed solution was allowed to stand in an ice bath for 5 min and reacted at 80°C 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 bBRM was analyzed by a modified method of Park and Yun [24]. Briefly, the bBRM was hydrolyzed with 2 M trifluoroacetic acid (TFA) for the neutral sugar and 6 N HCl for the amino sugar at 100°C. A Dionex CarboPac PA column (4.5×250 mm) was used, and the hydrolyzates were analyzed using Bio-LC DX-600 (Dionex, Sunnyvale, CA, U.S.A.) with an electrochemical detector (Dionex ED50). The column was eluted with 16 mM sodium hydroxide at a flow rate of 1 ml/min.

**Protein Analysis**

The total protein content of bBRM was determined by the method of Ohnishi and Barr [22]. The bBRM (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 bBRM was estimated by spectrophotometery at 752 nm, and bovine serum albumin (Sigma-Aldrich) was used as a standard protein. The amino acid composition of bBRM was carried out by the Pico-Tag method [29]. After hydrolysis with HCl at 110°C for 24 h, derivatization of the hydrolyzed bBRM 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, U.S.A.) 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°C.

**FT-IR Spectrum Analysis**

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

**Antibody to bBRM**

The bBRM was used as antigen. A rabbit was immunized subcutaneously (s.c.) with a mixture of the bBRM and complete (first week) or incomplete (the rest of 3 weeks) Freund's adjuvant (Sigma-Aldrich) once a week for 4 weeks. The antibody from the rabbit was referred to as anti-bBRM and stored at –20°C.

**Adherence of Anti-bBRM or Anti-KfspBRM to a Bacterial Slime Layer**

*B. licheniformis* E1 was cultured in BHI broth for 24 h and washed 3 times with saline. The bacterium was treated with 10% formaldehyde for 1 h, washed 3 times with saline, and reacted with either anti-bBRM, anti-KfspBRM, or control rabbit serum. After washing with saline, the bacteria were stained with FITC-conjugated goat anti-rabbit immunoglobulin (PharMingen) and analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, LA, U.S.A.) or captured using a confocal microscope with COMPIX software (Carl Zeiss, Oberkochen, Germany).

**Adherence of bBRM to Lymphocytes**

The fractionated T-cells or B-cells ($1\times10^6$ cells/ml) from a normal mouse were treated with 2 µg of bBRM or saline at 37°C for 1 h. After washing with phosphate-buffered saline, the cells were reacted with anti-bBRM at 4°C for 30 min, washed with PBS, and then stained with FITC-conjugated goat anti-rabbit immunoglobulin at 4°C for 30 min.

The cells were captured using a confocal microscope with COMPIX software.

### Statistical Analysis

All data were expressed as mean±SD of three independent experiments ($n$=3). The differences were analyzed using the Student's $t$-test, but the statistics did not show when the standard deviation divided by the mean was small and the difference between groups was large.

## RESULTS

### 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°C for 2 to 5 d under either aerobic or anaerobic conditions. The bacteria cultured 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), whereas anaerobic isolates appeared to include 4 kinds of $Gram^+$ bacteria (3 rod and 1 coccus forms) and 2 kinds of $Gram^-$ cocci.

### Selection of Bacteria that Produce Substance Reactive with Anti-KfspBRM

Among 31 isolates, only 24 kinds of Gram-positive bacteria were cultured in BHI broth under aerobic or anaerobic conditions, and their culture fluids were boiled at 100°C for 30 min. The serological responses of the concentrates from the bacterial culture fluids to anti-KfspBRM that were obtained from a rabbit immunized with KfspBRM 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-KfspBRM. Their reactions were more than 0.5 of optical density (O.D.). In contrast, those from the rest of the isolates (13 strains) showed low levels of O.D. (below 0.1) in the reaction with the antibody (data not shown).

**Table 1.** Isolation of bacteria from Kfsp[a] fermented for 1 year in nature environment.

| 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 that contained Kfsp.
[b]Isolation of bacteria was based on the morphology of colony and Gram's staining.

### The Biological Effect of the Bacterial Culture Concentrates, Reactive with Anti-KfspBRM, on the Proliferation of T-Cells or B-Cells

It was assessed that the concentrated culture fluids obtained from each of the 9 isolates strongly reacted with anti-KfspBRM. Therefore, to find out whether the concentrates would share the same biological function with KfspBRM, the effect of the concentrated culture fluid on the proliferation of B-cells and T-cells was examined by 6 h-$^3$H-TdR incorporations. As shown in Fig. 1, high levels of $^3$H-TdR incorporation by B-cells were shown in 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 from groups 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-cell 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 was similar to that of T-cells treated with the medium. Even if the

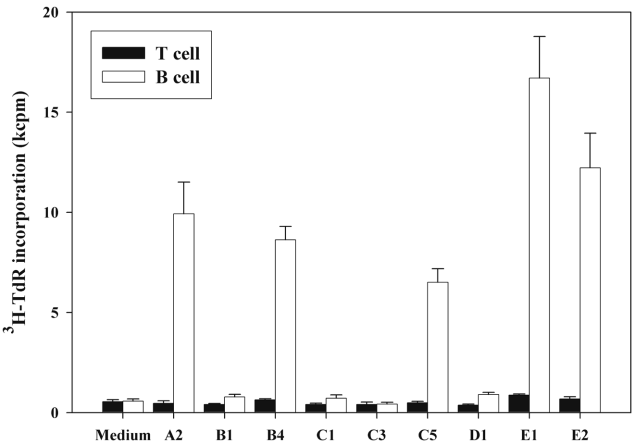


**Fig. 1.** The proliferative responses of T-cells or B-cells treated with the concentrates from the bacterial culture fluids that reacted with anti-KfspBRM.

T-cells and B-cells fractionated from mouse spleen were distributed into 96-well round-bottomed microtiter plates. Fifty μl of 5-fold diluted concentrates from each of the bacterial culture fluids was added to the wells, and the mixtures were incubated for 48 h. The cell proliferation was measured using the technique of 6 h $^3$H-TdR incorporation. Each value represents mean±SD of three independent experiments ($n$=3).

concentrated culture fluids from each of the 9 isolates showed the serologic responses to anti-KfspBRM in the ELISA test as shown above, their biologic 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 bBRM specific for B-cells, but not for T-cells.

### Identification of the Isolate Producing BRM Specific for B-cells

For further study of the bacterial BRM, we selected the isolate E1 that induced the highest level of $^{3}$H-TdR incorporation by B-cells, and performed the identification of the isolate. For macro- and microscopic examinations, the E1 isolate was grown in aerobic conditions and found to be Gram-positive spore-forming rod. In the biochemical test using API 50 CHB, the E1 isolate utilized 99.9% of carbohydrates used by *B. licheniformis* (data not shown). When the 16S rDNA gene sequence of the E1 isolate was compared with that of GenBank using the BlastN program, the E1 isolate 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*. Therefore, we referred to the E1 isolate as *B. licheniformis* E1.

### bBRM Activities of the Excreta and the Slime Layer of *B. licheniformis* E1

To investigate whether the bBRM originated 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, A and B: Group A was a mass of bacteria (5.4 g of wet weight) and group B was a culture fluid (3 l). Group A was suspended in 100 ml of saline, heated for release of water-soluble slime layer from the bacterium at 100°C for 30 min, and spun down. Group B was also heated at 100°C for 30 min and spun down. The supernatant harvested from each of the groups was concentrated to 50 ml by using a DIAFLO ultrafiltration membrane (Filter code: YM 100). Figure 3 shows the bBRM activities of the concentrates from groups A and B, estimated by proliferation of B-cells. B-cells treated with the diluted concentrates obtained from group B showed 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 with 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 significant compared with that of B-cells treated with medium as control. This means that both groups increased the proliferation of B-cells, suggesting that the bBRM might have originated from the slime layer of *B. licheniformis* E1.

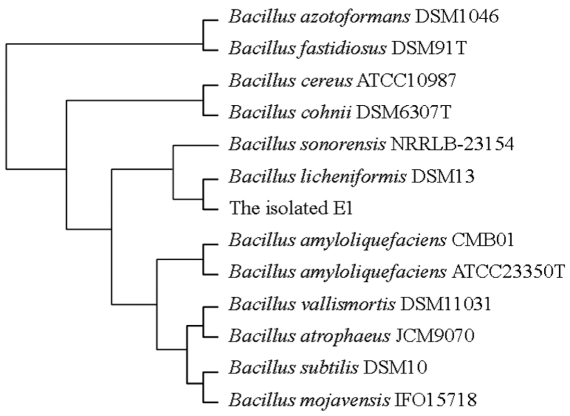


**Fig. 2.** The phylogenetic tree of the E1 isolate based on homology of the 16S rDNA gene sequence.

A 16S rDNA 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'. They were ligated into a T vector, and their products were 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.

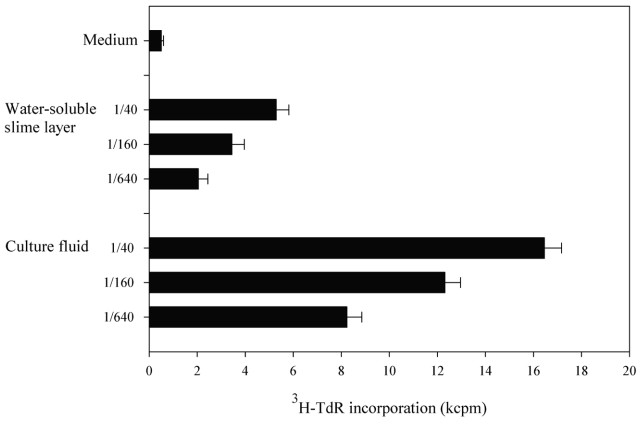


**Fig. 3.** BRM activity of a slime layer and a culture fluid obtained from *B. licheniformis* E1.

B-cells fractionated from mouse spleen were distributed into 96-well round-bottomed microtiter plates. Fifty μl of either the diluted concentrates from the culture fluid or the diluted concentrates from the slime layer were added to the wells, and they were incubated for 48 h. The cell proliferation was measured using the technique of 6 h $^{3}$H-TdR incorporation. Each value represents the mean±SD of three independent experiments ($n$=3).

### The Specific Binding of Anti-bBRM and Anti-KfspBRM to the Slime Layer of *B. licheniformis* E1

To further clarify that the origin of bBRM or KfspBRM was the slime layer of the bacteria, *B. licheniformis* E1 cultured in a BHI broth for 24 h was reacted with rabbit anti-bBRM, rabbit anti-KfspBRM, or control rabbit serum, stained with FITC-conjugated goat anti-rabbit immunoglobulin, and

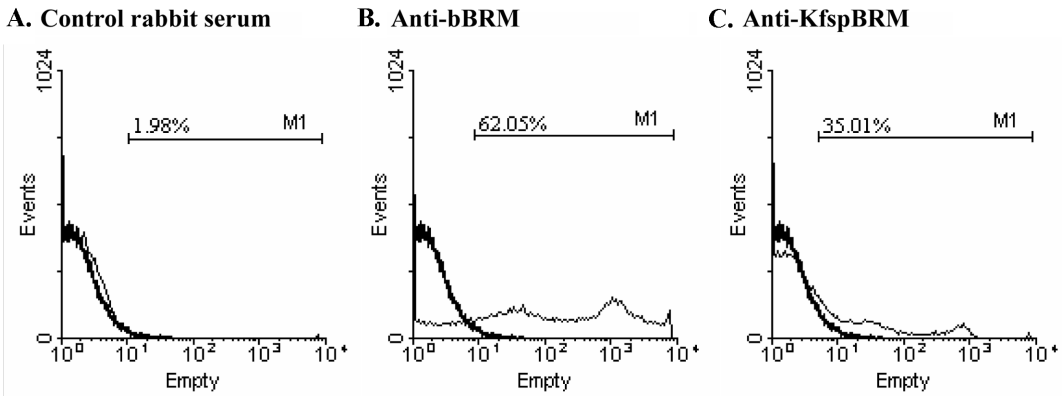


**Fig. 4.** The specific immunofluorescence of *B. licheniformis* E1 to anti-bBRM or anti-KfspBRM.
*B. licheniformis* E1 cultured for 24 h was reacted with anti-bBRM, anti-KfspBRM, or control rabbit serum, and stained with FITC-conjugated goat anti-rabbit immunoglobulin. The stained bacteria were analyzed by FACScan flow cytometry (A, B, and C).

analyzed by FACScan flow cytometry. As shown in Figs. 4A–4C, *B. licheniformis* E1 showing specific immunofluorescence increased in groups treated with anti-bBRM (62.05%) and anti-KfspBRM (35.01%), but not in those treated with control rabbit serum (1.98%), observed by confocal microscopy. *B. licheniformis* E1 treated with anti-bBRM or anti-KfspBRM showed specific immunofluorescence and the bacteria were agglutinated. In contrast, those treated with the control rabbit serum showed neither immunofluorescence nor the antibody-mediated aggregations (data not shown). The results indicate that both anti-bBRM and anti-KfspBRM bind to the slime layer of *B. licheniformis* E1, suggesting that the origin of bBRM is a slime layer of *B. licheniformis* E1 and that KfspBRM might also originate from *B. licheniformis* E1.

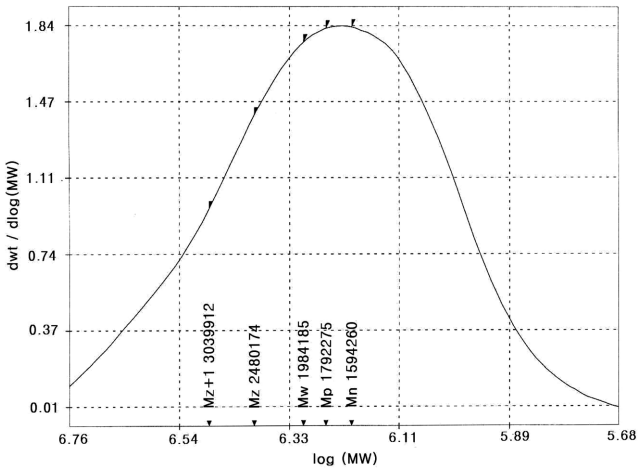


**Fig. 5.** Molecular weight of bBRM. The molecular weight of the bBRM was estimated by the GPC system with a column of ultrahydrogel 250, ultrahydrogel 500, and ultrahydrogel 1000.
The running sample was eluted with distilled water. Calibration of the columns was performed using dextrans (10–2,000 kDa) as reference molecular weight markers.

## Purification of bBRM from *B. licheniformis* E1 and its Molecular Weight

For a mass production of the bBRM, *B. licheniformis* E1 was cultured in BHI broth on a shaker for 48 h. Since the bBRM is heat-stable, the supernatant harvested from the bacterial culture was boiled in order to denature contaminated proteins. Purification of the bBRM from the supernatant was performed by the same method as that used to purify the KfspBRM from the Kfsp in the previous study [19]. Measurement of molecular weight of the purified bBRM was performed by the GPC system, and various molecular weights of dextrans were used for calibration of the column. As shown in Fig. 5, the bBRM appeared to be highly polymerized, having a molecular weight of 1,594 kDa.

## Chemical Composition of bBRM

Carbohydrates and proteins of bBRM were analyzed, and their contents were calculated in terms of percentages. The bBRM contained 33% (w/w) of reduced sugar (data not shown), and its sugars included glucosamine (35.1%), galactose (34.8%), galactosamine (22.6%), and glucose (7.5%) (Table 2). On the other hand, the protein content of the bBRM was shown to be 4.6% (w/w) (data not shown), and its major amino acids included serine (17.2%), proline (11.7%), tryptophan (11.5%), and alanine (10.1%) (Table 3).

**Table 2.** Sugar composition of bBRM.

| Sugar | pmol | mol % |
|---|---|---|
| Galactosamine | 255 | 22.6 |
| Glucosamine | 396 | 35.1 |
| Galactose | 392 | 34.8 |
| Glucose | 84 | 7.5 |
| Total | 1,127 | 100.0 |

**Table 3.** Amino acid composition of bBRM.

| Amino acid | pmol | mol % |
|---|---|---|
| Cys | 388 | 4.6 |
| Asp[a] | 625 | 7.5 |
| Glu[b] | 487 | 5.8 |
| Ser | 1,431 | 17.2 |
| Gly | 502 | 6.0 |
| His | 199 | 2.4 |
| Arg | 89 | 1.1 |
| Thr | 438 | 5.3 |
| Ala | 838 | 10.1 |
| Pro | 979 | 11.7 |
| Tyr | 406 | 4.9 |
| Val | 168 | 2.0 |
| Met | 209 | 2.5 |
| Cys2[c] | 50 | 0.6 |
| Ile | 178 | 2.1 |
| Leu | 209 | 2.5 |
| Phe | 83 | 1.0 |
| Trp | 955 | 11.5 |
| Lys | 100 | 1.2 |
| Total | 8,336 | 100.0 |

[a]Asp, amount of aspartate and asparagines.
[b]Glu, amount of glutamate and glutamine.
[c]Cys2, disulfide-linked cysteine.

## FT-IR Spectrum of bBRM

To further obtain molecular information, the bBRM was analyzed using FT-IR. As shown in Fig. 6, the FT-IR spectrum of the bBRM showed an 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 of absorption by the methyl group, and that at 1,737 $cm^{-1}$ is a typical spectrum of uronic acid. The absorption peaking at 1,581 $cm^{-1}$ and 1,414 $cm^{-1}$ indicate amide and carboxyl groups, respectively. This means that the bBRM includes protein. Therefore, the bBRM might be a glycoprotein.

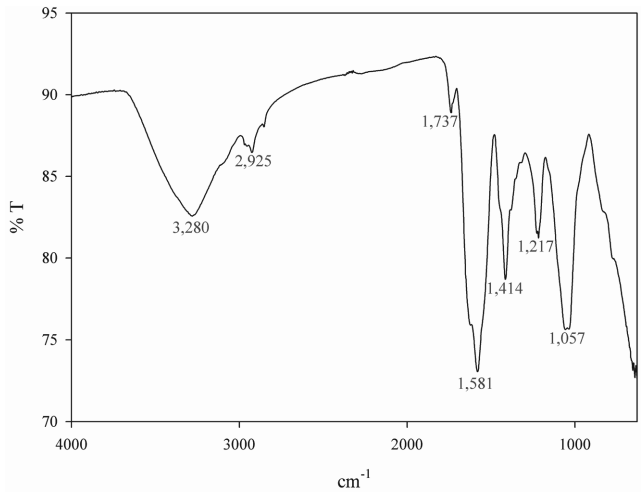


**Fig. 6.** Infrared absorption spectrum of bBRM. OH group of sugar, 3,280 $cm^{-1}$; methyl group, 2,925 $cm^{-1}$; uronic acid, 1,737 $cm^{-1}$; amide group, 1,581 $cm^{-1}$; carboxyl group, 1,414 $cm^{-1}$; ester group, 1,217 $cm^{-1}$.

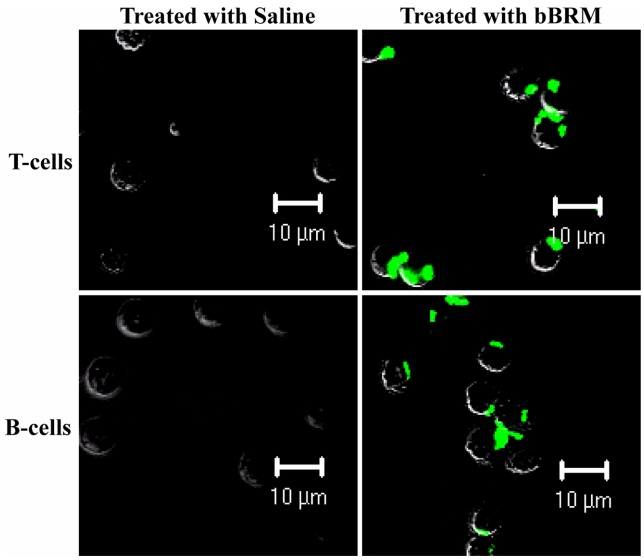


**Fig. 7.** Binding of bBRM to T-cells and B-cells.
T-cells or B-cells fractionated from mouse spleen were treated with the bBRM or saline at 37°C for 1 h. The cells were reacted with anti-bBRM, and then stained with FITC-conjugated goat anti-rabbit immunoglobulin. The cells were observed by confocal microscopy.

## Adherence of bBRM to T-Cells and B-Cells

The specific binding of bBRM to T-cells or B-cells was examined. Thus, the fractionated T-cells or B-cells treated with the bBRM or saline at 37°C for 1 h was reacted with anti-bBRM. After washing, the cells were stained with FITC-conjugated goat anti-rabbit immunoglobulin and then observed by confocal microscopy. As shown in Fig. 7, the specific immunofluorescence was detected on both T-cells and B-cells treated with anti-bBRM, but not on those treated with saline, and the bBRM-treated cells showed a tendency to aggregate with each other, compared with the saline-treated cells. This means that the bBRM binds to both B- cells and T-cells, thereby aggregating the cells.

## DISCUSSION

We isolated 5 kinds of bacteria producing B-cell-specific BRM from Kfsp, and found that physical and biological characteristics of the bBRM produced by one of the isolates were similar to those of KfspBRM. In a previous study, we reported that the BRM specific for B-cell is found in Kfsp, and suggested that bacteria involved in fermentation of Kfsp may produce the BRM, because it was found only in the fermented soybean paste, but not in

the non-fermented soybean paste. Therefore, we attempted to isolate bacteria from Kfsp that produce the BRM.

First of all, the bacteria in Kfsp were isolated, and a total of 31 kinds of bacteria including aerobes and anaerobes were isolated. In this experiment, the culture medium used was a BHI agar plate supplemented with 5% NaCl, since the concentration of NaCl in Kfsp is generally more than 5%. Therefore, the isolates are considered to be halophilic bacteria.

Since the cell envelope of Gram-negative bacteria contains lipopolysaccaride (LPS) that functions as B-cell mitogen [11, 20], Gram-positive bacteria of the isolates were selected by screening bacteria producing KfspBRM. The concentrates from the culture fluids of Gram-positive bacteria were heated to denature various proteins contaminated. This was possible because KfspBRM is heat stable, and their responses to anti-KfspBRM were measured by the ELISA method. In this experiment, we found that the concentrated culture fluids obtained from each of the 9 isolates strongly reacted with anti-KfspBRM. When the 9 concentrates were added to T-cells, no excess proliferation occurred. However, when they were added to B-cells, the enhanced proliferation of B-cells was observed in 5 of the 9 isolates. These results indicate that 5 kinds of bacteria isolated from Kfsp produce a B-cell-specific BRM shown in KfspBRM, because the concentrated culture fluids from those 5 kinds induced the proliferation of B-cells and their biological activities were heat stable.

Macro- and microscopic examinations revealed that the 5 isolates producing BRM specific for B-cells are aerobic Gram-positive spore-forming rods. Since the E1 isolate of the 5 isolates strongly increased the proliferation of B-cells, the isolate was selected for study of the characteristics of bBRM. The E1 isolate was identified as *B. licheniformis* and referred to *B. licheniformis* E1.

The examination of the origin of bBRM showed that both the concentrated culture fluid and the concentrated water-soluble slime layer of *B. licheniformis* E1 induced the proliferation of B-cells. However, the degree of proliferation was significantly higher in cells treated with the concentrated culture fluid than those treated with the concentrated water-soluble slime layer. These results suggest that bBRM originated from the slime layer of the bacteria. The reason why the activity of bBRM was higher in the concentrated culture fluid than in the concentrated water-soluble slime layer might be accumulation of bBRM released from the slime layer of the bacterium during cultivation.

To further confirm the fact that the origin of bBRM was a slime layer of the bacteria, the specific binding of anti-bBRM and anti-KfspBRM to the slime layer of *B. licheniformis* E1 was examined by immunofluorescent analysis. The result showed that specific immunofluorescence observed by FACScan flow cytometry and confocal microscopy was detected only in *B. licheniformis* E1 treated with anti-bBRM and anti-KfspBRM, but was not detected in the control rabbit serum. This confirms the above notion that bBRM originates from the slime layer of *B. licheniformis* E1, suggesting that KfspBRM may also originate from the slime layer of the 5 kinds of aerobic Gram-positive spore-forming bacilli that induced the proliferation of B-cells (Fig. 1).

bBRM was purified from the cultured fluid of *B. licheniformis* E1 by the same method used for that of KfspBRM from Kfsp in the previous study [19]. The molecular weight of the bBRM (1,594 kDa) differed only slightly from that of KfspBRM. The small difference between their molecular weights may be due to environment and different bacteria, including *B. licheniformis,* producing the BRMs. The difference may also be attributed to the fact that the bBRM and the KfspBRM were isolated from BHI broth cultured with *B. licheniformis* E1 and from the soybean paste fermented with many kinds of bacteria, respectively.

Chemical analysis showed that the bBRM contained 33% of reduced sugar and 7.5% of protein content, however, we did not determine the makeup of the remaining 59.5%. Accordingly, further analysis of the remaining parts of the bBRM will be needed in future. The sugar components of the bBRM appeared to be glucosamine, galactose, galactosamine, and glucose, and its major protein components were serine, proline, tryptophan, and alanine.

Further investigations, using FT-IR, revealed that the bBRM has a typical peak of OH stretching from the bound sugar group. The bBRM also showed peaks to indicate amide and carboxyl groups as well as acid sugar (uronic acid). The above results indicate that the bBRM contains not only neutral, acid, and amino sugars, but also includes proteins, suggesting that the bBRM is a glycoprotein.

Recently, it has been reported that microbial polysaccharides also show BRM activity [32]. Polysaccharide A (PS A) isolated from *Bacteriodes fragilis* induces abscess formation in animals, but also acts an as immunomodulator with activity specific for T-cells [22, 25]. β (1-3)-Glucan, glucose polymer, purified from fungi was reported to enhance macrophage phagocytosis [12, 13, 34] and to increase in the proportion of neutrophils and eosinophils [5, 33]. Mushrooms are also known to produce protein-bound polysaccharides that stimulated T-cell activation [21]. Furthermore, streptococci produce hyaluronic acid, which is a major carbohydrate component of the extracellular matrix of mammalian tissue, and has been shown to activate T-cell [27]. Most BRMs are shown to be specific for T-cells, neutrophils, and eosinophils, whereas only a few polymers are reported to be BRM specific for B-cells. These include proteoglycan isolated from *Phellinus linteus*, and polysaccharide from *Acanthopanax senticosus* [9, 14, 15].

In the present study, we demonstrated that bBRM, the product of *B. licheniformis* E1 and KfspBRM, functions as

a potent B-cell-specific BRM. The physiological and biochemical characteristics of γ-polyglutamic acid (PGA) produced by *B. licheniformis* 9945a have been reported [2]. It is an unusual naturally occurring anionic, water-soluble polyamide, synthesized as a bacterial slime layer by several *Bacillus* species including *B. licheniformis* 9945a [8, 10, 18, 30, 31]. Although both bBRM and PGA are protein-containing polymers originated from the slime layer of *B. licheniformis*, the sugar and amino acid components of the bBRM are different from those of PGA, and their biological functions are also different from each other.

To clearly prove that the bBRM is specific for B-cells but not T-cells, adherence of the bBRM to T-cells and B-cells was examined by confocal microscopy. The results showed that specific immunofluorescence was found not only on the surface of B-cells, but also on the surface of T-cells treated with bBRM, and that the aggregation of the cells occurred in groups treated with the bBRM, but not in groups treated with saline. These results indicate that the bBRM binds to both T-cells and B-cells, thereby inducing the aggregation of the cells. However, as shown in Fig. 1, the proliferation by the bBRM occurred only in B-cells and not in T-cells. This suggests that, although both T-cells and B-cells have receptors specific for bBRM, the receptor-mediated signal for activation of cells is induced only in B-cells [6].

In conclusion, we isolated BRM-producing *B. licheniformis* E1 from Kfsp, and found that the bBRM is a novel polymer inducing B-cell-specific proliferation, and that it originates from the slime layer of the bacteria. The bBRM reacted serologically with anti-KfspBRM, and its physical and biological characteristics were also found to be similar to those of KfspBRM.

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