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Characteristics of B Cell Mitogen Isolated from Korean-Style Fermented Soybean Paste

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Abstract Korean-style fermented soybean paste (KFSP), *Doenjang*, is a traditional food that is consumed as a protein source in Korea. Recently, efforts to identify biological response modifiers (BRMs) have been focused on food products. Accordingly, this study which isolated a biologically active substance from KFSP, named KFSP-BRM, was defined to be a heat-stable carbohydrate with a molecular weight of 2,000 kDa. The biological activity of KFSP-BRM was not inactivated by treatment with an anti-LPS antibody. The oral as well as intraperitoneal treatment of mice with KFSP-BRM 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*, KFSP-BRM was found to upregulate the production of interleukin-1 (IL-1) and IL-6 by macrophages and B cells but not the production of IL-2 by T cells. In conclusion, these data demonstrate the presence of a BRM in KFSP, which may provide an additional benefit to those consuming it as a food. KFSP-BRM is a novel B cell mitogen distinct from fresh soybean lectin or B cell mitogens, such as LPS and Streptococcus protein A. The major biological effects of KFSP-BRM would appear to be an increased production of IL-1 and IL-6 by macrophages and B cells, thereby enhancing the function of mature B cells.

Key words: KFSP, BRM, B cells, cytokine, mitogen, spleen index, immunoglobulin, LPS

Korean-style fermented soybean paste (KFSP), *Doenjang*, has been studied to detect agents that might function as BRM. Recently, fermented food products and/or microorganisms involved in the fermentation processes have been identified for their biologically active substances. These include *Lactobacillus acidophilus* from milk, which was reported to inhibit *Helicobactor pylori* adherence to glycolipid [12]. *Lactobacillus lactis* from Kimchi (Korean fermented vegetables) was reported to produce bacteriocin that inhibited the growth of pathogenic bacteria such as *Listeria monocytogenes*, *Staphylococcus aureus*, and *Bacillus cereus* [10]. Function of biological response modifiers (BRMs) of other lactic acid bacteria isolated from fermented milk have also been demonstrated by confirming induced interferons (IFN) [8, 16, 20, 21] and tumor necrosis factor-α (TNF-α) [20] by the bacteria isolated. *Saccharomyces cerevisiae* was also reported to produce β-glucan (free of mannoprotein) that activated macrophages to cause oxygen burst and to express cytokine genes such as INF-γ, TNF-α and IL-12 [6].

Interestingly, Japanese-style fermented soybean sauce (*shoyu*) and soybean paste (*miso*) have been found to inhibit Benzo(a)pyrene-induced mouse fore-stomach neoplasia [2, 3, 14] and decrease the occurrence of stomach and liver cancer [24], respectively. In addition, bacteria isolated from miso have been shown to inhibit the mutagenicity of a variety of chemical compounds [1].

KFSP has been a traditional food in Korea for centuries and is consumed as a protein source and spice.

KFSP is produced through the fermentation of soybeans by natural microbial flora for 1 year.

KFSP is also known to contain biologically active substances. Cytotoxic effect against carcinoma [4], antithrombotic peptides [19], angiotensin I converting enzyme inhibitory peptides [5, 18], antioxidants, a phenolic compound, and hydrophilic brown pigment [7, 11] have all been identified in KFSP.

BRMs are agents that modulate the host's immune response. These agents include a variety of biological molecules, cytokines, nucleic acids, thymic factors, monoclonal antibodies, and microbial components, yet the purified components of bacterial cell walls have led to the development of more effective adjuvants or stimulants of the immune response [23]. Because KFSP is made from soybeans fermented by natural microbial flora, it has been

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suggested that BRMs generated by microorganisms during fermentation may be present in KFSP.

Accordingly, this study, attempted to identify the BRMs from KFSP and investigate the biological effect of these BRMs in the functions of mouse immune cells.

MATERIALS AND METHODS

Mice

Female BALB/c mice between 6 and 7 weeks of age were obtained from the Korean Institute of Science Technology (Daejeon, Korea) and housed in a pathogen-free animal facility at Yonsei University College of Medicine, Seoul, Korea.

Reagents

The Lipopolysaccharide (LPS, from *E. coli*, serotype 055:B5) and soybean lectin (from *Glycine max*) were obtained from Sigma Chemical Co. (ST. Louis, U.S.A.). The Korean-style fermented soybean paste (KFSP) was purchased from Sunchang Traditional Foods Company (Soon Chang, Korea). This company manufactured the KFSP according to the following procedure: 1) Preparation of *Meju* cakes: Soybeans were soaked in water overnight, drained, and boiled for 5- 6 h. The cooked soybeans were cooled, crushed, and molded into cubical shapes (20×20× 10 cm) named *Meju* cakes. The *Meju* cakes were then dried under direct sunlight. Two days later, they were wrapped with rice straws, hung on a hook, and dried in indirect sunlight at 5- 10°C for 2 months. 2) Fermentation of *Meju* cakes: The fermented *Meju* cakes were placed in a cardboard box, covered with a thick blanket, and then incubated at 30°C for 2 weeks. 3) Ripening of *Meju*: The fermented *Meju* cakes were dried under direct sunlight for 2- 3 days, washed with water to remove any fungal hypha and spores on the surface, and dried again under direct sunlight for 2 days. The *Meju* cakes were placed in jars, then salt water (14% NaCl) twice the volume of the *Meju* cakes was added. The *Meju* paste was finally ripened at 20- 30°C for 3- 4 months.

Antibodies

The Purified mouse IgM (κ) and IgG (κ) antibodies (Abs), purified or horseradish peroxide (HRP)-conjugated goat anti-mouse IgM (μ chain specific) and IgG (H+L chain specific) Abs, and R-phycoerythein (PE)-conjugate rat anti-mouse CD3ε and fluorescein (FITC)-conjugate rat anti-mouse CD45R/B220 Abs were all purchased from Southern Biotechnology Associates, Inc. (Birmingham U.S.A.). The affiniPure rabbit anti-mouse IgM (μ chain specific) and IgG (H+L chain specific) Abs were purchased from ImmunoResearch Laboratories INC (West Grove, U.S.A.) and the FITC-conjugated goat anti-rabbit IgM+IgG (H+L chain specific) Abs was obtained from Southern Biotechnology Associates, In. The anti-LPS and anti-KFSP-BRM Abs were prepared from rabbits immunized with a mixture of LPS and complete/incomplete Freunds adjuvant and with a mixture of KFSP-BRM and complete/incomplete Freund's adjuvant, respectively.

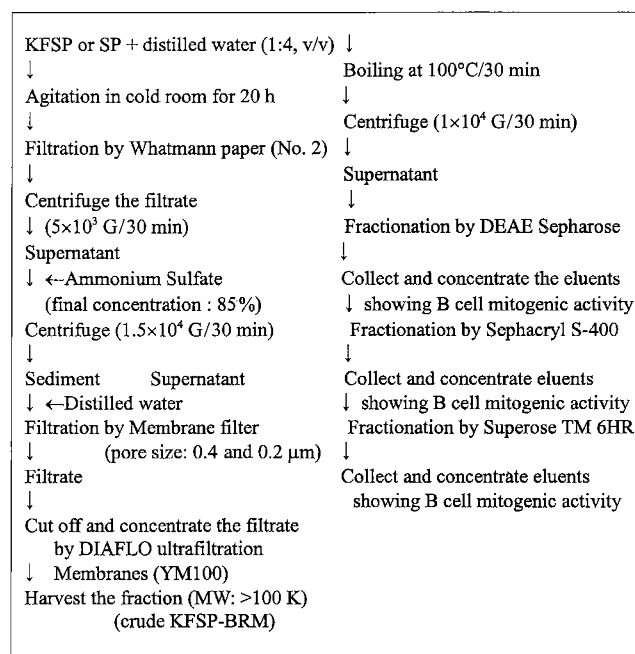

Fig. 1. Flow chart for purification of BRM from fermented soybean paste.
Korean style (KFSP) or non-fermented fresh soybean paste (SP).

Preparation of Crude Biological Response Modifier (cBRM) from KFSP

The extraction of B cell mitogen from KFSP was performed as shown in the flow chart presented in Fig. 1. KFSP and distilled water (1:4, v/v) were placed in a Waringblender and mixed at room temperature (RT) for 1 min. Non-fermented soybean paste (SP) was used as the control in contrast to KFSP. The mixtures were shaken for 18 h in a cold room and filtered by Whatman paper (No. 2). The filtered solutions were spun down using a Sorvall centrifuge (5×10^3 G/30 min). The supernatants (KFSP or SP extract) were harvested, saturated with ammonium sulfate (85%) for 16 h in a cold room, and spun down using a Sorvall centrifuge (1.5×10^4 G/30 min). The supernatants were discarded and the precipitates dissolved in distilled water. The dissolved precipitates were spun down again (1×10^4 G/ 30 min), and the supernatants harvested and filtered by a membrane (pore size: 0.2 μm). Larger than 100 kDa of molecular weight from the filtered solutions was cut off and concentrated using DIAFLO ultrafiltration membranes (YM 100) and Centricon Plus-80 (all from Amicon, Inc. Beverly, MA). The concentrated KFSP- and SP-extracts

were used as crude KFSP-BRM (KFSP-cBRM) and their biological activities measured by the proliferation of lymphocytes.

Purification of KFSP-cBRM

Since it is heat-stable, The cBRM from KFSP was boiled at 100°C for 30 min in order to remove any heat-labile proteins contaminated in the extract, then, it was spun down (1×10^4 G/30 min) and the supernatant harvested. Five ml of the supernatant was applied to a column (25×330 mm) of DEAE Sepharose Fast Flow (Pharmacia, Biotech. AB Uppsala, Sweden) previously equilibrated with a phosphate buffer (0.05 M, pH 7.4, PB) and the column was eluted with a linear gradient of 0- 1.0 M NaCl in PB over a period of 2 h at a flow rate of 2 ml/min. Fractions of 5 ml were collected, monitored at 280 nm, and assayed for BRMs. Those eluates showing the activity of a BRM were pooled and concentrated to an initial volume (5 ml) using Centricon Plus-80 (Amicon). One ml of the concentrated eluate was subjected again to a column (16×900 mm) of Sephacryl S-400 (Pharmacia Biotech) previously equilibrated with PB, and eluted with the same buffer at a flow rate of 1 ml/min. Three ml fractions were collected and monitored at 214 nm. Those fractions rich in BRMs were pooled and concentrated to 1 ml by Centricon Plus-20.

The concentrated eluate (1 ml) was subjected to a column of Superose TM 6HR (Pharmacia Biotech) previously equilibrated with PB and eluted with the same buffer at a flow rate of 1 ml/min. The elution was monitored at 214 nm. The predominant peak in each elution was collected and concentrated by Centricon Plus-20 (Fig. 1).

Spleen Index

The spleens from mice were weighed and the spleen index was calculated according to the formula [12]:

Spleen index=(spleen weight/whole body weight)×100

Isolation of Macrophages, and T and B Lymphocytes

The Macrophages were obtained from peritoneal exudate cells. Briefly, the peritoneal exudate cells were collected from normal mice that had been injected intraperitoneally with 10 ml of a serum- free RPMI 1640 medium using a syringe with a no. 18-gauge needle. The peritoneal cells were suspended in an RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 2.2 mM sodium bicarbonate, 100 units/ml penicillin, 100 μg/ml streptomycin (all from Life Technologies, Grand Island, U.S.A.; complete medium). Fifteen milliliters of the cell suspension (1×10^6 cells/ml) was added to a 75 cm^2 plastic culture flask (Costar, Cambridge, US.A.), which was then incubated for 3 h at 37°C in a humidified 5% CO_2 incubator (standard conditions unless otherwise stated). The non-adherent cells were removed by repeated washing with a serum-free RPMI 1640 medium. All the adherent peritoneal cells, harvested using a rubber policeman after leaving the flasks on ice for 30 min, were then used as macrophages. The lymphocytes were obtained from the spleen. The spleen was removed, a single-cell suspension was prepared by gently teasing between two glass slides, then the red blood cells were lysed using an ACK lysing buffer [9]. The macrophages of the cell suspension were then discarded by adherence to plastic as described above, while the non-adherent cells harvested after shaking the culture flask were used as the source of lymphocytes. The fractionation of the T or B cells from the spleen lymphocytes was performed by the negative selection of Thy 1.2- or B220-positive cells with Dynabeads mouse pan T or pan B (Dynal AS, Oslo, Norway) according to the manufacturer's direction.

Proliferation Assays

The fractionated T and B lymphocytes or non-fractionated lymphocytes were adjusted to 1×10^6 cells/ml in a complete RPMI medium, and 200 μl of cell suspensions were plated in 96-well round bottomed microtiter plates (Coatar). The cells were cultured in the presence or absence of KFSP-BRM or KFSP-BRM under standard conditions for 48 h. The Cell proliferation was measured using the technique of 6 h ^{3}H-thymidine (^{3}H-TdR, New England Nuclear, Boston, U.S.A.) incorporation.

Cytokine Production and Assay

For the production of IL-1 and IL-6, the prepared macrophages were adjusted to 1×10^6 cells/ml in a complete RPMI medium and one ml of the cell suspension was distributed into each of 24-well tissue culture plates (Coatar). Next, KFSP-BRM (5 μg/well) or a medium was added to the wells which were then cultured under the standard conditions for 24 h. The culture supernatants were harvested and used for the IL-1 and IL-6 assay. For the production of IL-2 and IL-6 by lymphocytes, T or B cells were suspended into a complete RPMI medium, and 2×10^6 cells in 1 ml were plated in each of 24-well tissue culture plates. KFSP-BRM (5 μg/well) or a medium was added to the wells, which were then cultured under standard conditions for 48 h. The culture supernatants were harvested and used for the IL-2 and IL-6 assay. The levels of cytokines in the supernatants were determined using murine IL-2 and IL-6 ELISA kits (Endogen Inc. Boston, U.S.A.).

Ig Measurements

The levels of total IgM and IgG Abs in the sera were measured using the capture-ELISA method [13] with 96-well microtiter plates (Nunc, Copen-hagen, Denmark). The plates were coated with 100 μl/well of affiniPure rabbit anti-mouse IgM (1 μg/ml) or IgG (2 μg/ml) Ab diluted with phosphate buffered saline (PBS) overnight at 4°C and then washed three times with PBS. The wells were blocked

with 150 μl of PBS containing 10% BSA (PBS-BSA) for 2 h at room temperature (RT) and washed three times with PBS containing 0.1% Tween 20 (PBST). The wells were filled with 100 μl/well of serum samples diluted by PBS-BSA and incubated for 2 h. The plates were washed five times, and was added to the wells, which were then 100 μl of 1/1,500 HRP-conjugated goat anti-mouse IgM or IgG Ab, incubated at RT for 1 h, washed five times with PBST, and reacted with a peroxidase substrate (Sigma) at 37°C for 30 min. The reaction was stopped by the addition of dilute sulfuric acid, and then measured in an ELISA plate reader at an optical density (OD) of 405 nm. Standard curves for IgM or IgG Ab were plotted using purified mouse IgM or IgG Ab.

Flow Cytometry

For a cytometric analysis of the splenic lymphocytes, the cells (5×10^5 cells) were incubated with 2 μg of PE-conjugated rat anti-mouse CD3e mAb or 2 μg of FITC-conjugate rat anti-mouse CD45R/B220 mAb. After washing three times with cold PBS, the stained cells were analyzed using an FACScan flow cytometer (Becton Dickinson, San Jose, U.S.A.). A Cytometric analysis of the phenotype of the splenic B cells was performed by an indirect method. B cells (5×10^5 cells) fractionated from splenic lymphocytes by negative selection with Dynabeads mouse pan T were incubated with 10 μl of affiniPure rabbit anti-mouse IgM (μ chain specific) or IgG (H+L chain specific) Ab at 4°C for 30 min. Normal rabbit serum was used as the control. After washing three times with cold PBS, the cells were stained again with 2 μg of FITC-conjugated goat anti-rabbit IgM+IgG (H+L chain specific) Ab at 4°C for 30 min and washed three times with cold PBS. The stained cells were analyzed as described above.

Analysis of Data

All data were expressed as the mean±SD. The differences were analyzed using the Student's T test. A p value of <0.05 was considered as statistically significant.

RESULTS

The Water Soluble Extract from KFSP Increased the Proliferation of Splenic Lymphocytes from Mice

To detect the substance that may act as a BRM, the lyophilized extracts (1 mg/ml) from KFSP or SP prepared as described in Fig. 1, were dissolved in saline and their biological activities evaluated by a proliferation assay of the lymphocytes. The splenic lymphocytes from normal BALB/c mice were plated in 96-well round bottomed microtiter plates and various concentrations of KFSP- or SP-extract were added to the wells. After incubation at 37°C for 48 h, the cell proliferation was measured by 6 h-^{3}H-TdR incorporation. As shown in Table 1, the ^{3}H-TdR incorporation by those lymphocytes cultured with 2 μg and 10 μg of SP-extract were 168±23 and 205±15 cpm, respectively, which was not different from that in the medium control (216±32 cpm). In contrast, those lymphocytes cultured with the KFSP-extract showed a significant increase in ^{3}H-TdR incorporation and their proliferative responsiveness was dependent on the dose of the KFSP-extract: 29,648±954 cpm and 154,065±3,552 cpm for 2 μg and 10 μg of KFSP-extract, respectively. These results demonstrate that a BRM inducing the proliferation of lymphocytes was present in the KFSP, but not in the fresh soybean paste. Accordingly, the BRM may originate from a fermentation products and/or microorganisms involved in the fermentation of soybean paste, since the induction of B cell proliferation failed in the culture with the SP-extract.

Table 1. Effect of water soluble extract from KFSP or SP on proliferation of splenic lymphocytes from BALB/c mice.

Stimulant	Conc. of extract	^{3}H-TdR incorporation (cpm) by lymphocytes
Medium		216±32
SP-extract	2 μg	168±23
	10 μg	205±15
KFSP-extract	2 μg	29,648±954
	10 μg	154,065±3,552

The KFSP- and SP-extracts were obtained from soybean paste fermented Korean style and non-fermented soybean paste, respectively. Splenic lymphocytes from normal mice were plated in a 96-well round bottomed microtiter plate (2×10^5 cells/200 μl/well), and various concentrations of KFSP- or SP-extract were added to the wells. The plates were incubated for 48 h and the cell proliferation was determined by 6 h-^{3}H-TdR incorporation. Data indicated are the mean±S.D. of three independent experiments.

Heat-Stability of KFSP-cBRM

To test whether the KFSP-cBRM was heat-stable, KFSP-cBRM dissolved in saline was heated at 60°C or 100°C for 30 min and the activities were measured by the 6 h-^{3}H-TdR incorporation of the splenic lymphocytes. The results (Table 2) illustrated that the biological function of KFSP-cBRM was not inactivated at either 60°C or 100°C. The 6 h-^{3}H-TdR incorporations by those lymphocytes cultured

Table 2. Heat stability of KFSP-cBRM.

Stimulant	^{3}H-TdR incorporation (1×10^3 cpm) by splenic lymphocytes cultured with KFSP-BRM treated at		
	Unheated	60°C/30 min	100°C/30 min
KFSP-BRM	25.1±1.0	24.0±0.9	22.9±0.7

The splenic lymphocytes from normal mice were plated in a 96-well round bottomed microtiter plate (2×10^5 cells/200 μl/well), and 2 μg of KFSP-cBRM heated at 60°C or at 100°C for 30 min was added to the wells. The plates were incubated for 48 h and the cell proliferation was determined by 6 h-^{3}H-TdR incorporation. Data indicated are the mean±S.D. of three independent experiments.

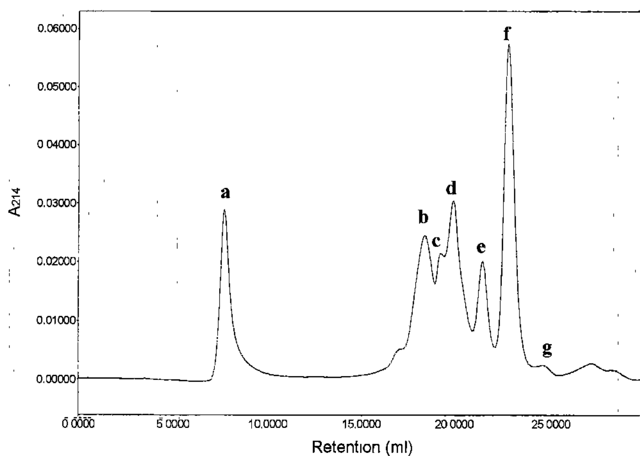

Fig. 2. Superose TM 6HR column chromatography of crude KFSP-BRM.
The fraction fractionated by Sephacryl S-400 and Superose TM 6HR column chromatography was subjected to a column of Superose TM 6HR, eluted with a phosphate buffer at a flow rate of 1 ml/mim, and monitored at 214 nm. The first peak (a) eluting from the column at 7.913 ml contained KFSP.

with 2 μg of KFSP-cBRM treated at 60°C and 100°C for 30 min were [24.0±0.9 (×10^3) cpm] and [22.9±0.7 (×10^3) cpm] respectively, which were not different from that in the unheated-KFSP-cBRM [25.1±1.0 (×10^3) cpm].

Purification of KFSP-cBRM

The KFSP-BRM was purified by two sequential stages of chromatography, ion exchange using DEAE Sepharose and HPLC gel filtration using Sephacryl S-400. Those fractions showing BRM activity were pooled and concentrated (Fig. 1).

For further purification, 100 μl of the concentrated fractions were subjected to a column of Superose TM 6HR. The elution was performed at a flow rate of 1 ml/min and monitored at 214 nm. As shown in Fig. 2, BRM activity was only detected in the fraction of the first peak (a) but not in the fractions of the other peaks (b, c, d, e, f, and g). The peak (a) fraction was termed as KFSP-BRM. The molecular weight of KFSP-BRM was estimated based on a standard curve which was constructed using Superose TM 6HR column and molecular weight markers, blue dextran (2,000 kDa), phosphorylase b (94 kDa), soybean trypsin inhibitor (20 kDa), and lactalbumin (14.4 kDa).

The elution was performed at a flow rate of 1 ml/min and monitored at 214 nm. KFSP-BRM was eluted in the fraction corresponding to about 2,000 kDa (data not shown). For qualitative analysis of KFSP-BRM performed using a Bradford reagent (Amaresco Inc. Solon, Ohio) and Amido black reagent (Sigma), KFSP-BRM was shown to be a heat-stable carbohydrate (data not shown).

KFSP-BRM Increased Proliferation of B Cells

Because the above result showed that KFSP-BRM increased the proliferation of splenic lymphocytes, the target cells proliferated from the splenic lymphocytes were investigated. The splenic lymphocytes from normal mice were fractionated into three cell populations; whole splenic lymphocytes, T cells, and B cells, as described in the Materials and Methods section. The cell populations (2×10^5 cells/well) were plated in 96-well round bottomed microtiter plates, and KFSP-BRM (0.5 μg/well) was added to the wells. The plates were incubated for 48 h under standard conditions and the cell proliferation was measured using 6 h-^{3}H-TdR incorporation. KFSP-BRM significantly increased the proliferation of whole cells (18,345±4,785 cpm) and B cells (24,113±4,252 cpm), yet not T cells (2,352±33 cpm), thereby indicating that KFSP-BRM is a B cell-specific mitogen (Table 3).

Table 3. KFSP-BRM enhanced proliferation of B cells from mice spleens.

Stimulant	^{3}H-TdR incorporation (cpm) by		
	Whole cells	T cells	B cells
Medium	315±52	226±33	204±29
KFSP-BRM	18,345±4,785	2,352±33	24,113±4,252

Splenic lymphocytes from normal mice were used in this experiment. Non-fractionated lymphocytes were used as whole cells and some of them were fractionated into T and B cells using Dynabeads mouse pan T or pan B, respectively. The fractionated or non-fractionated cells were seeded in a 96-well round bottomed microtiter plate (2×10^5 cells/100 μl/well), and 0.5 μg of KFSP-BRM or a medium was added to the wells. The plates were incubated for 48 h and the cell proliferation was determined by 6 h-^{3}H-TdR incorporation. Data indicated are the mean±S.D. of three independent experiments.

KFSP-BRM is Neither Soybean Lectin Nor Lipopolysaccharide (LPS) Derived from Gram Negative Bacteria

A number of plant lectins and other substances are known to be T or B cell mitogens, which have been employed in pheno-typing human and animal lymphocytes to assess their functions. The T cell mitogens, phytohemagglutinin (PHA) and concanavalin A (Con A), have been derived from *Phaseolus vulgaris* (kidney bean) and *Canavalia ensiformis* (jack bean), respectively, whereas the B cell mitogens, lipopolysaccharide (LPS) and *Staphylococcus* protein A, originated from the cell walls of gram-negative and -positive bacteria, respectively [22]. Soybeans also include lectin. Accordingly, to define whether KFSP-BRM originates from soybean lectin or bacterial LPS, the effect of soybean lectin on the proliferation of B cells and the effect of anti-LPS Ab on the biological activity of KFSP-BRM were investigated. As shown in Fig. 3, KFSP-BRM significantly increased the proliferation of B cells [25.14± 2.15 (×10^3) cpm], however, the cells cultured with soybean lectin [0.25±0.09 (×10^3) cpm] were not proliferated compared to those cultured with only a medium [0.27±0.03 (×10^3) cpm]. Because KFSP-BRM and LPS both increased

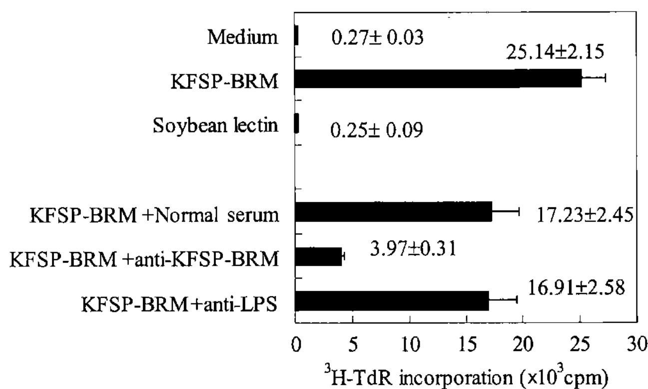

Fig. 3. KFSP-BRM is a novel B cell mitogen distinct from soybean lectin or LPS.

Splenic lymphocytes from normal mice were seeded in a 96-well round bottomed microtiter plate (2×10^5 cells/100 µl/well), then 100 µl of KFSP-BRM (0.5 µg), soybean lectin (2 µg), or a medium added to one plate and 100 µl of a mixture of a medium (50 µl) and normal rabbit serum (50 µl), a mixture of KFSP-BRM (0.5 mg) and rabbit anti-KFSP-BRM serum (50 µl), or a mixture of KFSP-BRM (0.5 µg) and rabbit anti-LPS serum (50 µl) was added to another. The mixtures were pre-incubated at room temperature before any addition. The plates were then incubated for 48 h and the cell proliferation was determined by 6 h-^{3}H-TdR incorporation. Data indicated are the mean±S.D. of three independent experiments.

the proliferation of B cells, their mitogenic effects were compared to the ability of anti-KFSP-BRM or anti-LPS Ab in blocking the biological function of KFSP-BRM. It was found that the proliferative responsiveness of those B cells stimulated with KFSP-BRM was significantly blocked by anti-KFSP-BRM Ab [3.97±0.31 ($\times10^3$) cpm], yet not by either anti-LPS Ab [16.91±2.58 ($\times10^3$) cpm] or the corresponding normal rabbit serum [17.23±2.45 ($\times10^3$) cpm]. These results indicate that KFSP-BRM is a novel B cell mitogen distinct from soybean lectin or LPS.

KFSP-BRM Increases Size of Mouse Spleen

To examine whether the enhanced proliferation of lymphocytes by stimulation with KFSP-BRM could also be reproduced in an *in vivo* system, normal mice were treated orally or intraperitoneally with KFSP-BRM (10 µg/mouse). Two days later, its effect was measured using a spleen index, as described in Materials and Methods. As shown in Fig. 4, a significant increase in the size of the spleens was observed in those mice treated with KFSP-BRM. The spleen indexes of the mice treated orally and intraperitoneally with KFSP-BRM were 1.5-fold (0.51±0.04%, $p<0.05$) and 3-fold (0.94±0.08%, $p<0.02$), respectively, higher than those of the corresponding control mice (0.33±0.02%). These results indicated that KFSP-BRM can also induce the proliferation of splenic lymphocytes *in vivo* and can be absorbed in the gastrointestinal tract without degradation by gastric acid or enzymes.

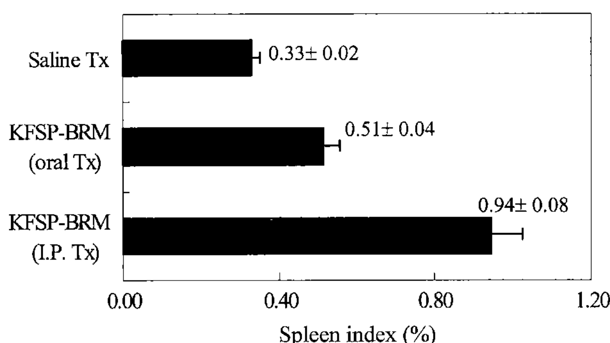

Fig. 4. Mice treated orally or intraperitoneally with KFSP-BRM (10 µg/mouse) or saline.

Three days after treatment the spleens were removed and weighed. The splenic index was calculated as described in materials and methods.

Treatment of Mice with KFSP-BRM Increases Proliferation of Splenic B Cells

To confirm whether the cell population in the enlarged spleens induced by KFSP-BRM was due to increase of B cells, normal mice were treated i.p. with KFSP-BRM (10 µg/mouse) or saline, and two days later their splenic lymphocytes were analyzed by flow cytometry using PE-conjugate rat anti-mouse CD3ε and FITC-conjugate rat anti-mouse CD45R/B220 mAbs. As shown in Fig. 5, the cells expressing CD3ε and CD45R/B220 in the control mice treated with saline were 17.1% and 13.1%, respectively, whereas those expressing CD3ε and CD45R/B220 in the KFSP-BRM-treated mice were 6.5% and 36.9%, respectively, thereby indicating that KFSP-BRM significantly increased the number of splenic B cells *in vivo*. In contrasts, the phenotypes of the B cell population increased by the treatment of KFSP-BRM were analyzed by flow cytometry using affiniPure rabbit anti-mouse IgM, -mouse IgG and FITC-conjugated goat anti-rabbit IgM+IgG Abs. As shown in Fig. 6, the flow cytometry analysis showed a significant

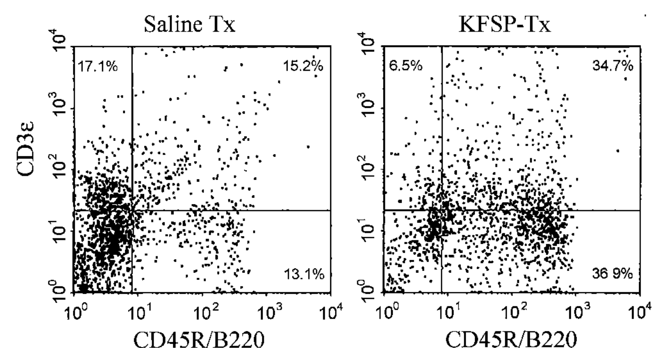

Fig. 5. Flow cytometric analysis of splenic lymphocytes isolated from mice treated with KFSP-BRM or saline.

The mice were injected i.p. with KFSP-BRM (10 µg/mouse) or saline. Three days later, 3 mice from each group were sacrificed and the lymphocytes isolated from their spleen were pooled. An FACS analysis was performed using double staining for CD3ε-PE and CD45R/B220-FITC. The result of a representative experiment from three independent experiments are shown.

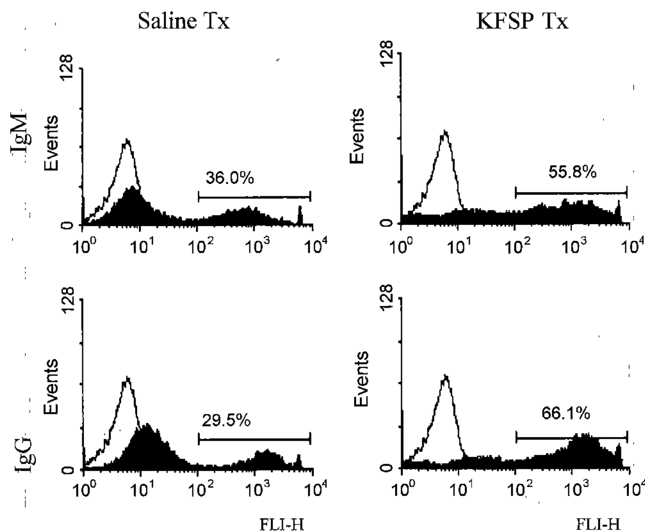

Fig. 6. Flow cytometric analysis of phenotype of splenic B cells isolated from mice treated with KFSP-BRM or saline.
The mice were injected i.p. with KFSP-BRM (10 μg/mouse) or saline. Three days later, 3 mice from each group were sacrificed and their lymphocytes pooled. The B cells of the splenic lymphocytes were isolated by the depletion of the Thy 1.2 positive cells with Dynabead mouse pan T. The B cells were stained with FITC-conjugated goat anti-mouse IgM or -mouse. IgG and then analyzed by flow cytometry. The results of a representative experiment from three independent experiments are shown.

increase in cells expressing IgM and IgG in mice treated with KFSP-BRM (55.8% and 66.1%, respectively) as compared to the corresponding control mice (36.0% and 29.5%, respectively). This was statistically significant ($p<0.05$ in the case of IgM; $p<0.05$ in the case of IgG). The degree of increase was much more pronounced in those cells expressing IgG rather than IgM.

KFSP-BRM Increases Non-Specifically the Production of IgM and IgG *In Vivo*

The fact that splenic B cells were significantly increased in mice treated with KFSP-BRM and that the phenotypes were B cells expressing IgM and IgG suggest that KFSP-BRM may non-specifically induce the production of immunoglobulins *in vivo*. Accordingly, the levels of total IgM and IgG in sera from the mice treated with KFSP-BRM were investigated. Normal mice were treated i.p. with KFSP-BRM (10 μg/mouse) or saline, and five days later their bloods were collected. The immunoglobulins in their sera were assayed by ELISA using purified or HRP-conjugated goat anti-mouse IgM (μ chain specific) and IgG (H+L chain specific) Abs. As expected, the levels of total IgM and IgG were significantly increased in the sera of KFSP-BRM-treated mice (3.35±0.95 and 41.74±0.99 mg/ml, respectively) compared with that of the corresponding control mice (0.14±0.19 and 19.33±0.2 mg/ml, respectively). The degree of increase was higher in IgM than in IgG. These results suggest that the KFSP-BRM not only

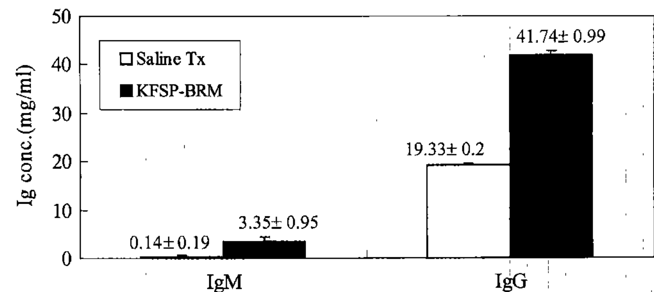

Fig. 7. KFSP-BRM increased the levels of serm IgM and IgG.
The mice were injected i.p. with KFSP-BRM (10 μg/mouse) or saline. Three days later, 3 mice of each group were sacrificed, and their bloods were harvested. The levels of immunoglobulins in their sera were measured by ELISA. Data indicated are the mean±S.D. of three independent experiments.

increased the proliferation of the mature B cells but also increased the production of immunoglobulins by plasma cells (Fig. 7).

Effect of KFSP-BRM on Profiles of Cytokines by Immune Cells

Many kinds of cytokines, including interleukin-1 (IL-1), IL-4, IL-5, and IL-6, are involved in B cell proliferation and antibody production by B cells, whereas the proliferation of T cells is regulated by IL-2. In the results shown above, KFSP-BRM increased the proliferation of splenic B cells but not T cells. Therefore, the effects of KFSP-BRM on the production of IL-1 and IL-6 by macrophages, IL-2 and IL-6 by T cells, and IL-6 by B cells were investigated. Macrophages, and T and B cells isolated from mouse peritonium and spleen, respectively, were cultured with KFSP-BRM (1 μg/well) or a medium for 6 h (in the case of macrophages) or 48 h (in the case of T and B lymphocytes), and the levels of cytokines in their culture

Table 4. Effect of KFSP-BRM on production of cytokines by peritoneal macrophages and splenic T and B cells.

Cell population	Stimulant	IL-1 (pg/ml)	IL-2 (pg/ml)	IL-6 (pg/ml)
Macrophages	Medium	248±39	NT	1,950±322
	KFSP-BRM	3,135±428	NT	21,832±2,754
T cells	Medium	NT	48±8	25±4
	KFSP-BRM	NT	62±12	158±28
B cells	Medium	NT	NT	35±5
	KFSP-BRM	NT	NT	532±317

The Macrophages were obtained from peritoneal exudate cells and isolated by adherence to plastic. The T cells and B cells were isolated from the spleen using Dynabeads mouse pan T or pan B. One ml of the cell suspensions (1×10^6 macrophages/ml, 2×10^6 T cells/ml, or 2×10^6 B cells/ml) was plated into 24-well tissue culture plates and cultured with KFSP-BRM (1 μg/well) or a medium for 6 h (in the case of macrophages) or 24 h (in the case of T cells and B cells). The levels of cytokines in the culture supernatants were measured using ELISA kits.
NT: not tested.

supernatants were assayed by ELISA. As shown in Table 4, KFSP-BRM appeared to significantly increase the production of IL-1 and IL-6 by macrophages and IL-6 by T and B cells, yet not IL-2 by T cells. The levels of IL-1 (3,135±428 pg/ml) and IL-6 (21,835±2,754 pg/ml) in the culture supernatant of macrophages stimulated with KFSP-BRM were approximately 15-fold ($p<0.01$) and 11-fold ($p<0.01$), respectively, higher than those of the medium control (248±39 and 1,950±322 pg/ml, respectively). The IL-6 production by T and B cells stimulated with KFSP-BRM was 158±28 and 532±317 pg/ml, respectively, which were approximately 6-fold ($p<0.05$) and 9-fold ($p<0.01$) higher than those of the medium controls (25±4 and 35±5, respectively). Although T cells stimulated with KFSP-BRM produced IL-6, the level was approximately 4-fold lower than that of B cells stimulated with KFSP-BRM. In contrast, the level of IL-2 (62±12 pg/ml) in the culture supernatant of T cells stimulated with KFSP-BRM was not increased, which was similar to the level in the culture supernatant of medium-stimulated T cells (48±8 pg/ml). These results suggest that the increased proliferation of B cells and high level of antibody production in mice treated with KFSP-BRM may be associated with the increased production of IL-1 and/or IL-6 by macrophages and B cells.

Discussion

The purpose of this work was to search for BRMs in KFSP and to define their biological functions. In the screening test for BRMs, it was demonstrated that a biologically active substance that increased the proliferation of mouse splenic lymphocytes was present in the extract from KFSP, but not in that from non-fermented fresh soybean paste. This result suggests that the microorganisms fermenting the soybeans would appear to play an important role in the production of the BRM. The BRM from the KFSP extract was purified and named as KFSP-BRM. KFSP-BRM was established as a heat-stable carbohydrate, which increased the proliferation of splenic lymphocytes as shown in the extract of KFSP. To define the increased cell population of splenic lymphocytes due to KFSP-BRM stimulation, T and B cells from the splenic lymphocytes were fractionated and their proliferative response to KFSP-BRM evaluated. It was found that KFSP-BRM significantly increased the proliferation of B cells but not T cells, thereby indicating that KFSP-BRM is a B cell mitogen. So far, most B cell mitogens have been found in plant lectins (pokeweed), bacterial cell wall components (LPS or *Streptococcus* protein A), or parasites (proline racemase from Trypanosome) [17]. To investigate the origin of KFSP-BRM, the mitogenic activities of KFSP-BRM and soybean lectin were compared with splenic B cells from mice. The comparison of the biological properties of KFSP-BRM and LPS was evaluated based on the blocking effect of anti-KFSP-BRM and an anti-LPS antibody on the proliferation of B cells stimulated by KFSP-BRM, since LPS increases the proliferation of B cells. The results revealed that the mitogenic activity of KFSP-BRM was not blocked by the anti-LPS antibody, and stimulation with soybean lectin did not induce the proliferation of B cells. *Streptococcus* protein A is also a B cell mitogen, however, it is heat-labile. Therefore, these results suggest that KFSP-BRM is a novel B cell mitogen distinct from soybean lectin or B cell mitogens, such as LPS and *Streptococcus* protein A, implying that KFSP-BRM may originate from another bacteria. Accordingly, further studies to investigate the origin of KFSP-BRM are ongoing.

To investigate whether KFSP-BRM is absorbed in the gastrointestinal tract and its function can be also reproduced *in vivo* was considered to be important for the evaluation of KFSP as a food. Therefore, normal mice were treated orally or i.p. with KFSP-BRM and two days later their spleen indexes were measured. As shown in Fig. 4, both oral and intraperitoneal treatment of KFSP-BRM significantly increased the spleen indexes of the mice. The degree of their increase was 1.5–3 fold higher than that of the saline-treated mice. These findings indicate that KFSP-BRM can increase the proliferation of splenic lymphocytes *in vivo* and that it can be absorbed in the gastrointestinal tract without degradation by gastric acid or enzymes, suggesting that KFSP would appear to prove an additional benefit for those consuming it as a food.

To confirm whether the increased size of the spleen in those mice treated with KFSP-BRM was associated with the proliferation of B cells, the ratio of B cells to T cells was compared in the spleen of KFSP-BRM-treated mice and the saline-treated ones. A significantly increased ratio of B cells to T cells was found in the splenic lymphocytes of the KFSP-BRM-treated mice compared with that of the saline-treated ones.

Accordingly, the phenotype of the increased number of B cells proliferated by KFSP-BRM was investigated. B cells fractionated from the spleens of mice treated with KFSP-BRM or saline were analyzed by flow cytometry using rabbit anti-mouse IgM or -mouse IgG and FITC-conjugated goat anti-rabbit IgM+IgG. Although the phenotypes of B cells expressing each immunoglobulin class were not examined, it was found that an increased number of B cells expressing surface IgM and IgG, respectively, was found in the spleen of the KFSP-BRM-treated mice compared with that in the saline-treated ones. When considering these results together, it was concluded that the increased size of the spleen was due to the increased proliferation of B cells, in addition to B cell types expressing both surface IgM and IgG were significantly increased by KFSP-BRM.

Mature B cells express immunoglobulins and can be grouped into two different forms, namely, resting B cells and activated B cells. The former are virgin or memory B lymphocytes and only express immunoglobulins on the cell surface, while the latter are effector cells of the B cell lineage (called plasma cells), which are uniquely specialized in secreting immunoglobulins. Therefore, the fact that splenic B cells expressing surface IgM or IgG (Fig. 6) and the levels of serum IgM or IgG (Fig. 7) were significantly increased in those mice treated with KFSP-BRM suggests that KFSP-BRM would appear to be involved in the differentiation of resting B cells to plasma cells, the proliferation of plasma cells and also the production of antibodies by plasma cells.

Cytokines are regulatory molecules that increase or inhibit the differentiation, proliferation, or activation of cells, through which immune and inflammatory responses, hematopoiesis, and many other biologic responses are regulated. Cytokines are produced by particular cell types in response to a variety of stimuli, for example, IL-2 produced by type 1 helper T cells (Th 1 cells) is a major growth factor for the proliferation of T cells. In contrast, IL-6 produced by a variety of cells including type 2 helper T cells (Th 2 cells), activated B cells, and macrophages, is associated with the differentiation and proliferation of B cells and antibody production by plasma cells. IL-1 produced by antigen-presenting cells including, macrophages acts as a costimulatory factor for the activation of T and B cells. In regards to the effect of KFSP-BRM on the production of cytokines by T and B cells, and macrophages, it was found that KFSP-BRM significantly increased the production of IL-1 and IL-6 by macrophages and IL-6 by B cells, yet not the production of IL-2 by T cells. T cells stimulated with KFSP-BRM also increased the production of IL-6, however, this level of IL-6 was still significantly lower than the level produced by B cells. These results suggest that the increased number of splenic B cells expressing surface IgM and IgG and enhanced level of serum IgM and IgG in mice treated with KFSP-BRM was due, at least in part, to IL-1 and IL-6 produced by macrophages or IL-6 produced by B cells.

The present study demonstrated that the BRM isolated from KFSP is a novel B cell mitogen distinct from fresh soybean lectin or B cell mitogens, such as LPS and *Streptococcus* protein A. KFSP-BRM increased the production of IL-1 and IL-6 by macrophages and B cells, thereby, enhancing the proliferation of mature B cells and increasing the levels of serum antibodies.

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