Food Sci. Biotechnol. Vol. 17, No. 6, pp. 1279 ~ 1284 (2008)

Food Science and Biotechnology

The Korean Society of Food Science and Technology

Differences in Manufacturing Process and Quality between *Cheonggukjang* for Use in the Raw and *Cheonggukjang* for Stew

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Abstract When *cheonggukjang* was manufactured using a *Bacillus subtilis* CH10-1 starter culture, a short-term fermentation for 14-18 hr appeared to be the optimal for the raw *cheonggukjang* to avoid the formation of a bitter taste and to contain a high concentration of free sugars, whereas a long-term fermentation for more than 4 days was the optimal for the *cheonggukjang* for stew in order to contain a high concentration of free amino and organic acids, which are responsible for sweet, savory, and bitter tastes present in stewed *cheonggukjang*. During activation of murine splenic T cells with phytohemagglutinin (PHA), the presence of either poly-γ-glutamic acid (γ-PGA) or partially hydrolyzed γ-PGA resulted in reduction in the level of interferon-γ production and enhancement in the level of interleukin-5 production, possibly due to suppression of Th1 activity and augmentation of Th2 activity. Taken together these results indicate that the raw *cheonggukjang* and the *cheonggukjang* for stew are different in their quality and taste as well as immunomodulating activity.

Keywords: raw *cheonggukjang, cheonggukjang* for stew, manufacturing process, quality characteristics, immunomodulating activity of poly-γ-glutamic acid

Introduction

Although *cheonggukjang* has traditionally been prepared for the use of stew (i.e., the *cheonggukjang* for stew) in Korea, *cheonggukjang* has recently begun to be eaten in the raw as Japanese *natto*. The raw *cheonggukjang*, prepared for the use in the raw, is manufactured using the same process as the Japanese *natto*, which often results in a bitter taste. Since the butyric acid and 3-methyl butanoic acid contained in the raw *cheonggukjang* are responsible for its unpleasant odor and unique taste, it is necessary for a process that removes these compounds to be developed during fermentation period (1,2). In addition, because *cheonggukjang* is often manufactured using environmental microorganisms, extra care must be taken during its production to avoid sanitary problems that could lead to food poisoning or formation of biogenic amines.

In relation to *cheonggukjang*, studies evaluating poly-γ-glutamic acid (γ-PGA) that is present in the slime layer of the starter *Bacillus subtilis* (3-5) culture used to ferment *cheonggukjang*, as well as studies on the fermentation and fermenting cell used to produce *cheonggukjang* (6-8), studies on the taste and odor of *cheonggukjang* (1,2,9,10), and studies on the functional enforcement of *cheonggukjang* (11-13) have already been conducted. However, the difference in the manufacturing conditions, the taste compositions, and the conditions that result in the formation of bitter and savory tastes during manufacturing of the raw *cheonggukjang* and the *cheonggukjang* for stew have not been elucidated in detail. Furthermore, little is known about the functional difference in immunomodulating activity of γ-PGA or partially hydrolyzed γ-PGA, which is obtained from these two different types of *cheonggukjang*.

In the present study, differences in manufacturing process and quality between the raw *cheonggukjang* and the *cheonggukjang* for stew were evaluated. The effect of γ-PGA or partially hydrolyzed γ-PGA on activation of murine T cells and B cells *in vitro* was further investigated to evaluate a merit of *cheonggukjang* as a functional food.

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Received May 20, 2008; Revised July 12, 2008;
Accepted July 17, 2008

Materials and Methods

Starter and incubation The 16s rRNA sequence of the starter culture used in this study is the same as that of AB055853 in GeneBank. According to the National Center for Biotechnology Information (NCBI), this organism is a species of *B. subtilis* that can be used to manufacture *cheonggukjang* (6). The starter (*B. subtilis* CH10-1 that was isolated from traditional *cheonggukjang*) was initially cultured in Luria-Bertani (LB) broth (Difco Lab., Detroit, MI, USA) medium at 30°C for 1 day. Soybeans that had been soaked in water for 1 day at 4°C were then added to this mixture in a 1:10 ratio with water and boiled. The boiled soybean juice was then filtered and autoclaved at 121°C for 15 min to prepare the soybean extract medium, which was then inoculated with the 1 day old starter culture and incubated at 37°C for 24 hr.

***Cheonggukjang* manufacturing** Soybeans (250 g), which had been soaked in water for 1 day at 4°C, were added into 500-mL of polypropylene container and then autoclaved at 121°C for 60 min. The sterilized soybeans were then inoculated with the starter that had been incubated in the soybean extract medium to give a final concentration of 0.01%(v/w). The soybean inoculated with the starter was then incubated at 39°C for 2 hr, followed by incubation at 40°C for 6 hr. Sequentially, the temperature was increased

to 42°C for 6 hr, after which it was again cooled to 40°C, at which point the soybean sample was considered to be *cheonggukjang*.

To discriminate between raw and stew *cheonggukjang*, we incubate both at same production conditions and a sensory test to evaluating the different flavors.

Sensory evaluation The sensory evaluations were conducted as described previously (14). Briefly, 18 trained sensory panels (10 undergraduate school students and graduate school students along with 8 panel members who were between 50 and 60 years old) evaluated the *cheonggukjang* using a scoring method that employed a 5-point scale, with odors being ranked as one of the following: very bad (1 point), slightly bad (2 points), common (3 points), good (4 points), and very good (5 points).

Identification of spores and cells Spores and vegetative cells were identified using 5%(w/v) malachite green staining solution and safranin staining solution, respectively, as described previously (15).

Determination of viscosity After agitation of *cheonggukjang* cultures, we use forceps to pull up a bean at a velocity of 2 cm/sec and measure the extended length of its viscous substance. This process was repeated 5 times at room temperature.

Extraction and purification of taste compositions The extraction of taste compositions was conducted using the method described elsewhere (16). The compounds responsible for taste, including free amino acids, free sugars, and organic acids, were then purified using the Amberlite IR-120 (Sigma-Aldrich, St. Louis, MO, USA) and Amberlite IRA-400 (Sigma-Aldrich) as described previously (17,18). Free amino acids were analyzed using an amino acid autoanalyzer (L-8800; Hitachi, Tokyo, Japan) with an ammonia filter column (ammonia, 4.6×60 mm; main column, 2622SC-PE; 4.6×60 mm). The autoanalyzer was configured to the reaction detection method (Ninhydrine) specified in the PF-STD method. The free sugars were analyzed by high performance liquid chromatography (HPLC) (600E; Waters, Milford, MA, USA), Sugar-Pak I column (φ6.5×300 mm) under the following conditions: column temperature, 90°C; mobile phase, Ca-ethylene-diaminetetraacetic acid (Ca-EDTA, Sigma-Aldrich) buffer (50 mg Ca-EDTA/1 L dH_2O); flow rate, 0.5 mL/min; injection volume, 10 μL. The nonvolatile organic acids were analyzed by methylating the samples according to the method described by Schlenk and Gellerman (19), and then extracting the volatile organic acids using the method described by Kageyama *et al.* (20). The extracted volatile organic acids were then analyzed by gas chromatography under conditions that have been previously described (17,21).

Purification of γ-PGA or partially hydrolyzed γ-PGA from *cheonggukjang* After fermentation for 14 hr, the raw *cheonggukjang* was mixed with 2 volume of distilled water (DW) and incubated at 4°C overnight with gentle shaking. The supernatant was harvested by centrifugation at 600×g and then mixed with 9 volume of cold ethanol. The mixture was incubated at −20°C for 30 min to precipitate γ-PGA. The precipitated γ-PGA was collected by centrifugation at 600×g and washed with cold ethanol twice. After the precipitated γ-PGA was dissolved in DW, the solution was centrifuged to remove insoluble contaminant as pellets, and then the supernatant was lyophilized to obtain γ-PGA powder.

In order to harvest partially hydrolyzed γ-PGA from the *cheonggukjang* for stew, the *cheonggukjang* following fermentation for 4 days was mixed with 2 volumes of DW and incubated at 4°C overnight with gentle shaking. After centrifugation, the partially hydrolyzed γ-PGA contained in the supernatant was adsorbed in the IRA410 column and then eluted using $(NH_4)_2CO_3$. The partially hydrolyzed γ-PGA was lyophilized to dry and then washed with ethyl ether twice. After the obtained sample was dissolved in DW, the solution was centrifuged to remove insoluble contaminant as pellets, and then the supernatant was lyophilized to obtain partially hydrolyzed γ-PGA powder.

Animals C57BL/6 male mice, 4 to 6 month old, were purchased from the Animal Resources Center at Korea Research Institute of Bioscience and Bioengineering under specific-pathogen free conditions.

Isolation of murine splenocytes and activation with phytohemagglutinin (PHA) Single cell suspensions were prepared by mechanical dissociation of individual spleens in 1× Hanks' balanced salt solution (HBSS, Sigma-Aldrich) and the cells were pelleted by centrifugation at 100×g (22). Erythrocytes were removed by lysis by suspending in a 1× erythrocyte lysing buffer (B&D Systems, Minneapolis, MN, USA) for 10 min. For activation of T cells with PHA (Sigma-Aldrich), the splenic cells, which were suspended at a concentration of 4×10^6 cells/mL in RPMI 1640 medium containing 10%(v/v) fetal bovine serum (FBS, Sigma-Aldrich), 20 mM HEPES, 5×10^{-5} M β-mercaptoethanol, and 100 μg/mL of gentamycin, were treated with 2 μg/mL of PHA for 48 hr.

Cytokine quantitative analysis Maxisorp immunoplates (NUNC, Napeville, IL, USA) were coated with monoclonal anti-interferon-γ (IFN-γ) and anti-interleukin-5 (IL-5) antibodies (BD Biosciences Pharmingen, San Diago, CA, USA). After blocking, samples and serial dilutions of standards were added to duplicate wells and incubated overnight for 4°C. The wells were washed and further incubated with biotinylated anti-IFN-γ and anti-IL-5 antibodies. After washing, peroxidase-labeled anti-biotin antibody (BD Biosciences Pharmingen) was added and developed with 2,2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)-containing H_2O_2. Absorbance was read at 430 nm on an enzyme-linked immunosorbent assay (ELISA) plate reader (BD Biosciences Pharmingen).

Statistical analyses The results were expressed as the mean±standard deviation (SD). An analysis of variance (ANOVA), using SPSS v 12.0 (SPSS Inc., Chicago, IL, USA) software, was used to make a statistical comparison among the groups.

Table 1. The changes in taste and odor determined by sensory evaluation and in viscosity of *cheonggukjang* during fermentation period

Culture time (hr)	Taste (characteristic)	Aroma (characteristic)	Viscosity (cm)[1)]
12	bad (bean taste)	bad (bean aroma)	13±1.9
13	bad (bean taste)	bad (bean aroma)	27±2.1
14	very good (savory taste)	very good (savory aroma)	31±2.2
15	very good (savory taste)	very good (savory aroma)	20±1.2
16	very good (savory taste)	very good (savory aroma)	15±0.7
17	very good (savory taste)	very good (savory aroma)	7±1.2
18	bad (weakly bitter taste)	good (weakly savory aroma)	3±0.8
19	bad (weakly bitter taste)	good (weakly savory aroma)	3±0.5
20	bad (weakly bitter taste)	bad (weakly bitter aroma)	3±0.5
21	bad (weakly bitter taste)	bad (weakly bitter aroma)	2±0.4
24	bad (weakly bitter taste)	bad (bitter aroma)	-
26	bad (bitter taste)	bad (bitter aroma)	-
28	bad (bitter taste)	bad (bitter aroma)	-
30	bad (bitter taste)	very bad (very bitter aroma)	-
32	bad (bitter taste)	very bad (very bitter aroma)	-
36	bad (bitter taste)	very bad (very bitter aroma)	-
40	very bad (very bitter taste)	very bad (very bitter aroma)	-
42	very bad (very bitter taste)	very bad (very bitter aroma)	-
44	very bad (very bitter taste)	very bad (very bitter aroma)	-
48	very bad (very bitter taste)	very bad (very bitter aroma)	-

[1)]Mean±SD.

Results and Discussion

Kinetic analysis of taste, odor, and viscosity of *cheonggukjang* during fermentation period In order to examine the optimal fermentation periods for the raw *cheonggukjang* and the *cheonggukjang* for stew, time kinestics of the changes in the taste, ordor, and viscosity of *cheonggukjang* were investigated following inoculation of the sterilized soybean with a starter culture of *B. subtilis* CH10-1. As shown in Table 1, the sensory evaluation for the taste as well as odor of *cheonggukjang* indicated that the *cheonggukjang* had a soybean taste and odor until 12-13 hr after incubation. The taste and odor of the *cheonggukjang* was found to be more savorious after incubation for between 14 and 17 hr. However, during the incubation period from 18 to 48 hr, the *cheonggukjang* developed a bitter taste and odor. On the other hand, the viscosity of *cheonggukjang*, which reflects the content of γ-PGA, reached a maximum at 14 hr, and then decreased rapidly so that it was not detectable after fermentation for 24 hr. It has been reported that the γ-PGA is the major component of slime layer of the bacterial starter, *B. subtilis*, which is generated by γ-polyglutamate synthetase (3). It has also been reported that the γ-PGA produced by *B. subtilis* is degraded by its own exoenzymes, such as γ-D,L-glutamyl hydrolase and γ-Glu-X carboxypeptidase (carboxypeptidase G; E.C. 3.4.19.9) (23). These previous and current results regarding the generation and degradation of the γ-PGA by *B. subtilis* suggested that there was a rapid and dynamic change in the content of γ-PGA in *cheonggukjang* by the action of γ-PGA-synthesizing as well as γ-PGA-degrading enzymes during fermentation period. To investigate further the changes in the taste and odor of *cheonggukjang* depending on the incubation period, the sensory evaluation was conducted for the individual *cheonggukjangs* manufactured for long-term periods ranging up to 120 hr, and also the changes in the taste and odor of these *cheonggukjangs* were compared between before and after boiling. Although the taste and odor of the raw *cheonggukjang* were evaluated as good at 14 hr after incubation, since then it became bad regardless of the length of incubation period (Table 2). However, the taste and odor of the raw *cheonggukjang* appeared to be changed following boiling so that the quality of the *cheonggukjang* manufactured for 96 or 120 hr was evaluated as very good or good after boiling (Table 3). To understand chemical changes underlying these time-dependent changes in the taste as well as odor of the *cheonggukjang*, we have compared the levels of free amino acids, volatile organic acids, non-volatile organic acids, and free sugars in the *cheonggukjang* manufactured

Table 2. The sensory evaluation of the raw *cheonggukjang*

Culture time (hr)	Sensory evaluation			
	Taste		Aroma	
	Score[1)]	Result	Score[1)]	Result
14	4.6±2.5	very good	4.1±2.1	very good
48	1.8±1.8	very bad	2.1±1.7	very bad
72	1.6±3.0	very bad	1.8±2.6	very bad
96	1.5±1.8	very bad	1.9±2.1	very bad
120	1.6±2.3	very bad	1.6±2.3	very bad

[1)]Mean±SD.

Table 3. The sensory evaluation of boiled *cheonggukjang*

Culture time (hr)	Sensory evaluation			
	Taste		Aroma	
	Score[1)]	Result	Score[1)]	Result
14	1.6±2.3	very bad	1.1±3.0	very bad
48	1.8±2.6	very bad	2.4±2.3	very bad
72	2.9±3.0	bad	3.3±1.8	good
96	3.6±2.1	very good	3.4±2.3	very good
120	3.0±1.5	good	3.5±2.2	very good

[1)]Mean±SD.

for 14 hr with those in the *cheonggukjang* manufactured for 96 hr. As shown in Table 4, the contents of amino acids with a sweet taste (alanine, glycine, lysine, and threonine) and amino acids with a savory taste (aspartic acid, glutamic acid, and cysteine) increased after 96 hr incubation as compared to the contents detected after 14 hr incubation. In particular, the levels of glutamic acid and cysteine in the *cheonggukjang* 14 hr after incubation appeared to increase 18.3 and 5.5-fold at 96 hr, indicating a contribution of these amino acids to the acquisition of savory flavor of the *cheonggukjang* manufactured by 96 hr incubation. At the same time, among the bitter-tasty amino acids tested, the leucine and phenylalnine were not detected in both *cheonggukjangs* manufactured for 14 and 96 hr, and only the contents of isoleucine and methione increased 2.6 and 2.8-fold, respectively. Since *B. subtilis* used for the starter culture of *cheonggukjang* is a potent producer of protein-hydrolyzing enzymes including proteinases, amino-peptidases, and carboxypeptidases (24), it was likely that the degradation of soybean proteins by these protein-hydrolyzing enzymes caused the change in the levels of free amino acids, which can be directly associated with the taste and odor of *cheonggukjang*. Under these conditions, although the content of a volatile organic acid, propionic acid, was higher in the *cheonggukjang* manufactured for 14 hr, the content of butyric acid and 3-methyl butanoic acid, both of which cause a bad odor, was higher in the *cheonggukjang* manufactured for 96 hr than that in the counterpart (Table 5). Among the non-volatile organic acids tested, the levels of fumaric acid and glutaric acid were higher in the *cheonggukjang* manufactured for 14 hr, whereas the level of oxalic acid was higher in the *cheonggukjang* manufactured for 96 hr (Table 6). In addition, the contents of free sugars including maltose, glucose, and fructose were higher in the *cheonggukjang* manufactured for 14 hr than in the *cheonggukjang* manufactured for 96 hr (Table 7).

To investigate of the alteration in the metabolic activity of the starter culture, *B. subtilis* CH10-1 during the incubation period, the ratio of endospores to vegetative cells were investigated at various time points after incubation. As shown in Fig. 1, the *B. subtilis* existed as intact vegetative cells in the *cheonggukjang* until 48 hr after incubation. The endospores began to be detected at 72 hr and then increased in a time-dependent manner until 120 hr after incubation, when only endospores in the absence of vegetative

Table 4. The contents of free amino acids in the raw *cheonggukjang* and the *cheonggukjang* for stew (mg/kg)

Culture time (hr)	Sweet savory								
	Ala	Gly	Lys	Thr	Total	Asp	Glu	Cys	Total
14	0.26	0.17	0.31	0.18	0.92	0.03	0.10	0.00	0.13
96	0.86	0.52	1.09	0.46	2.93	0.12	1.89	0.01	2.02

Culture time (hr)	Bitter				
	Ile	Leu	Met	Phe	Total
14	0.28	0.00	0.15	0.00	0.43
96	0.75	0.00	0.42	0.00	1.17

Table 5. The contents of volatile organic acids in the raw *cheonggukjang* and the *cheonggukjang* for stew (mg/kg)

Culture time (hr)	Acetic acid	Propionic acid	Butyric acid	3-Methyl butanoic acid	Total
14	0.00	7.94	0.00	0.30	8.24
96	3.88	0.26	0.25	14.25	18.64

Table 6. The contents of non-volatile organic acids in the raw *cheonggukjang* and the *cheonggukjang* for stew (μg/kg)

Culture time (hr)	Lactic acid	Oxalic acid	Malonic acid	Furmaric acid	Succinic acid	Glutaric acid	Citric acid	Total
14	0.00	0.23	Trace	0.31	Trace	0.46	0.00	1.00
96	0.00	3.56	Trace	Trace	Trace	0.31	0.00	3.87

Table 7. The contents of free sugars of raw *cheonggukjang* and the *cheonggukjang* for stew (mg/kg)

Culture time (hr)	Sucrose	Maltose	Glucose	Galactose	Fructose	Total
14	0.00	0.31	0.94	0.04	0.58	1.87
96	0.00	0.18	0.02	0.07	0.00	0.27

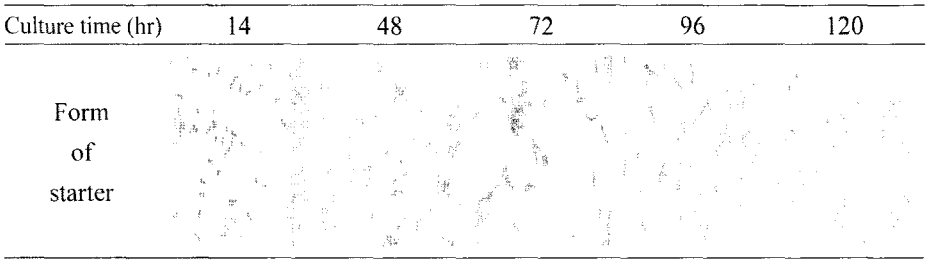

Fig. 1. Form of *cheonggukjang* starter depending on manufacturing periods of *cheonggukjang*.

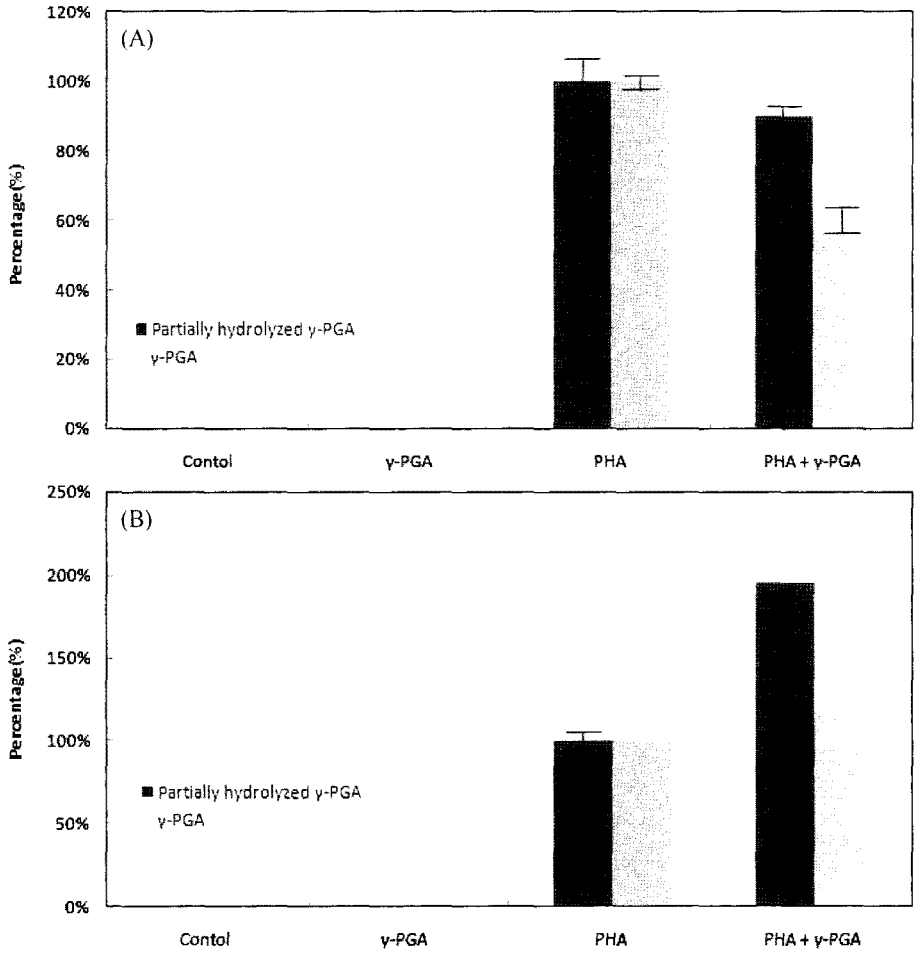

Fig. 2. Effect of γ-PGA or partially hydrolyzed γ-PGA on PHA-induced production of interferon-γ (A) and interleukin-5 (B) in murine splenic T cells *in vitro*.

cells were detectable in the *cheonggukjang*. These results suggested that the digestion of soybean components during first 48-hr incubation was mainly mediated by the action of metabolic activity and exoenzymes of the vegetative cells of *B. subtilis*, whereas the digestion process after 72 hr incubation was mediated by action of a combination of exoenzymes, which were secreted from the vegetative cells, and endoenzymes, which could be released from the cells via their burst along with the release of endospores.

Current results indicated that, based on the sensory evaluation, the *cheonggukjang* manufactured by incubation for 14 to 17 hr was good for the use in the raw like the Japanese *natto*, in that it appeared to have the most favorable taste and odor with the highest viscosity reflecting the content of γ-PGA, and that extension of the incubation time period to longer than 18 hr caused development of a bitter taste. In addition, although the *cheonggukjang* was manufactured by incubation for longer period led to development of an off-flavor possibly due to the accumulation of volatile organic acids, butyric acid, and 3-methyl butanoic acid, a significant enhancement in the contents of sweet-tasty amino acids (alanine, glycine, lysine, and threonine) and savor-tasty amino acids (aspartic acid, glutamic acid, and cysteine) following 96-hr incubation could confer a potent favorable flavor to the *cheonggukjang* for stew. Consequently, these results suggest that a short-term fermentation for 14-18 hr is the optimal for the raw *cheonggukjang* to avoid the formation of a bitter taste and to contain a high level of γ-PGA and free sugars, and a long-term fermentation for more than 4 days

was the optimal for the *cheonggukjang* for stew to accumulate a high level of free amino acids with savory and sweet taste.

In vitro* immunomodulating activity of γ-PGA or partially hydrolyzed γ-PGA purified from *cheonggukjang In order to examine an immunomodulating activity of γ-PGA, we decided to investigate whether γ-PGA could exert regulatory effect toward murine splenic T lymphocytes *in vitro*. Since the viscosity of *cheonggukjang*, which is directly associated with the presence of γ-PGA, was maximal at 14 hr and then became undetectable at 24 hr after incubation, it seemed likely that a degradation of γ-PGA occurred following a maximal accumulation of γ-PGA during the fermentation period of *cheonggukjang*. In this context, γ-PGA was isolated from the *cheonggukjang* manufactured for 14 hr and partially hydrolyzed γ-PGA was isolated from the *cheonggukjang* manufactured for 96 hr. From 250 g of the individual *cheonggukjangs* manufactured for 14 or 96 hr, 20 g of γ-PGA or 12 g of partially hydrolyzed γ-PGA could be obtained. When murine splenic T cells were activated by PHA (2 μg/mL) in the presence or absence of either γ-PGA (100 μg/mL) or partially hydrolyzed γ-PGA (100 μg/mL), the production of IFN-γ by T cells following activation with PHA was reduced in the presence of γ-PGA or partially hydrolyzed γ-PGA (Fig. 2A). However, the production of IL-5 was enhanced in the presence of γ-PGA or partially hydrolyzed γ-PGA (Fig. 2B). On the other hand, when the effect of the γ-PGA or partially hydrolyzed γ-PGA on lipopolysaccharide (LPS)-mediated activation of murine splenic B cells, the B-cell activation was not affected in the γ-PGA or partially hydrolyzed γ-PGA (data not shown). Under these activation conditions, treatment of murine T cells and B cells with either γ-PGA or partially hydrolyzed γ-PGA alone resulted in no response of T cells and B cells. Since cytokine INF-γ is synthesized by Th1 cells and IL-5 is synthesized by Th2 cells (25), these results suggest that γ-PGA or partially hydrolyzed γ-PGA might regulate helper T-cell activation through suppression of Th1 activity and augmentation of Th2 activity.

In conclusion, these results demonstrate that the content of free sugars and γ-PGA was higher in the raw *cheonggukjang*, which was fermented for 14-18 hr, whereas the content of amino acids that confer the savory taste, and the content of off-flavor organic acids such as butyric acid and 3-methyl butanoic acid were higher in the *cheonggukjang* for stew, which was fermented for more than 4 days, than the counterpart. Additional results show that γ-PGA may have an immunomudulating activity via suppressing Th1 activity and augmenting Th2 activity of murine T cells.

Acknowledgments

This research was supported by the Yeungnam University research grants in 2007.

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