Food Eng. Prog.
Vol. 18, No. 4. pp. 276~281 (2014.11)
DOI http://dx.doi.org/10.13050/foodengprog.2014.18.4.276
ISSN 1226-4768 (print), ISSN 2288-1247 (online)

청국장에 대한 ε-Poly-L-lysine 혼합제재의 항 미생물 효과 및 혼합비율의 최적화

이동화 · 백무열 · 최성원[1] · 이은정[2] · 김병용*
경희대학교 생명자원과학연구원 및 식품생명공학과, [1]오산대학교 호텔조리계열, [2]해강바이오

Antimicrobial Effect of ε-Poly-L-lysine Mixture on *Cheonggukjang* and Optimization of the Mixing Ratio

Dong Hwa Lee, Moo Yeol Baik, Sung Won Choi[1], Eun Jung Lee[2], and Byung Yong Kim*

Department of Food Science and Biotechnology, Institute Life Science and Resources, Kyung Hee University
[1]Department of Food and Culinary Arts, Osan University
[2]Hae Kang Bio

Abstract

The objectives of this study were to evaluate the antimicrobial effects of ε-poly-L-lysine mixture (EPM) on *cheonggukjang* and to determine the optimal mixing ratio in *cheonggukjang*. Increasing the EPM up to 2% decreased viable cell counts at both 15°C and 30°C storage. The pH of *cheonggukjang* without EPM increased slowly over the storage periods, but *cheonggukjang* with EPM showed a slight increasing tendency, with the highest pH at 2% EPM. The sensory scores, such as texture and flavor, were highest in samples containing 2% EPM; however, overall preference was not significantly different when compared to the control. For the optimum *cheonggukjang* processing conditions, 13 experimental points were selected. Soybean and EPM were chosen as independent variables. Viable cell count, pH, texture, and overall preference were measured. The optimum formulation of *cheonggukjang* using the numerical analysis was found to be 98.52% soybean and 1.48% EPM, resulting in a 0.722 desirability value.

Key words: *Cheonggukjang*, ε-poly-L-lysine mixture (EPM), optimization

Introduction

Cheonggukjang is a traditional Korean fermented food made using soybeans as the main ingredient. Compared to other sauces such as *gochujang* and *doenjang*, it has a higher fat and protein content, and beneficial nutritional and physiological aspects (Kim et al., 1999). During the fermentation process used in the production of *cheonggukjang*, *Bacillus subtilis* produces sticky substance, which is composed of a polymer of the levan form of fructan and polyglutamate derived from soybean carbohydrates and proteins (Lee et al., 1992). Due to its thrombolytic, antitumor, and immunopotentiation effects, as well as antioxidant activity, consumption of *cheonggukjang* has gradually increased recently (Lee et al., 1992; Kim et al., 1999). Because of the diversity of the inoculated microorganisms, technical difficulties can be encountered during fermentation. Among these difficulties, film and gas are generated during the distributional fermentation period, and the acidity of the mixture is dramatically increased by the continued growth of lactic acid bacteria (Yoo et al., 1998).

As a way to overcome these problems, many studies evaluating the addition of functional preservatives are in progress on these sauces. These preservatives have functional properties that do not interfere with the unique taste and flavor of the sauce. In accordance with an improvement in living standards and consumers interest in health, people tend to prefer natural rather than synthetic preservatives (Cho et al., 2005). Lysozyme (Hughuey & Johnson, 1987), lactoferrin (Orman & Reiter., 1968), chitosan (Oh et al., 1999), polyphenol (Sakanara et al., 1996), acetic acid (Beuchat & Golden, 1989), polylysine (Ko & Kim, 2004), and bacteriocin (Jack et al., 1966) are currently used in the food industry as natural preservatives.

ε-Poly-L-lysine is typically produced as a homo-polypeptide

*Corresponding author: Byung-Yong Kim, Department of Food Science and Biotechnology, Institute Life Science and Resources, Kyung Hee University, Yongin, Gyeonggi 449-701, Korea
Tel: +82-31-201-2627; Fax: +82-31-202-0540
E-mail : bykim@khu.ac.kr
Received February 24, 2014; revised May 19, 2014; accepted May 20, 2014

of approximately 25-30 L-lysine residues (Kahar et al., 2002). As polylysine is stable against pH changes and heat, and doesn't affect the flavor of food, it is currently widely used in many foods (Shima et al., 1984). The amino groups of polylysine act as a cationic surfactant in water and have antimicrobial activity by absorbing to the cell walls of microorganisms (Kahar et al., 2001).

In this study, effect of ε-poly-L-lysine mixture (EPM) with ethanol upon the deterioration of *cheonggukjang* was investigated, and the optimal mixing ratio for *cheonggukjang* preparations was studied using an optimization process.

Materials and Methods

Materials

Raw soybeans were purchased from Dalseong-Nonghyup (Daegu, Korea). *Bacillus subtilis* KCCM-1089P used in the fermentation of *cheonggukjang* was obtained from KCCM (Seoul, Korea). ε-Poly-L-lysine and ethanol were obtained from Shinseung Hichem (Seoul, Korea). 98.2% ethanol and 0.8% ε-poly-L-lysine (EPM) were mixed to determine the antibacterial effect upon *cheonggukjang.*

Three kilograms of soybeans were put in water at 20°C for 12 h, steamed for 30 min, and cooled at 40°C. 20 mL of *B. subtilis* and 400 mL of water were blended with the soybeans to produce the culture solution. This solution was cultured at 42°C for 24 h and *cheonggukjang* was subsequently manufactured (Kim et al., 2008). Various amounts of EPM (0-2% by weight of *cheonggukjang*) were commingled with prepared *cheonggukjang.*

Measurement of pH and color

Ten gram of crushed sample in 90 mL of water was filtered through filter paper (Whatman No.5). pH was measured using an Orion 710 A+ pH meter (Thermo Fisher Scientific, Beverly, MA, USA). The color of the samples was measured using a Colorimeter (JC-801, Color Techno System. Co. Ltd, Tokyo, Japan) and L (lightness) was determined.

Measurement of the antimicrobial activity of EPM and viable cell counts

Antimicrobial activity was measured using *Escherichia coli* KCTC-1039 or *Bacillus cereus* KCTC-1021. 50 μL of 0-2% EPM was spread on an LB agar plate. A 7 mL layer of top agar (0.8%) containing 100 μL each of *E. coli* or *B. cereus* in culture medium was then added to the plate and the plate was incubated at 37°C until a clear zone appeared.

In order to measure viable cells, 10 g of the sample in 90 mL of water was homogenized using an ACE homogenizer (Ultra-Turrax T25, Janke and Kunkel, Brussels, Belgium) set at 15,000 rpm. 1 mL of the homogenized sample was spread on potato dextrose agar (Difco, Detroit, MI, USA) and colony number was measured after 48 h at 30°C.

Sensory analysis

Sensory analysis was carried out by 10 expert panelists. Each panelist evaluated 10 g of boiled sample to which 1 g of salt had been added. A nine-point hedonic scale of 1 (disliked extremely) to 9 (liked extremely) was used by the panelists to evaluate taste, texture, flavor and overall preference.

Experimental design for determining the optimal mixing ratio

The experimental mixture design and statistical analysis were performed using Design Expert software version 7 (Stat-Easy Co., Minneapolis, MN, USA). A D-optimal design consisting of 13 experimental runs, including five replicates at the center point, was chosen to evaluate the combined effect of two independent variables; *Cheonggukjang* (X_1) and EPM (X_2). Based on preliminary experiments, the ranges of the two samples were $0.98 \le X_1 \le 1$ and $0 \le X_2 \le 0.02$, respectively. The response values utilized were viable cells, pH, texture, and overall preference. The effect and regression coefficients of individual linear, interactive, and cubic terms were determined. Results were considered to be statistically significant if they had a probability (p) of 0.05. The numerical optimization was performed using the canonical model basis with a set response goal area and desirability was predicted.

Statistical analysis

All data analysis was performed using SPSS (Statistical Package for the Social Sciences v20.0, Chicago, IL, USA). The level of significance among samples was tested with analysis of variance (ANOVA) and Duncan's multiple range tests performed at the $p<0.05$ level.

Results and Discussion

pH of *Cheonggukjang*

pH of *cheonggukjang* mixed with EPM stored at two temperatures, 15 and 30°C, was measured over a 192 h period, as shown in Fig. 1. The pH of *cheonggukjang* with 2% EPM increased from 6.33 to 6.59 and from 6.31 to 7.19 when stored for 192 h at 15 and 30°C, respectively. The pH of the control without EPM was the lowest after 192 h, having decreased from 6.38 to 6.00 and from 6.34 to 5.55 after storage at 15 and 30°C, respectively. In general, pH levels were maintained or

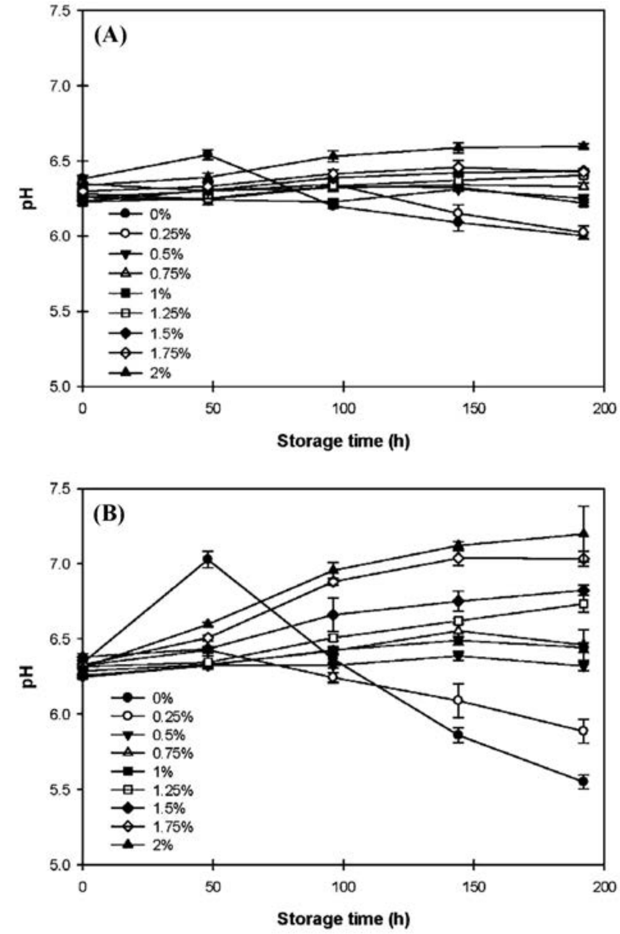

Fig. 1. Changes in pH of *cheonggukjang* with different EPM concentrations during storage at 15°C (A) and at 30°C (B).

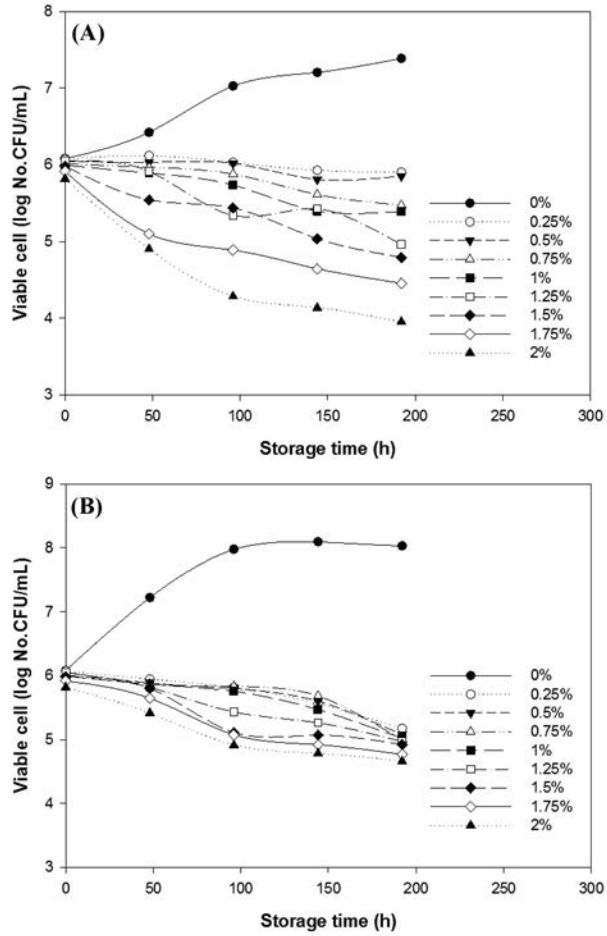

Fig. 2. Viable cell count in *cheonggukjang* with different EPM concentrations during storage at 15°C (A) and at 30°C (B).

slightly increased in samples containing between 0.5 and 1.5% EPM, whereas the control samples showed an increase in pH during the initial 48 h period followed by a continuous decrease in pH over the remainder of the 192 h period. This increase in pH during the initial 48 h storage period might be due to the production of ammonia gas during initial fermentation while the subsequent gradual decrease in pH of *cheonggukjang* may be due to an increase in acidity resulting from the growth of lactic acid bacteria (Suh et al., 1982; Sung et al., 1984). In general, the pH of the *cheonggukjang* with EPM showed a trend of increasing or maintained pH during storage at either 15 or 30°C.

Effect on the growth inhibition of microorganisms

The antimicrobial activity of EPM was tested by evaluating the growth of *E. coli* and *B. cereus*. Potato dextrose agar plate was used for incubation of those strains. Growth of *E. coli* was restricted at 0.4 mg/L EPM, whereas 0.2 mg/L was the minimum concentration for the restriction on growth of *B. cereus*, demonstrating that higher level of EPM was needed for growth restriction of *E. coli* than of *B. cereus*. The viable cell counts in *cheonggukjang* with 0-2% added EPM stored at two temperatures (15 and 30°C) are shown in Fig. 2. Higher EPM concentrations resulted in greater microbial growth inhibition. The most significant reduction in viable cell count was seen with 2% EPM, with a reduction from 6.6×10^5 CFU/mL to 8.95×10^3 CFU/mL at 15°C (Fig. 2A) and 6.6×10^5 CFU/mL to 4.6×10^4 CFU/mL at 30°C (Fig. 2B). Addition of 1.5-2% EPM at 15°C resulted in viable cell counts below 10^5 CFU/mL. These concentrations of EPM showed relatively higher bacterial growth inhibition than lower EPM concentrations (below 1.5%) at both storage temperatures. When 1.0-1.5% EPM and fermented vinegar mixtures were added to *yakbab*, yeast growth was most effectively inhibited (Kim, 2004). Park (2000) also reported that polylysine mixtures inhibited microbial growth and that fermentation occurred slowly in

Table 1. Changes in L values during storage of *cheonggukjang* with different EPM concentrations at 15 and 30°C.

Temp.	Content (%)	Storage time (h) 0	48	72	144	192
15°C	0	53.41±0.14[b]	52.47±0.14[c]	51.60±0.38[a]	50.26±0.14[b]	49.23±0.47[b]
	1	55.69±0.31[a]	54.53±0.30[a]	52.15±0.13[a]	52.48±0.30[a]	52.24±0.83[a]
	2	55.40±0.41[a]	53.28±0.14[b]	52.11±1.23[a]	50.84±0.14[b]	49.69±0.41[b]
30°C	0	57.33±0.08[a]	55.47±0.48[a]	55.36±0.31[a]	48.33±0.48[b]	47.69±1.02[b]
	1	56.85±0.22[a]	55.03±0.55[a]	54.91±0.20[a]	52.38±0.55[a]	50.75±0.38[a]
	2	56.23±0.09[a]	51.46±0.92[b]	50.23±0.73[b]	47.86±0.92[b]	47.69±1.05[b]

Values are mean±standard deviation for n=3
[a-c]Mean within each column with no common superscripts are significantly different ($p < 0.05$)

kimchi. More viable cells remained at higher EPM concentrations following 30°C storage than following 15°C storage due to the optimal growth temperature of viable cells (20-40°C).

Measurement of colors (L value)

The color changes in terms of L values (lightness) of the *cheonggukjangs* at 15 and 30°C are shown in Table 1. L values in samples without EPM decreased from 53.41 to 49.23 at 15°C and also decreased from 57.33 to 47.69 at 30°C. Furthermore, L values in samples with 2% EPM decreased from 55.40 to 49.69 at 15°C and from 56.23 to 47.69 at 30°C, indicating that *cheonggukjang* turned dark during the fermentation time with or without EPM. This is similar to the report of Choi et al. (1998) in that as fermentation time increased, lightness of *cheonggukjang* decreased (i.e.: turned dark). When the EPM content was increased, L values were not changed substantially except in the 72 h storage time point at 30°C. With respect to the effect of temperature, L values at 30°C (49.23-52.24 at 192 h) were generally lowered (darker) than at 15°C (47.69-50.75 at 192 h).

Sensory analysis

The mean sensory scores for taste, texture, flavor, and overall preference of *cheonggukjang* with different EPM concentrations (0-2%) fermented at 30°C for 24 h are shown in Table 2. The control samples without EPM received the lowest flavor (2.4) and texture scores (3.4), while increasing EPM levels received increasing flavor and texture scores. 2% EPM received the highest scores (3.5 and 5.3). Consequently, the specific flavor and texture of *cheonggukjang* was improved by the addition of EPM. Taste was not found to be significantly different among EPM concentrations. A similar finding was reported by Ko & Kim (2004) in that there was no significant difference in taste regardless of the polylysine concentration utilized. But the flavor that was reason for the reluctance of *cheonggukjang* was improved by the addition of EPM. Overall preference was also not significantly different between different EPM concentrations.

Table 2. Sensory evaluation of *cheonggukjang* with different EPM concentrations.

	Taste	Texture	Flavor	Overall preference
0%	4.2±0.74[NS]	3.4±0.48[a]	2.4±0.49[a]	5.8±0.6[a]
0.25%	4.1±0.83	4.6±0.91[bc]	3.2±0.87[bc]	5.4±0.8[ab]
0.50%	4.2±0.87	3.5±0.67[a]	2.7±0.64[ab]	5.4±0.49[ab]
0.75%	4±1	3.9±0.83[ab]	2.4±0.8[a]	6±0.77[a]
1%	4±0.77	3.8±0.74[a]	2.5±0.67[ab]	5.4±0.66[ab]
1.50%	4±1.18	4.8±0.74[c]	2.7±0.64[ab]	4.9±0.94[bc]
1.75%	4.1±0.94	4±0.63[ab]	2.9±0.7[abc]	5.6±0.34[ab]
2%	4±1.18	5.3±0.78[c]	3.5±0.8[c]	5.5±0.8[ab]

[1)]Values are mean±standard deviation for n=10.
[2)]NS=Not significant.
[a-c] Mean within each column with no common superscripts are significantly different ($p < 0.05$).

Design point selection for mixture design, modeling and analysis

Mixture design is an important methodology for experiments in which the variable factors are the proportions of the components of a mixture and the response variables vary as a function of these proportions (Choi et al., 2006). The range of mixture ratios of *cheonggukjang* and EPM was 98-100% and 0-2% (w/w), respectively. The total content of mixed *cheonggukjang* (sum of all variables) was 100% (w/w), without the inclusion of the fixed variables. In order to allocate the points for the mixture within the feasible design region, a modified distance design was applied. Thirteen total design points were set for the experimental designs: three experimental points, five points for calculating the lack of fit, and five replication points (Table 3). In order to remove division errors

Table 3. Quality characteristics of *cheonggukjang* under various conditions by optimal design.

No	Run	Cell count (log CFU/mL)	pH	Sensory characteristics	
				Texture	Overall preference
1	8	4.75	4.77	4	4.6
2	10	8.13	8.02	3.4	5.8
3	1	5.19	5.17	4.6	4.4
4	13	5.06	5.00	3.9	6
5	7	4.69	4.71	5.3	5.5
6	12	8.22	8.04	3.4	5.8
7	3	4.66	4.66	5.3	5.5
8	6	8.06	8.03	3.4	5.8
9	9	4.94	4.92	4.8	4.9
10	4	5.02	5.02	3.8	5.4
11	5	5.06	5.05	3.8	5.4
12	2	5.10	5.08	3.5	5.4
13	11	4.60	4.61	5.3	5.5

Table 4. Analysis of selected models and regression of polynomial equations for the 4 responses.

Response	Model	Prob>F	Equation in term of pseudo components
Cell count	Quadratic	0.0025	7.88A+4.57B-5.75AB-9.46AB(A-B)
pH	Cubic	0.0126	5.57A+7.22B+0.55AB+1.32AB(A-B)
Texture	linear	0.0007	3.43A+4.99B
Overall preference	linear	0.074	5.53A+5.23B

[1)]A: *Cheonggukjang*, [2)]B: EPM

for the order of the actual experimental designs, all experimental orders were performed at random. According to a mixture design, four kinds of responses, including viable cell count, pH, texture and overall preference, were chosen.

In order to analyze the interaction effect on each *cheonggukjang* mixture, modeling was necessary for each response (Han & Kim, 2003). Analysis of the selected models and regressions of the polynomial equations are shown in Table 4. The significance of the selected models was determined by the F-test. Viable cell count and pH were each selected to a quadratic and cubic non-linear model, respectively (0.0025 and 0.0126 probability values). But texture and overall acceptability selected to linear model meant independent contribution. Each coefficient determined at the predicted canonical equation showed the effect of each *cheonggukjang* on each response as a numerical value.

Optimization of mixture ratio for manufacturing *cheonggukjang*

The optimum mixture ratio of *cheonggukjang* with EPM was determined using the optimization process suggested by Derringer and Suich (1980). While texture and overall preference were set to the maximized value, viable cells and pH were each set to the minimized value and target 6.5 values, respectively (Table 5). The desirability constraint used the criterion of degree of optimization. From the numerical optimization results, the optimum *cheonggukjang* formulation was determined to be 98.52% *cheonggukjang* and 1.48% EPM. The optimum process resulted 72.2.% desirability and the predicted response values for this mixture ratio showed that the viable cell, pH, texture, and overall preference scores were 1.56×10^5 CFU/mL, 6.5, 4.38 and 5.34, respectively (Table 5).

Table 5. Optimum constraints using numerical optimization method.

Constraint name	Goal	Numerical optimization solution
Cheonggukjang	is in range	98.52%
EPM	is in range	1.48%
Cell count	minimize	$1.56*10^5$ CFU/mL
pH	is target = 6.5	6.5
Texture	maximize	4. 38
Overall preference	maximize	5.34
Desirability		0.722

Acknowledgement

The research was partially supported by the Shin Seung Hichem in 2013, which is gratefully acknowledged.

References

Beuchat LR, Golden DA. 1989. Antimicrobials occurring naturally in foods. Food Technol. 43: 134-142.

Cho MH, Bae EK, Ha SD, Park JY. 2005. Application of natural antimicrobials to food industry. Korean J. Food Sci. Technol. 38: 36-45.

Choi ID, Son JR, Hong HC, Kim KJ. 2006. Consumer acceptance of three rice varieties formulated by a simplex-lattice mixture design. J. Food Sci. Nutr. 11: 78-83.

Choi UK, Ji WD, Chung YG. 1998. Characteristics of *chunggugjang* produced by *Bacillus subtilis* DC-2. J. Korean Soc. Food Sci. Nutr. 27: 846-851.

Derringer G, Suich R. 1980. Simultaneous optimization of several response variables. J. Qual. Technol. 12: 214-219.

Han GH, Kim BY. 2003. Optimization of the extrusion processing conditions of soymilk residue and corn grits mixture. J. Korean Soc. Food Sci. Nutr. 32: 1270-1277.

Hughuey VL, Johnson EA. 1987. Antimicrobial activity of lysozyme bacteria involved in food spoilage and food borne disease. Appl. Environ. Microbiol. 53: 2165-2170.

Jack RW, Wan J, Gorden J, Harmark K, Davidson BE, Hiller AJ, Wettenhall RE, Hickey MW, Conventry MJ. 1996. Characterization of the chemical and antimicrobial properties of piscicolin 126, a bacteriocin produced by *Carnobacterium piscicola* JG126. Appl. Environ. Microbiol. 62: 2897-2903.

Kahar P, Iwata T, Hiraki J, Park EY, Okebe M. 2001. Enhancement of ε-polylysine production by *Streptomyces albulus* strain 410 using pH control. J. Biosci. Bioeng. 91: 190-194.

Kahar P, Kengo K, Iwata T, Hiraki J, Kojima M, Okebe M. 2002. Production of ε-polylysine in an airlift bioreactor (ABR). J. Biosci. Bioeng. 93: 274-280.

Kim JH. 2004. Food Microbiology and Fermentation Food Science Experiments. Jigu Publishing Co. pp253-261, Seoul, Korea.

Kim MH, Lee NH, Choi UK. 2008. Fermentation characteristics of *cheonggukjang* made of germinated soybean under light condition. J. Life Sci. 18: 1420-1425.

Kim SH, Yang JL, Song YS. 1999. Physiological functions of *cheonggukjang*. Food lnd. Nutr. 4:40-46.

Ko EM, Kim BY. 2004. Antimicrobial activity of ε-polylysine mixtures against foodborne pathogens. J. Korean Soc . Food Sci. Nutr. 33: 705-710.

Lee YL, Kim SH, Choung NH, Yim MH. 1992. A study on the production of viscous substance during *cheonggukjang* fermentation. J. Korean Agric. Chem. Soc. 35: 202-209.

Oh SW, Hong SP, Kim HJ, Choi YJ. 1999. Antimicrobial effects of chitosan on *Escherichia coli* O157:H7, Staphylococcus and Candida albicans. Korean J. Food Sci. Technol. 31: 1667-1678.

Orman JD, Reiter B. 1968. Inhibition of bacteria by lactoferrin and other iron chelating agents. Biochem. Biophys. Acta. 170: 351-354.

Park KH. 2000. A study on the effect of ε-polylysine on the shelf-life extension of *Kimchi.* MS Thesis, Korean Nation Univ. of Transportation, Chungju, Korea.

Sakanara S, Aizawa M, Kim M, Yamamoto T. 1996. Inhibitory effects of green tea polyphenols on growth and cellular adherence of an oral bacterium, *Prophyromonas gingivalis*. Biosci. Biotechnol. Biochem. 60: 745-749.

Shima S, Matsuoka H, Iwamoto T, Sakai H. 1984. Antimicrobial action of ε-poly-L-lysine. J. Antibiotics. 37: 1449-1455.

Suh JS, Lee SG, Ryu MK. 1982. Effect of *Bacillus* strains on the *cheonggukjang* processing-II. Changes of the components and enzyme activities during the storage of *cheonggukjang*. Korean J. Food Sci. Technol. 14: 309-314.

Sung NJ, Ji YA, Chung SY. 1984. Changes in nitrogenous compounds of soybean during *cheonggukjang* koji fermentation. J. Korean Soc. Food Sci. Nutr. 13: 275-284.

Yoo CK, Seo WS, Lee CS, Kang SM. 1998. Purification and characterization of fibrinolytic enzyme excreted by *Bacillus subtilis* K-54 isolated from *cheonggukjang*. Korean. J. Appl. Microbiol. Biotechnol. 26: 507-514.