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Development of a Functional Chungkookjang (Soybean Paste Fermented for 2-4 Days) with Anti-AGS Human Gastric Cancer Cell Properties

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Abstract

To develop a functional chungkookjang; the anticancer effects of chungkookjangs prepared with different varieties of soybeans, starters, fermentation periods and seasoning additive ratios; were studied against AGS human gastric adenocarcinoma cells using the MTT assay, at different stages chungkookjang processing. The chungkookjang samples exerted different antiproliferative effects according to the variety of soybeans used. The chungkookjangs manufactured with soybean var. manrikong exhibited the highest cytotoxicity against AGS human cancer cells. The chungkookjangs fermented with rice straw and *B. licheniformis* strongly inhibited the growth of the AGS human cancer cells. All fermented chungkookjangs had a strong inhibitory effect on the growth of the cancer cells; however, the non-fermented soybean (chungkookjang) showed a low inhibition rate. The fermented chungkookjangs mixed with red pepper powder (RPP) and garlic exhibited strong antiproliferative effect against the cancer cells, and chungkookjang prepared with 1.1% RPP and 1.1% garlic showed the highest cytotoxicity against the cancer cells. The functional chungkookjang fermented with soybean variety of manrikong and *B. licheniformis* for 3 days at 40°C and then mixed with 7.9% salt, 1.1% RPP and 1.1% garlic, exhibited a higher antiproliferative effect than the chungkookjangs prepared by traditional or modified methods, according to the MTT assay. The functional chungkookjang exhibited a similar anticancer effect to the traditional doenjang. These results indicate that the fermentation period and the ratio of seasoning additives, as well as the variety of soybeans and starter cultures may affect the degree of the anticancer effect of chungkookjang.

Key words: functional chungkookjang, AGS human gastric cancer cells, anticancer, MTT assay

INTRODUCTION

Chungkookjang (soybean paste fermented only a few days) is a traditional fermented food, along with doenjang (soy paste fermented for more than 4 months), kanjang (soy sauce) and kochujang (red pepper soybean paste) in Korea. It has been an important source of essential amino acids and fatty acids, supplementing rice and barley proteins in the Korean diet (1). *Bacillus subtilis* or *Bacillus natto* are the primary microorganisms responsible for the fermentation of chungkookjang. After fermentation for 3 days at 40°C, the intact soybeans are covered with a viscous substance and have a soft texture and unique flavor (2).

During the fermentation of chungkookjang, *B. subtilis*- or *B. natto*-, etc derived enzymes hydrolyze the soy proteins into easily digestible peptides and amino acids (3). The fermented chungkookjang contains functional components such as the trypsin inhibitor (3-6), isoflavones (7,8), phytic acid (9), saponins (10), lignin (11), vitamin E (12) and unsaturated fatty acids (13); all of which have demonstrated antimutagenic and anticancer effects.

Natto in Japan, is similar to chungkookjang in Korea, but has a sticky and viscuous polysaccharide with a cheesy texture. The process for making natto is to wash, soak, and steam the small-size soybeans, allow them to cool down and mix with a starter of *Bacillus natto* for a 16~24 hour fermentation process at 40~50°C. For making chungkookjang, large or medium sized cooked soybeans are fermented at 40°C for 3 days with a small amount of rice straw added as a starter or no-starter and then mixed with salt, garlic, red pepper, green onion or ginger, which are then post-fermented to prolong their edible periods. However, this later process is not required for the preparation of natto. Chungkookjang is traditionally served as ggikae (pot stew), while fresh natto is used as a topping for rice with soy sauce or mustard (14,15).

Chungkookjang is traditionally prepared at home by different types of processes depending on the region. The quality of chungkookjang varies considerably with vari-

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eties of soybean, fermenting bacterial strains, fermentation time, the ratio of subingredients, etc. In order to develop a functional chungkookjang, the *in vitro* anticancer effects of chungkookjangs prepared with different varieties of soybeans, starters, fermentation periods and subingredient ratios were investigated during the processing of chungkookjang using AGS human gastric cancer cells in an MTT assay. From these results, the processing method of a functional chungkookjang was established and the anticancer effects were also evaluated and compared with doenjang and natto.

MATERIALS AND METHODS

Soybeans

Soybean var. joonjuri (10 g/100 grains, 41% crude protein, 19% crude lipid), soybean var. manrikong (20 g/100 grains, 41% crude protein, 20% crude lipid) and soybean var. hwangkeumkong (25 g/100 grains, 41% crude protein, 20% crude lipid) and US No. 1 soybean (imported from USA, 16 g/100 grains, 35% crude protein, 18% crude lipid) were obtained from the National Yeongnam Agricultural Experiment Station, Milyang, Korea), the National Crop Experiment Station (Suwon, Korea) and the Korea Soy Sauce Industrial Cooperative (Seoul, Korea), respectively.

Bacterial strains

Bacillus subtilis (KCCM 11315) and *Bacillus licheniformis* CN-115 (16) were obtained from the Korea Culture Center of Microorganisms (Daejeon, Korea) and the Dept. of Food Science and Technology, Yeongnam University, Kyeongnam, Korea, respectively. The medium used for these strains was nutrient broth (Difco, USA) supplemented with 0.05% glucose. The cells were prepared by the incubation at 37°C for 24 hrs and diluted in sterile soybean soup to prepare in a suspension (OD_{600} 0.8) for inoculation of the cooked soybeans.

Preparation of chungkookjang

Traditional chungkookjang and modified chungkookjang were prepared by standardized methods from published literature (17). To prepare traditional chungkookjang, soybeans were washed, soaked in water and then cooked for 4 hrs at atmospheric pressure. The cooled soybeans were fermented with cut rice straw. In the modified method of making chungkookjang, soybeans were cleaned with water and then soaked in 1.5-folds of water at 15°C for 12 hrs and drained for 45 min. The soaked soybeans were cooked in an autoclave (1.0 ~ 1.5 kg/cm^2) for 60 min. After cooling to 50°C, the soybeans were inoculated with 1.0% *Bacillus subtilis* and incubated at 40°C for 72 hrs. The fermented chungkookjang was then crushed and mixed with 7.9% salt. A functional chungkookjang was also prepared from cooked soybeans were inoculated with *Bacillus licheniformis* CN-115, incubated at 40°C for 72 hrs, and then crushed and mixed with 7.9% salt, 1.1% red pepper powder and 1.1% crushed garlic.

Doenjang and natto samples

Traditional doenjang was obtained from Sungchang Traditional Kochujang Village (Moonokrye Food Co., Sunchang, Choenbuk, Korea). Natto (Daie Ltd., Shinko, Japan) was purchased from a local market in Busan, Korea.

Extraction

Freeze dried and powdered chungkookjangs, doenjang and natto samples were extracted with methanol (MeOH, 20-fold) three times by shaking for 8 hrs and then used as a MeOH extract. Each extract was dried by a rotary vacuum evaporator (Buchi 011 & 461, Switzerland) at 45°C and then dissolved in dimethyl sulfoxide (DMSO, Sigma Chemical Co., USA) for the experiment.

Cancer cells and MTT assay

RPMI 1640, fetal calf serum (FCS), trypsin-EDTA and penicillin-streptomycin were purchased from GIBCO Co. (Gaithersburg, MD, USA). AGS human gastric adenocarcinoma cells were obtained from Korea Cell Line Bank (KCLB, Seoul, Korea). The cells were cultured in RPMI 1640 and supplemented with 1% penicillin-streptomycin and 10% FCS. The media were changed two or three times every week. After six or seven days, the cultured cancer cells were washed with PBS. The cells were dissociated with 0.05% trypsin-0.02% EDTA and 180 μL of the cell suspensions (1×10^4 cells/mL) were seeded in each well of 96-well microtitre plates with various concentrations of each extract (20 μL). After 70 hours incubation, 20 μL of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution (2.5 mg/mL PBS) was added. After an additional 4 hours incubation, the supernatant medium was carefully removed. The formazan dye was solubilized by adding 150 μL DMSO to each well followed by gentle shaking. The optical densities were read on an Elyza reader at 540 nm (18-20).

Statistical analysis

Data analyses were performed using SAS 6.0 (SAS Institute, Cary, NC, USA). ANOVA was used to determine possible differences among groups; Duncan's multiple range test was used for *post hoc* comparisons if significant group differences were found.

RESULTS AND DISCUSSION

Effect of soybean varieties on the growth of AGS cells

Most soybeans used for chungkookjang preparation in

Korea are large size. However, small size soybeans have also been used to increase the absorbability of water, for softening of texture and for improved growth of *Bacillus subtilis* or *Bacillus natto* (21). Imported soybeans (US No. 1 from USA) are used to manufacture commercial chungkookjang and doenjang. To determine the best soybean variety for preparing a functional chungkookjang, soybean var. joonjuhri (small size), soybean variety hwangkeumkong (large size), US No. 1 (imported soybean) and soybean var. manrikong (medium size), that has already been demonstrated to have antimutagenic and anticancer activity (21-23) were compared.

Chungkookjang samples exerted different antiproliferative effects depending on the variety of soybeans (Table 1). The chungkookjang prepared with soybean var. joonjuhri and marikong effectively inhibited the proliferation of AGS human gastric cancer cells in the MTT assay, where 51% and 61% inhibition rates were observed with the addition of 0.05 mg/mL, respectively. The chungkookjang manufactured with var. manrikong exhibited the highest cytotoxicity against AGS cancer cells. Even at 0.1 mg/mL, chungkookjang made from soybean var. hwangkeumkong or US No. 1 soybean-used chunkookjang samples inhibited the growth of AGS cancer cells by less than 50%.

We had already reported that the methanol extracts of chungkookjang prepared with soybean var. manrikong exhibited higher antimutagenic activities than those of chungkookjang manufactured with soybean var. hwangkeumkong or US No. 1 soybean against AFB_1 and MNNG in the Ames test using *Salmonella typhimurium* TA100, and MNNG in the SOS chromotest using *E. coli* PQ37 (22,23). Ko (24) also indicated that chungkookjangs exerted different antiproliferative effects depending on the variety of soybeans in the MTT assay. The chungkookjang prepared with soybean var. of manrikong showed the highest cytotoxicity against HT-29 human colon cancer cells. It is unknown what components in var. manrikong may cause or enhance the anticancer effect of chungkookjang, but further studies are needed to elucidate the active components.

Effect of starters on the growth of AGS cells

Cooked soybeans are fermented under ambient environmental conditions or by using rice straw in the traditionally prepared chungkookjang. However, *Bacillus subtilis* or

Table 1. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay of methanol extracts from chungkookjangs that fermented with different kinds of soybeans against AGS human gastric adenocarcinoma cells

Soybean var. \ Treatment (mg/mL)	OD_{540} 0.05	OD_{540} 0.1
Control (PBS)	1.066 ± 0.04^{a}	1.066 ± 0.04^{a}
Joonjuhri[1)]	0.519 ± 0.04^{d} (51)[5)]	0.379 ± 0.02^{c} (64)
Manrikong[2)]	0.414 ± 0.04^{e} (61)	0.254 ± 0.05^{c} (76)
Hwangkeumkong[3)]	0.844 ± 0.01^{b} (21)	0.576 ± 0.14^{b} (46)
US No.1[4)]	0.722 ± 0.05^{c} (32)	0.677 ± 0.04^{b} (36)

[1-4)]The size of the soybeans. [1)]10 g/100 grains. [2)]20 g/100 grains. [3)]25 g/100 grains. [4)]16 g/100 grains.

[5)]Inhibition rate (%) $= \frac{OD_{540} \text{ of control} - OD_{540} \text{ of sample}}{OD_{540} \text{ of control}} \times 100$

[a-c]Means with the different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

Table 2. MTT assay of methanol extracts from chungkookjangs fermented with different starters against AGS human gastric adenocarcinoma cells

Starter \ Treatment (mg/mL)		OD_{540} 0.05	OD_{540} 0.1
Control (PBS)		1.066 ± 0.04^{a}	1.066 ± 0.04^{a}
Natural starter	No inoculum	0.582 ± 0.05^{b} (45)[3)]	0.413 ± 0.06^{b} (61)
	Rice straws	0.273 ± 0.03^{d} (74)	0.229 ± 0.09^{d} (79)
Cultured starter	*B. subtilis*[1)]	0.519 ± 0.04^{b} (51)	0.379 ± 0.02^{bc} (64)
	B. licheniformis[2)]	0.393 ± 0.05^{c} (63)	0.298 ± 0.03^{cd} (72)

[1)]*Bacillus subtilis* KCCM 11315.

[2)]*Bacillus licheniformis* CN-115.

[3)]Inhibition rate (%) $= \frac{OD_{540} \text{ of control} - OD_{540} \text{ of sample}}{OD_{540} \text{ of control}} \times 100$

[a-d]Means with the different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

Bacillus licheniformis is frequently used for fermentation in the modified chungkookjang (17). Table 2 shows the inhibitory effect of chungkookjang prepared with different starters on the growth of the cancer cells. The chungkookjangs fermented with rice straw or a starter culture of *B. licheniformis* showed a higher inhibitory effect on the growth of the cancer cells than chungkookjangs prepared by natural fermentation or with *B. subtilis*. Especially, chungkookjangs fermented with rice straw or *B. licheniformis* as a starter inhibited the growth of the cell growth by more than 60% at the 0.05 mg/mL concentration.

Youn et al. (21) reported that the concentrations of components producing offensive odors, such as alkyl pyrazines and benzaldehyde were lower in *B. licheniformis* inoculated chungkookjang than in those inoculated with *B. subtilis* or *B. natto*.

From these results, rice straws or inoculation of *B. licheniformis* to ferment chungkookjang increases the *in vitro* anticancer effect, and the inoculation of *B. licheniformis* is more desirable because it produces a more consistent and sanitary product than using rice straw.

Effect of fermentation periods on the growth of AGS cells

Table 3 shows the effect of fermentation time in preparing chungkookjangs on cytotoxicity against AGS human gastric cancer cells. All fermented chungkookjangs strongly inhibited the growth of AGS cancer cells; however, the non-fermented chungkookjang (cooked soybeans) exhibited less inhibition than the raw soybeans in the MTT assay. There was no significant difference ($p<0.05$) in the antiproliferative effects according to the lengths of the fermentation.

Therefore, we demonstrated that fermented chungkookjang has stronger anticancer properties than non-fermented chungkookjang. Several studies have indicated that fermented doenjang or chungkookjang showed stronger antimutagenic effects than non-fermented cooked soybeans (17,22,24). Ko (24) also reported that fermented chungkookjang exhibited stronger inhibitory effects on mutagenicity induced by AFB_1 and MNNG and on the growth of AGS human gastric cancer cells and HT-29 human gastric cancer cells than non-fermented chungkookjang. Kwon (17) reported that fermentation of chungkookjang for more than 4 days at 40°C produced an unpleasant off-flavor by decreasing γ-GTP activity and increasing the ammonia-type nitrogen level.

These results demonstrate that fermenting chungkookjang fermented for 3 days at 40°C increases *in vitro* antimutagenic and anticancer effects while optimizing sensory quality.

Effect of ingredient ratios on the growth of AGS cells

Traditional chungkookjang is made from fermented soybeans, salt seasoned with RPP and garlic, but modified or commercial chungkookjangs are prepared with only the addition of salt. From the literatures, we standardized the subingredient kinds and ratios that are used in the preparation of traditional chungkookjang. The ratio was 7.9% salt, 1.1±0.9 of red pepper powder (RPP) and 1.1±0.9 of garlic in proportion to 100 fermented soybeans (17).

Table 4 shows the inhibitory effect of chungkookjangs prepared with different seasoning ratios on the AGS human garstric cancer cells. The chungkookjangs with RPP and garlic exhibited a strong antiproliferative effect against the AGS cells, whereas that made with only salt only weakly inhibited the growth of the AGS cells. The chungkookjang prepared with 1.1% RPP and 1.1% garlic exhibited the highest cytotoxicity against the cancer cells.

We previously reported that RPP exhibited antimutagenic activity against AFB_1 (25-27). Capsaicin, vitamin C and carotenoids in red pepper powder are believed to be the compounds that exert antimutagenic and anticancer effects (28-30). Garlic extract and allyl sulfide in garlic

Table 3. MTT assay of methanol extracts from chungkookjangs fermented for different time periods at 40°C against AGS human gastric adenocarcinoma cells

Treatment (mg/mL) / Fermentation time	OD_{540} 0.05	OD_{540} 0.1
Control (PBS)	1.066±0.04[a]	1.066±0.04[a]
Raw soybeans	0.645±0.09[c] (39)[2)]	0.552±0.01[b] (52)
0 day-fermented[1)]	0.809±0.08[b] (24)	0.609±0.05[b] (43)
3 day-fermented	0.519±0.04[cd] (51)	0.379±0.02[c] (64)
5 day-fermented	0.481±0.06[d] (55)	0.221±0.04[d] (79)
3 day-fermented & 15 day-ripened	0.581±0.10[cd] (45)	0.334±0.02[c] (69)

[1)]The cooked soybeans.

[2)]Inhibition rate (%) $= \frac{OD_{540} \text{ of control} - OD_{540} \text{ of sample}}{OD_{540} \text{ of control}} \times 100$

[a~d]*Means with the different letters are significantly different* ($p<0.05$) by Duncan's multiple range test.

Table 4. MTT assay of methanol extracts from chungkookjangs prepared with different levels of seasoning additives against AGS human gastric adenocarcinoma cells

Subingredient \ Treatment (mg/mL)	OD_{540} 0.05	OD_{540} 0.1
Control (PBS)	1.066±0.04[a]	1.066±0.04[a]
Salt[1)]	0.681±0.10[b] (36)[3)]	0.534±0.02[b] (50)
Salt+1.1% RPP[2)]+1.1% garlic	0.438±0.04[c] (59)	0.386±0.02[c] (64)
Salt+2.0% RPP+0.2% garlic	0.561±0.06[b] (47)	0.459±0.08[bc] (57)
Salt+0.2% RPP+2.0% garlic	0.655±0.03[b] (39)	0.482±0.05[bc] (55)

[1)]7.9% addition.
[2)]Red pepper powder.
[3)]Inhibition rate (%) = $\frac{OD_{540} \text{ of control} - OD_{540} \text{ of sample}}{OD_{540} \text{ of control}} \times 100$
[a-c]Means with the different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

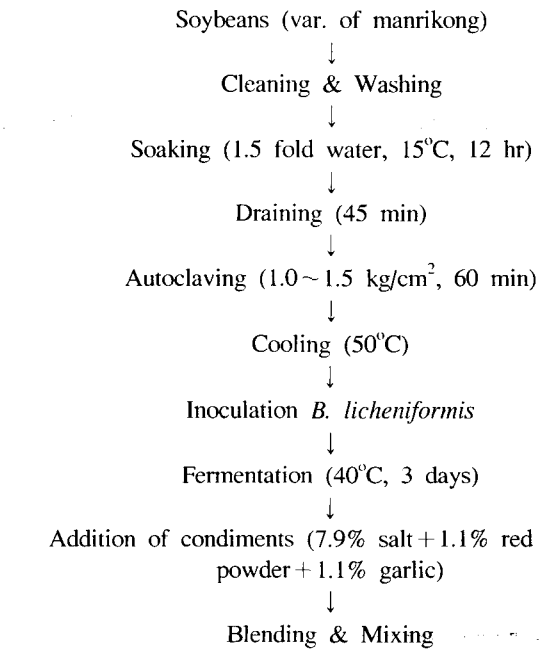

Fig. 1. A diagram for the preparation of a functional chungkookjang developed in this study.

have been shown to convert ultimate mutagens into nontoxic products by increasing glutathione *S*-transferase activity and nonprotein-SH in the microsomal enzyme system of mouse liver (31,32). Lim and Kim (33) and Park et al. (34) also reported that garlic showed antibacterial activity and greatly inhibited the growth of HCT-15 and HT-29 human colon carcinoma cells. This data suggests that the *in vitro* anticancer effects of chungkookjangs can be increased by the addition of seasonings such as RPP and garlic.

From these results, a method for preparing a functional chungkookjang was diagramed as shown in Fig. 1. Soybeans var. of manrikong were cleaned, soaked in 1.5-folds of water at 15°C for 12 hrs and drained for about 45 min. The soaked soybeans were cooked by using autoclave (1.0 ~1.5 kg/cm^2) for 60 min. After cooling to 50°C, the soybeans were inoculated with 1.0% *B. licheniformis* and incubated at 40°C for 3 days. The functional chungkookjang was prepared by crushing and mixing with 7.9% salt, 1.1% RPP and 1.1% crushed garlic.

Table 5. MTT assay of methanol extracts from chungkookjang and other soybean products against AGS human gastric adenocarcinoma cells

Fermented soybeans \ Sample (mg/mL)	OD_{540} 0.05	OD_{540} 0.1
Control (PBS)	1.489±0.09[a]	1.489±0.09[a]
T[1)]- chungkookjang	1.062±0.04[b] (29)[4)]	0.876±0.14[b] (41)
M[2)]- chungkookjang	1.019±0.04[b] (32)	0.759±0.02[b] (49)
F[3)]- chungkookjang	0.833±0.05[c] (44)	0.498±0.03[c] (67)
Doenjang	0.955±0.15[b] (36)	0.501±0.02[c] (66)
Natto	0.962±0.04[b] (35)	0.706±0.14[b] (53)

[1)]Traditionally prepared chungkookjang.
[2)]Chungkookjang prepared with modified method.
[3)]Functional chungkookjang prepared in this experiment.
[4)]Inhibition rate (%) = $\frac{OD_{540} \text{ of control} - OD_{540} \text{ of sample}}{OD_{540} \text{ of control}} \times 100$
[a-c]Means with the different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

Anticancer effect of the functional chungkookjang

The functional chungkookjang prepared from manrikong soybeans and fermented with *B. licheniformis* for 3 days at 40°C and then mixed with 7.9% salt, 1.1% RPP and 1.1% garlic exhibited a stronger antiproliferative effect than the traditional or modified chungkookjangs in the MTT assay (Table 5). The inhibitory effect of natto on the growth of the cancer cells was similar to that of the modified chungkookjang. The functional chungkookjang exhibited a similar anticancer activity to that of doenjang which is known as an anticancer functional food (34-39).

These results indicate that the degrees of the anticancer effect of the chungkookjangs can be altered by manipulating the fermentation period and subingredient ratio as well as by varying the soybean and starter cultures. Although a functional chungkookjang was developed using cancer cells *in vitro*, further studies are needed on the *in vivo* anticancer effects of functional chungkookjang and to clarify its precise molecular mechanisms.

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