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Properties of *Cheonggukjang* Fermented with *Bacillus* Strains with High Fibrinolytic Activities

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Abstract

We previously isolated *Bacillus* strains with high fibrinolytic activities (FAs) from *cheonggukjang* prepared by traditional ways. To test their potential as starters for *cheonggukjang*, soybean was fermented for 72 hr at 37°C with each isolate and a control lab strain: *B. subtilis* CH3-25 (BS3-25), *B. amyloliquefaciens* CH51 (BA51), *B. amyloliquefaciens* CH86-1 (BA86-1), and *B. subtilis* 168 (BS168, control, lab strain). Viable cell numbers of all *cheonggukjang* samples rapidly increased and reached about 10^9 CFU/g after 6 hr. During 72 hr, the initial pH of 6.3 rapidly increased to 8.1~8.2 for *cheonggukjang* fermented with BS3-25 or BA86-1, and 7.3 for those with BA51 or BS168. FAs and protease activities (acid, neutral, and alkaline) rapidly increased in *cheonggukjang* fermented with BS3-25, BA51, or BA86-1 during the first 12 hr. On the other hand, those of *cheonggukjang* fermented with BS168 slightly increased during the first 36 hr. There were significant changes in acid and neutral protease activities in *cheonggukjang* fermented with BA51 or BA86-1 during the 24 hr. Rapid increases of β-glucosidase activity corresponded well with rapid increases of α-amylase and α-galactosidase activities in addition to increases in antioxidant activities and the TPCs (total phenolic contents). The highest increase in the TPCs was observed in *cheonggukjang* fermented with BA86-1 while the least was that fermented with BS168.

Key words: *cheonggukjang*, *Bacillus amyloliquefaciens*, *Bacillus subtilis*, fibrinolytic enzymes, functionalities

INTRODUCTION

Fermented soy foods have been consumed for a long time in many Asian countries and the most well-known examples include Korean *cheonggukjang* (1), Doenjang, Japanese Natto (2), and Chinese Douchi (3). Bacilli, secreting several proteases and amylases into culture media, play key roles during soy food fermentations (4). *Cheonggukjang* has been known to possess some health promoting effects such as anticancer activity, blood pressure lowering effect, FAs, and probiotic effect (5). Although the consumption of *cheonggukjang* has been increased due to enhanced recognition of health benefits by the public, the quality of *cheonggukjang* produced by traditional methods is not satisfactory often. In traditional methods, fermentation is carried out by natural microflora associated with rice straw, which often contaminated with undesirable organisms such as toxin producing *Bacillus cereus* (6).

Bacilli strains with desirable properties must be used as starters if *cheonggukjang* with good quality is produced in a large scale. The desirable properties for starters include the ability to grow fast on soy and to produce desirable metabolites, thus enhancing the functionality of *cheonggukjang*. Starters should not produce bad odor or taste during fermentation. Ideal starters enhance the functionalities of foods when grown on foods by producing bioactive compounds or transforming less bio-active compounds into more active ones. Conversion of isoflavone glucosides into more active aglycone forms by lactic acid bacteria is an example (7) and FAs shown by some bacilli is another example (8).

We previously isolated several bacilli with strong FAs from *cheonggukjang* traditionally prepared at Sunchang county, North Jeolla province (9). In this paper, we prepared *cheonggukjang* by inoculating each from three isolates with high FAs and a control lab strain. General properties of *cheonggukjang* were compared in addition to their TPCs and antioxidant activities. Carbohydrate-cleaving enzyme activities (galactosidase, protease, amylase, glucosidase) were also measured to see the possible relationship with TPCs or antioxidant activities of

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cheonggukjang.

MATERIALS AND METHODS

Preparation of *cheonggukjang*

Tae-Kwang variety soybean grown in Gyeongbuk province (2004 crop year) was used for *cheonggukjang* preparation. Whole beans were washed and soaked in distilled water for 12～18 hr at room temperature (RT). After water was decanted, soybean was autoclaved for 20 min at 121°C. Soybean was inoculated with 2% each culture (dry soybean weight, v/w): BS3-25, BA51, BA86-1, and BS168 (control lab strain, ATCC 23857). Fermentation was proceeded for 3 days at 37°C. Samples were taken at different time points (0, 6, 12, 18, 24, 36, 48, 60, and 72 hr) for analyses.

Cell numbers and pH of *cheonggukjang*

Cheonggukjang samples were transferred into the BagFilter® (Interscience, France) with 50 mL of 0.1% peptone water. Then samples were homogenized using Seward stomacher® Lab Blender (Seward, England) for 1 min. Homogenized *cheonggukjang* sample (1 mL) was serially diluted with 0.1% peptone water and then 100 μL of diluted samples were spread onto LB (Bacto tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L) plates. After incubation for 18 hr at 37°C, single colonies were counted. All measurements were done in triplicates and the mean values were represented. pH of the samples were measured by using a pH meter (DMS, Korea).

FA assay

FAs were measured by comparing the area of lysis zone on a fibrin plate with those caused by known concentrations of plasmin (0.003U, Sigma, USA) as standards (10). In a petri dish, 7 mL of 1.2% (w/v) fibrinogen solution in 1 M phosphate-buffered saline (PBS) was mixed with the same volume of 2% (w/v) agarose solution along with 0.1 mL of thrombin solution (100 NIH units/mL). The petri dish was then left for 1 hr at RT to allow a fibrin clot layer to form. Next, 10 μL of the sample solution was dropped into holes that had been made on a fibrin plate by using a capillary glass tube, and the plate was incubated for 10 hr at 37°C. The size of the clear zone formed by the sample was converted into plasmin unit by comparing with those by known plasmin units. A standard curve showing relationship between the size of clear zones and plasmin units was drawn in the range of 0.94～15 mU of plasmin units.

Protease activity assay

Protease activities of *cheonggukjang* samples were determined by modified methods of Oh and Eom (11) and Kim et al. (12) using 1.0% casein (sodium casein, w/v) as the substrate. Freeze-dried *cheonggukjang* sample (-70°C) was homogenized in sterile water, followed by centrifugation (12,000×*g*, 15 min, 4°C). The pellet was resuspended in 1 mL of 0.4 M lactic acid buffer (pH 3.0, for acid protease), 0.5 M sodium phosphate buffer (pH 6.0, for neutral protease), or 0.2 M McIlvaine buffer (pH 8.0, for alkaline protease), and then subjected to sonication for 30 sec followed by cooling on ice for 30 sec (total 3 cycles). Casein was dissolved in one of the above three buffers depending on the type of protease. 1 mL of casein solution, 100 μL of sample extract, and 20 μL of 0.01 M $CaCl_2$ were mixed and the mixture was incubated for 15 min at 37°C in a water bath. Reaction was stopped by addition of 2 mL of 5% trichloroacetic acid. After mixing, each tube was centrifuged at 3,000 rpm for 10 min and 1 mL of supernatant was taken into a glass tube. Then, 2 mL of 0.5 M NaOH was added to each tube. After mixing, 0.1 mL of Folin-Ciocalteu reagent was added and then A_{550} was read after 10 min. One unit of protease activity was defined as the amount of enzyme that developed the color equivalent to 1 μmol of tyrosine per min under standard conditions.

Carbohydrate degrading enzyme assays

α-Amylase activity of *cheonggukjang* was determined according to the method of Miller (13). Freeze-dried *cheonggukjang* sample (-70°C) was homogenized in sodium acetate buffer (pH 5.4), followed by centrifugation (12,000×*g*, 15 min, 4°C). The pellet was resuspended in 1 mL of sodium acetate buffer (pH 5.4), and then subjected to sonication for 30 sec followed by cooling on ice for 30 sec (total 3 cycles). The reaction mixture consisted of distilled water (500 μL), 1% NaCl (1 mL), sodium acetate buffer (pH 5.4, 2 mL), 0.5% soluble starch (5 mL), and sonicated sample (500 μL). The mixture was incubated for 30 min at 55°C, and then filtered through a Whatman No.4 (Waters, USA). One mL of supernatant was mixed with 3 mL of DNS (dinitrosalicylic acid) reagent and boiled for 5 min. The absorbance of the resulting solution was measured at 550 nm. One unit of α-amylase activity was defined as the amount of enzyme that developed the color equivalent to 1 μmol of maltose per min under standard conditions.

α-Galactosidase activity was determined according to the method of Church et al. (14). Freeze-dried *cheonggukjang* sample (-70°C) was homogenized in PBS buffer, followed by centrifugation (12,000×*g*, 15 min, 4°C). The pellet was resuspended in 1 mL of PBS buffer, and then sonicated as described above. The reaction mixture consisted of 50 μL of 10 mM *p*-nitrophenyl-α-galactoside, 50 μL of 100 mM McIlvaine buffer (pH 5.8), and

100 μL of sonicated sample. After 15 min at 45°C, the reaction was stopped by adding 3 mL of 0.25 M sodium carbonate. The absorbance of the resulting solution was measured at 600 nm. One unit of α-galactosidase activity was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol from the substrate (*p*-NPG) per minute under the assay conditions.

β-Glucosidase activity was assayed by the method of Bahl and Aqrawal (15). Reaction mixture was consisted of 0.1 mL of 10 mM *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG), 0.35 mL of 0.2 M sodium acetate buffer (pH 5.5), and 0.05 mL of sonicated sample solution. After 30 min at 50°C, the reaction was stopped by the addition of 0.5 mL of 1 M sodium carbonate. Released *p*-nitrophenol was measured at 400 nm. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol per minute under the assay conditions.

TPC assay

TPCs were determined according to the method of Sato et al. (16). One gram of freeze-dried *cheonggukjang* was homogenized with 10 mL of 95% ethanol for 2 hr at 60°C and then filtered through a 0.45 μm membrane filter (Waters, USA). Briefly, 1 mL of sample extract was mixed with 1 mL of 95% ethanol and 5 mL of distilled water. Five milliliters of 50% (v/v) Folin-Ciocalteu reagent was added to the mixture, and then 1 mL of 5% Na_2CO_3 was added after 1 min. The mixture was shaken and left for 60 min in the dark at RT. A phenolic content standard curve equation was generated by assaying gallic acid ethanolic solutions with the concentrations of 25～200 μg/mL and plotting the concentration versus absorbance at 725 nm. Results were expressed as μg gallic acid equivalents/g of dried sample.

DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity

Antioxidant activity of *cheonggukjang* was estimated by measuring DPPH radical scavenging activity according to the method of McCue and Shetty (17). 0.2 mL of the sample ethanolic extract (control was 95% ethanol) was mixed with 0.8 mL of 0.2 mM DPPH in 95% ethanol and the mixture was left in the dark for 30 min at RT. The absorbance of the resulting solution was measured at 517 nm. The capability to scavenge DPPH radical was calculated as follows:

$$\text{DPPH scavenging activity (\%)} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

Statistical analysis

Statistical analysis was performed using analysis of variance (ANOVA) followed by Duncan's multiple range test at α=0.05. The values are mean±SD for three measurements in each group.

RESULTS AND DISCUSSION

Viable cell number and pH of *cheonggukjang*

Fig. 1 shows the viable cell numbers in *cheonggukjang* during fermentation at 37°C. All 4 inoculated bacilli showed relatively good growth and no significant differences in cell numbers were observed. Cell numbers increased rapidly from the initial number of 10^7 CFU/g of sample and reached 10^9 CFU/g of sample in all *cheonggukjang* samples within the first 24 hr. After 24 hr, cell numbers remained constant in all *cheonggukjang* samples. *Cheonggukjang* fermented with BA51 showed lower cell number than others but the difference was not significant. Pack et al. (18) prepared *cheonggukjang* by inoculating 3 *Bacillus* strains individually and incubating at 40°C. The cell numbers were 10^9 CFU/g of sample after 72 hr. We observed similar results through this work (18). Fig. 2 shows the pH change of *cheonggukjang* during fermentation. The initial pH was 6.3 and then gradually increased as fermentation proceeded, reaching 8.2 in *cheonggukjang* fermented with BS3-25 at 72 hr. *Cheonggukjang* fermented with BS3-25 or BA86-1 showed higher pHs than that fermented with BA51 or BS168. Different pHs reflected different capabilities of strains to produce metabolites such as ammonia, responsible for pH increase in *cheonggukjang*. The pH values were in the same range as reported by others (18).

FAs of *cheonggukjang*

Fig. 3 shows the changes in FAs of *cheonggukjang* during fermentation. FAs increased significantly during

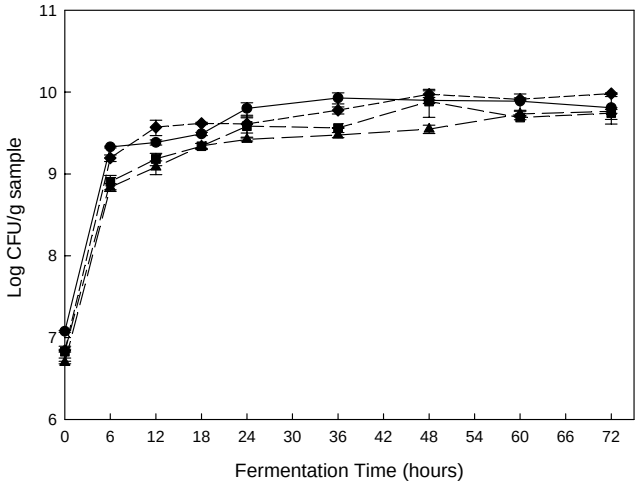

Fig. 1. Viable cell numbers of *cheonggukjang* during fermentation with BS3-25 (●), BA51 (▲), BA86-1 (■), and BS168 (◆) at 37°C for 72 hr.

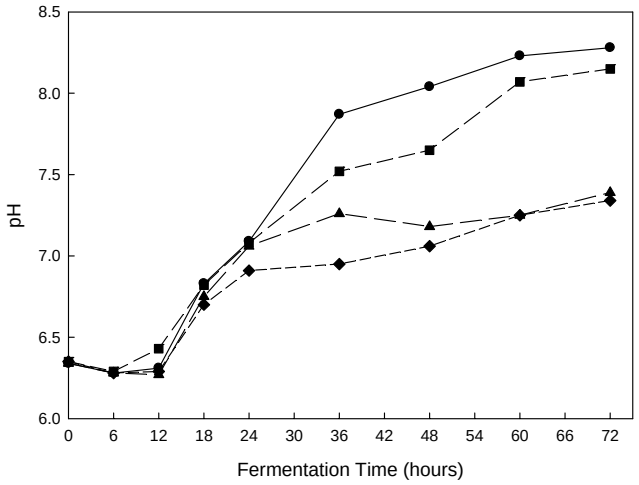

Fig. 2. pH of *cheonggukjang* during fermentation with BS3-25 (●), BA51 (▲), BA86-1 (■), and BS168 (◆) at 37°C for 72 hr.

the first 24 hr of fermentation ($p<0.05$) (Fig. 3) and *cheonggukjang* fermented with BA86-1 showed the highest value (16.5±0.5 U/g of dried sample) at 24 hr and then maintained stably at a reduced level during the rest of fermentation. *Cheonggukjang* fermented with control lab strain, BS168, did not show activity until 36 hr, then the activity rapidly increased, and after 48 hr, reached in the same range with other *cheonggukjang* samples. It was suspected that some protease(s) with FA became active from inactive state or its synthesis induced upon entering into the stationary growth phase. It has been known that during stationary phase, proteolytic activities are increased to degrade inactive or denatured proteins (19). Several proteases are involved for the proteolysis and some of them might be responsible for the sudden increase of FAs. Obviously the fibrinolytic enzymes from 3 strains are different from those of BS168

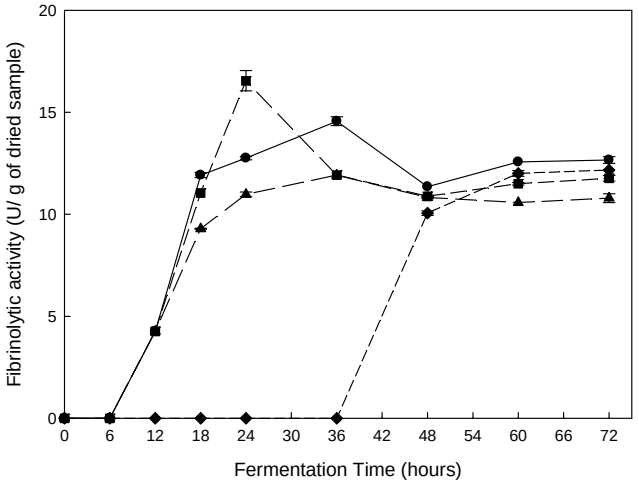

Fig. 3. Fibrinolytic activities of c*heonggukjang* during fermentation with BS3-25 (●), BA51 (▲), BA86-1 (■), and BS168 (◆) at 37°C for 72 hr.

because the activities appeared during the exponential growth phase. Further studies are necessary to characterize them and maximize the activities for the preparation of *cheonggukjang*.

Protease activities of *cheonggukjang*

Changes in acid, neutral, and alkaline protease activities of *cheonggukjang* during fermentation are shown in Fig. 4. Acid protease activities gradually increased in all *cheonggukjang* samples during 72 hr of fermentation. *Cheonggukjang* fermented with either BS3-25, BA51, or BA86-1 showed significantly higher activities than that with BS168 up to 60 hr (Fig. 4(a)). Neutral protease activities increased in a similar way as fermentation proceeded (Fig. 4(b)). *Cheonggukjang* fermented with BA51 showed the highest activity (11.5 U/g of dried sample) at 60 hr, followed by that with BA86-1 (11.2 U/g of dried sample) at 36 hr. *Cheonggukjang* fermented with either BA86-1 or BA51 had higher activities than those with *B. subtilis* strains. The patterns of acid protease and neutral protease activities were similar with those for *cheonggukjang* prepared by germinated soybean (20). Although the activity values were not the same probably because of differences in the methods, acid and neutral protease activities of our samples showed a similar pattern of gradual increase during fermentation like *cheonggukjang* prepared by other researchers (20). *Cheonggukjang* fermented with BA86-1 showed the highest alkaline protease activity (8.3 U/g of dried sample, Fig. 4(c)) at 36 hr. The results indicated that *B. amyloliquefaciens* strains had generally higher enzyme activities than *B. subtilis* strains used for this work (see below for description of other enzymes).

α-Amylase activity of *cheonggukjang*

Changes in α-amylase activities of *cheonggukjang* samples are shown in Fig. 5. *Cheonggukjang* fermented with either BA 86-1 or BA51 showed significant increases in α-amylase during the first 24 hr and then the activity gradually increased for the next 24 hr. The highest α-amylase activity was observed in *cheonggukjang* fermented with BA86-1 for 48 hr (67±1.8 U/g of dried sample). *Cheonggukjang* fermented with BS168, a control, did not show any activity until 36 hr and then activity appeared and gradually increased. Compared to control *cheonggukjang*, *cheonggukjang* fermented with BS3-25 showed slightly higher α-amylase activity, but its level was much lower than those for *cheonggukjang* fermented with *B. amyloliquefaciens* strains. Similarly low α-amylase activities were reported for *cheonggukajng* prepared by using rice straw or inoculated with *B. natto* (20), and the activities were in the same range

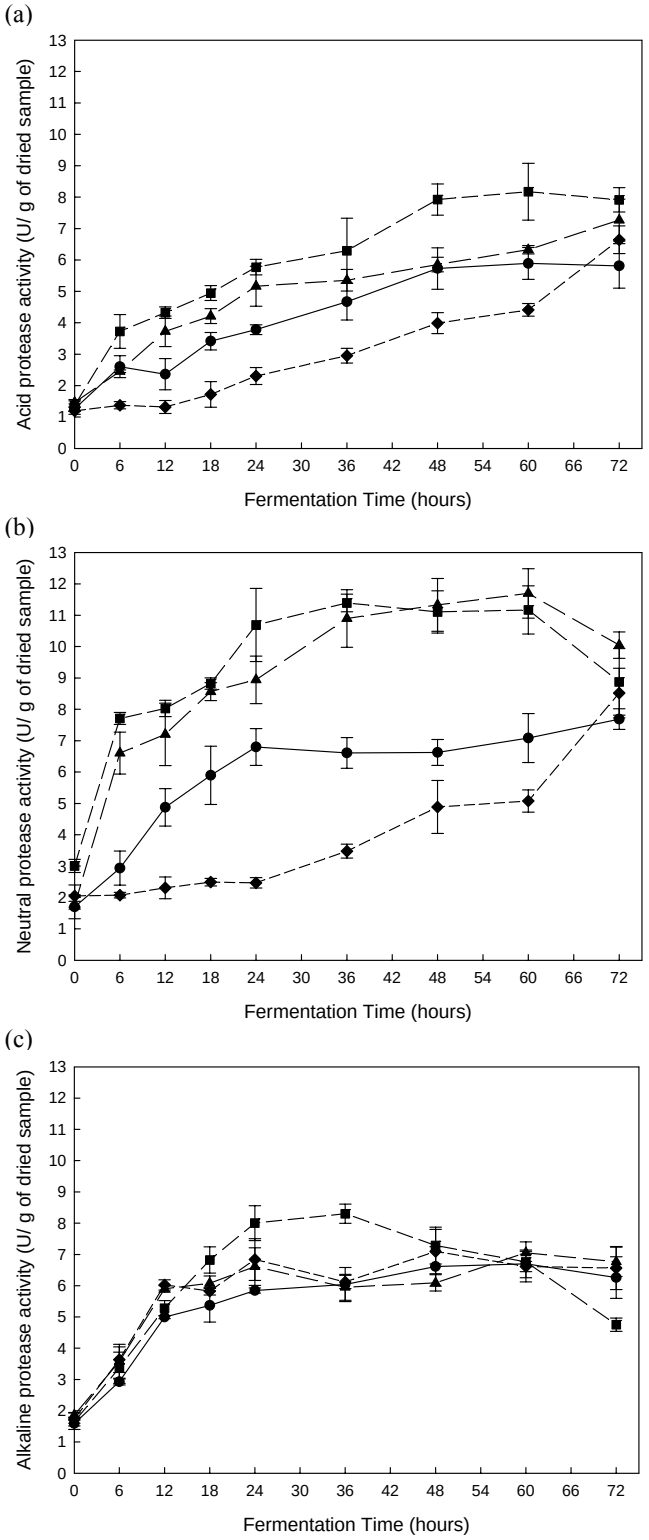

Fig. 4. Acid, neutral, and alkaline protease activities of *cheonggukjang* during fermentation with BS3-25 (●), BA51 (▲), BA86-1 (■), and BS168 (◆) at 37°C for 72 hr. (a), (b), and (c) were measured at pH 3, 6, and 8, respectively.

of *cheonggukjang* prepared with BS168 or BS3-25. The results indicated that some *B. subtilis* strains or bacilli naturally present in rice straw did not have strong α-

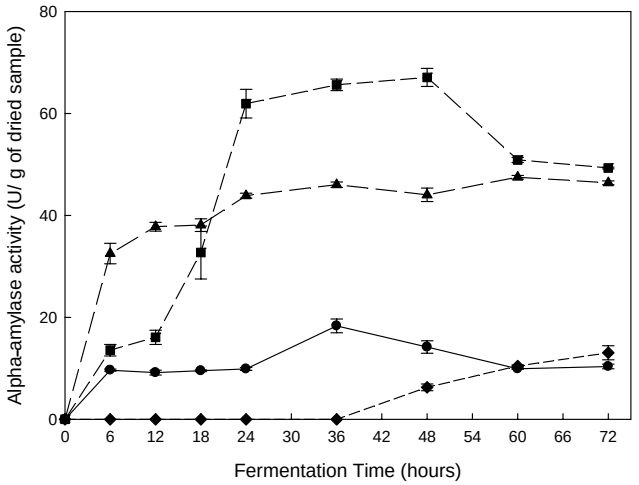

Fig. 5. α-Amylase activity of *cheonggukjang* during fermentation with BS3-25 (●), BA51 (▲), BA86-1 (■), and BS168 (◆) at 37°C for 72 hr.

amylase activity. α-Amylase cleaves the α-1-4 glucosidic bonds in starch randomly, generating small amounts of glucose, maltose, and α-limit dextrin (21). Higher α-amylase activities could help producers grow on starchy food materials and produce metabolites.

β-Glucosidase activity of *cheonggukjang*

Changes in the β-glucosidase activities of *cheonggukjang* samples are shown in Fig. 6. Significant increase in β-glucosidase activity was observed during the 18 hr of fermentation ($p<0.05$). *Cheonggukjang* fermented with BA51 and BA86-1 showed rapid increases in the activity for the first 18 hr, reaching 378.9±11.1 and 486.9±19.6 mU/g of dried sample, respectively. The highest activity was observed in *cheonggukjang* fermented with BA86-1 (539.9±3.0 mU/g of sample) for 36 hr and the lowest activity in that fermented with

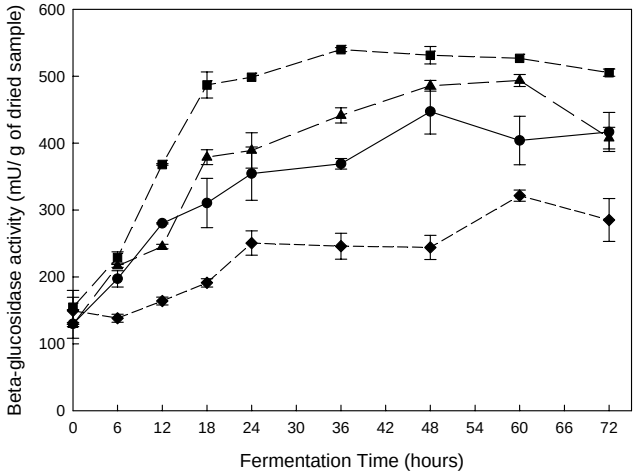

Fig. 6. β-Glucosidase activity of *cheonggukjang* during fermentation with BS3-25 (●), BA51 (▲), BA86-1 (■), and BS168 (◆) at 37°C for 72 hr.

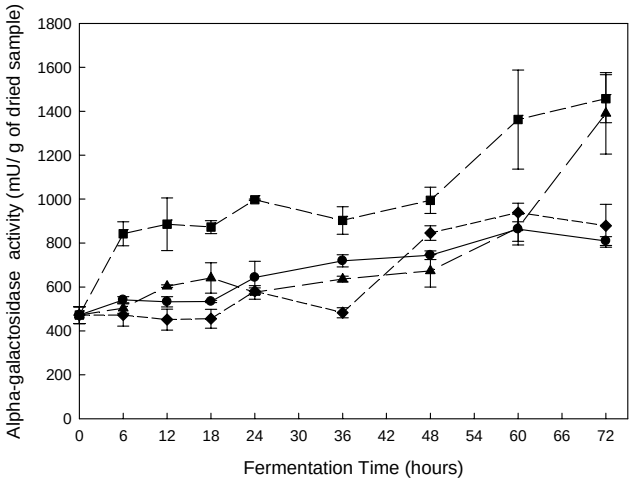

Fig. 7. α-Galactosidase activity of *cheonggukjang* during fermentation with BS3-25 (●), BA51 (▲), BA86-1 (■), and BS168 (◆) at 37°C for 72 hr.

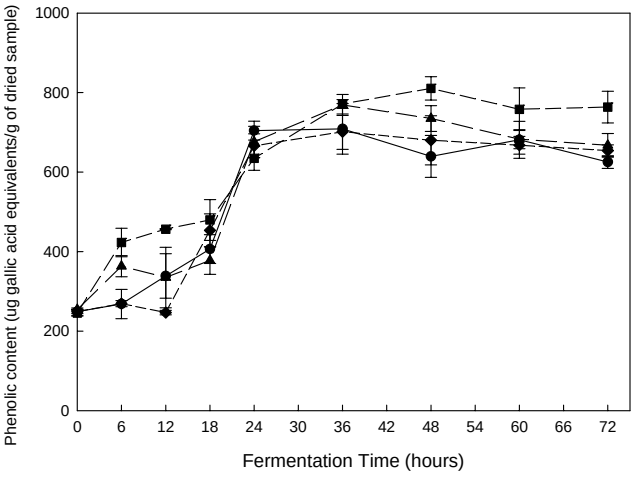

Fig. 8. TPC of *cheonggukjang* during fermentation with BS3-25 (●), BA51 (▲), BA86-1 (■), and BS168 (◆) at 37°C for 72 hr.

BS168 (245.9±19.3 mU/g of sample).

β-Glucosidase is the enzyme responsible for the conversion of glycosidic compounds such as soy isoflavonoids into aglyconic forms. Aglycones such as genistein and daizein are more easily absorbed inside body than glycosidic counterparts and thus functionally more active than glycosides such as genistin and daidzin (7). β-Glucosidase activity is thus desirable for *Bacillus* starters.

α-Galactosidase activity of *cheonggukjang*

Fig. 7 shows the changes in α-galactosidase activity of *cheonggukjang*. *Cheonggukjang* fermented with BA86-1 for 72 hr showed the highest activity (1457.4±109.4 mU/g of dried sample) and that fermented with BA51 the second highest activity (1390.5±185.7 mU/g of dried sample). *Cheonggukjang* fermented with BS3-25 showed the lowest activity (809.4±20.2 mU/g of dried sample) at the same time point.

α-Galactosidase is the enzyme which cleaves the α-1-6 bond in raffinose and stachyose, releasing galactose and sucrose. Both raffinose and stachyose are the causing agents for flatulence when soy and soyfoods are consumed, which is one of the big problems hindering consumption of soyfoods (22). BA86-1 with high α-galactosidase activity might be useful for reduction of flatulence factors during *cheonggukjang* fermentation.

TPC of *cheonggukjang*

Changes in the TPC of *cheonggukjang* samples are shown in Fig. 8. TPC in all *cheonggukjang* gradually increased during the first 24 hr and then remained until the end of fermentation period. It may be due to rapid growth of *Bacillus* strains releasing carbohydrate-cleaving enzymes at the early stage of fermentation. *Cheonggukjang* fermented with BA86-1 had the highest TPC throughout the fermentation and the highest value was observed at 48 hr, 810.6±29.2 μg gallic acid equivalents/g of dried sample, possibly due to high carbohydrate-cleaving enzyme activities (galactosidase, amylase, glucosidase).

DPPH radical scavenging activity of *cheonggukjang*

Antioxidant activity of *cheonggukjang* measured by DPPH scavenging assay gradually increased with fermentation time up to 48 hr and then remained or decreased during the rest of fermentation period (Fig. 9). The highest DPPH scavenging activities were observed in *cheonggukjang* fermented with BA86-1 for 36 hr (92.9±1.7%) and BA51 for 60 hr (92.4±7.2%). On the other hand, *cheonggukjang* fermented with *B. subtilis* strains showed significantly higher DPPH scavenging ac-

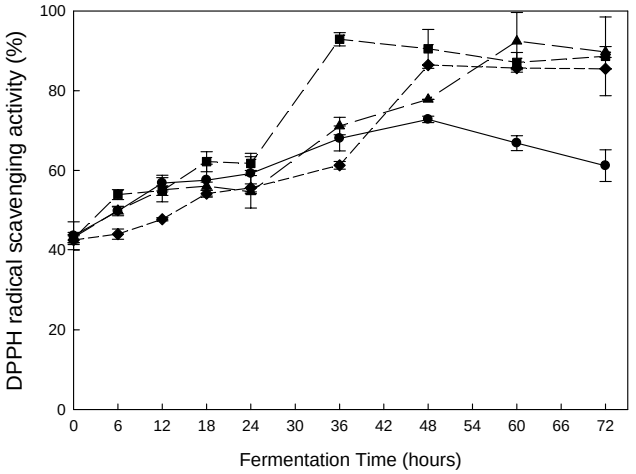

Fig. 9. DPPH scavenging activity of *cheonggukjang* during fermentation with BS3-25 (●), BA51 (▲), BA86-1 (■), and BS168 (◆) at 37°C for 72 hr.

tivities at 48 hr; 86.4±0.05% for 168 and 72.8±0.8% for BS3-25. These values were much higher than those reported previously. Pack et al. reported that the DPPH scavenging activities of *cheonggukjang* fermented with *B. subtilis* or *Bacillus* strains were in the range between 18.95 and 23.37% (18). Thus it seemed that the strains used in this work were better in terms of generating compounds responsible for the activities.

In this study, *cheonggukjang* was prepared by inoculation of each strain from two *B. amyloliquefaciens* (BA51, BA86-1) and two *B. subtilis* strains (BS3-25, BS168). BA51, BA86-1, and BS3-25 were isolates from traditional *cheonggukjang* with strong FAs whereas BS168 was a control, lab strain. BA51 and BA86-1 showed higher β-glucosidase, α-amylase, and α-amylase activities than two *B. subtilis* strains. Higher TPC and DPPH radical scavenging activities of *cheonggukjang* fermented with BA51 or BA86-1 could be due to the higher enzyme activities since the enzymes generate various smaller molecules which contribute to the TPC and antioxidative capacities of *cheonggukjang*. McCue and Shetty similarly reported that lignin degrading enzymes increased TPC and antioxidant capacity during whole soybean processing (17). Therefore, BA86-1 and BA51 seem desirable starters for *cheonggukjang* fermentation judged from these results. Although the results obtained through this work indicated the advantages of *cheonggukjang* prepared by inoculation of starter(s) over *cheonggukjang* traditionally prepared, the effects of starters must be evaluated carefully through animal or human trials. Through animal or human tests, the improved functionalities of *cheonggukjang* by controlled fermentation including fibrinolysis and antioxidative activities could be assessed accurately. In addition to functionalities and safety issues, further studies should be done on the sensory properties of *cheonggukjang* prepared by either *B. amyloliquefaciens* strain. Also, the effects of mixed culture on the quality of *cheonggukjang* are examined to know if there will be some synergistic effects between different starters for the functionalties of *cheonggukjang*. As a conclusion, both BA51 and 86-1 are promising as starters for the production of *cheonggukjang* with enhanced functionalities. Future studies are required to prove their usefulness as starters.

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