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Quality Characteristics of *Kochujang Meju* Prepared with *Aspergillus* Species and *Bacillus subtilis*

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Abstract To standardize a manufacturing method and improve the quality of traditional *kochujang*, eight-types of *meju* with different shapes (brick, grain) were prepared using *Aspergillus oryzae* (A.o) or *Aspergillus sojae* (A.s) alone or in combination with *Bacillus subtilis* (B.s). The physicochemical characteristics and enzyme activities of the various *meju* were compared during fermentation for 12 days at 28°C. The moisture content of both the brick- and grain-shaped *meju* were gradually decreased from an initial content of 50.47 to 54.89% to a content of 12.91 to 16.25% on day 12 of fermentation. The neutral protease activities of the brick-shaped *meju* ranged from 1.19±0.12 to 1.25±0.28 unit/mL, and were similar for all treatments. The α-amylase activities in A.s+B.s treatment of brick-shaped and grain-shaped *meju* were the highest, 11±0.6 and 9±0.7 unit/mL, respectively. The β-amylase activities ranged from 1.53±0.01 to 1.56±0.02 unit/mL, and were similar for all treatments. The amino type nitrogen content of A.o+B.s brick-shaped *meju* was the highest, 0.39±0.03%. We confirmed that the brick-shaped *meju* prepared with *A. oryzae* and *B. subtilis* could be used to prepare traditional *kochujang* to improve the quality of the product.

Keywords: *meju*, *Aspergillus* sp., *Bacillus subtilis*, enzyme, *meju* shape

Introduction

Kochujang, a fermented red pepper-soybean paste, is a popular spicy condiment common in Korean, and is characterized by a hot, sweet, and savory taste. Red pepper powder (greater than 6%, w/w) and salt may be mixed with the *meju*, which is fermented by natural microflora or by the use of pure strains, mostly *Aspergillus* and *Bacillus* sp. and the enzymes produced, and boiled rice before or after the fermentation process.

Kochujang can be prepared by the traditional method (at home using *meju*) or by a mass-production commercial process in factories using *Aspergillus oryzae*. In the case of traditional *kochujang*, various naturally occurring bacteria and fungi proliferate in the *meju* and require a long fermentation and aging period (1). The traditional processes often generate an 'off-flavor' and unacceptable taste because of contaminant microorganisms (2). In attempts to improve the flavor and taste of *kochujang*, controlled fermentation and the additional enzyme activities of known microflora (*A. oryzae*) have been managed commercially, and the process has been accelerated (3).

The quality of *kochujang*, however, depends not only on the unique ratio of the ingredients, the processing methods, and the conditions of aging, but also on the types of *meju* or *koji* products that are desired (4). *Meju* is one of the most important ingredients in the *kochujang* preparation and directly influences the quality of the final *kochujang* product. Therefore, an excellent *meju* is required for the preparation of high-quality *kochujang*. The most important microorganisms present in the *meju* of traditional *kochujang* preparations (where various microorganisms participate during fermentation) are the bacteria and fungi, which both yield their own special flavors and tastes; the bacteria influence flavor on the inside of the *meju*, while fungi influence the outer surface of the *meju* (5, 6). Enzymes produced by both bacteria and fungi in the *meju* hydrolyze the proteins and the carbohydrates of the soybean, which are the basis of traditional *kochujang*. Therefore, the control of *meju* fermentation, whether by commercial or traditional at-home methods, is important for the unique taste of a *kochujang* (7). Many studies on the improvement of manufacturing methods and the selection of strains for *meju* preparation have been conducted (4, 8-10). To produce a traditional *kochujang* with standardized qualities, however, we selected a manufacturing process using *Aspergillus* fungi and *Bacillus* sp. bacteria.

In this study to optimize the production process for a high quality *kochujang* prepared by traditional fermentation method, eight-types of *meju* (brick-shape, grain-shape) were prepared with *Aspergillus oryzae* (A.o) and *A. sojae* (A.s) alone or in combination with *Bacillus subtilis* (B.s). We evaluated the *meju* based on their physicochemical properties and enzyme activities as determined during a 12-day fermentation process.

Materials and Methods

Materials A Taekwang variety of soybean harvested in autumn 2004 in Sunchang (Jeonbuk, Korea) were used for the *meju* preparations. An Ilmi variety of non-glutinous rice, also harvested in autumn 2004 in Buan (Jeonbuk, Korea), was used for the *koji* and *meju* preparation.

Microorganisms *A. oryzae* and *A. sojae* used for the *koji* preparation were obtained from the Hagyeong Fermentation

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Research Center (Gyeonggi, Korea). The *B. subtilis* for *meju* preparation was obtained from Daesang Foods Co. (Sunchang, Korea).

***Koji* preparation** The *koji* preparation procedures are outlined in Fig. 1. Non-glutinous rice was soaked for 12 hr at 20°C, drained, and steam-heated for 30 min at 121°C. The *koji* was then cooled to approximately 30°C, inoculated with a 3%(v/w) spore suspension of either *A. oryzae* or *A. sojae* and incubated for five days at 25°C (11). The spore suspension was made by homogenizing the spores with 10 mL of 0.1% peptone water containing 0.1% Tween 80 in the plate count agar medium.

***Meju* preparation** The soybean used for the *meju* preparation contain approximately 10.5% moisture, 17.9% oil, 38.7% protein, 26.2% carbohydrate, and 5.4% ash. Eight types of *meju* were prepared according to their physical shapes (brick or grain), the two strains of fungi used (*A. oryzae, A. sojae*) and one bacterium used (*B. subtilis*). The *meju* preparation procedures are outlined in Fig. 2 and Table 1 (12). Soybean were soaked in water for 9 hr at 20°C, allowed to drain for 1 hr and steam-heated for 30 min at 121°C. The soybeans were then cooled to about 30°C and ground. The non-glutinous rice was soaked in water for 12 hr at 20°C and treated as described for the soybeans. For *meju* prepared with *A. oryzae* or *A. sojae*, the mixture of steam-heated soybean and non-glutinous rice (6:4) was inoculated with 0.5%(v/w) of non-glutinous rice *koji* prepared with *A. oryzae* or *A. sojae*, mixed and then molded in bamboo trays (18×10×3 cm). The soybean and non-glutinous rice mixture was ground for the preparation of brick-shaped *meju*, but not for the grain-shaped *meju*. For *meju* prepared with *B. subtilis*, the mixture of steam-heated soybean and non-glutinous rice (6:4) was inoculated with 0.5%(w/w) of the culture medium of *B. subtilis* together with 0.5%(v/w) of non-glutinous rice *koji* prepared with *A. oryzae* or *A. sojae*, mixed and then molded in bamboo trays (18×10×3 cm). *Meju* prepared with *A. oryzae* and *A. sojae* with or without *B. subtilis* were fermented for 12 days at 28°C and dried for 3 days in a drying room. The *meju* was ground finely with a 425-μm sieve (Laboratory test sieve, Endecotts Ltd., England) for subsequent analysis (13).

Chemical analysis Mashed *meju* (5 g) mixed with 45 mL distilled water was prepared to measure the pH of the *meju* with a pH meter (Orion SA520; Orion Research Inc., Beverly, MA, USA) (14). The titratable acid content was determined as the titration volume in mL of 0.1 N NaOH needed to bring the pH to 8.3 after homogenization, filtering and a 10-fold dilution of the *meju* powder (15). Moisture content was determined through a 105°C drying method (16).

Enzyme activities The extracts of 10 g of *meju* powder were prepared by shaking in 100 mL of distilled water for 4 hr at room temperature, followed by centrifugation at 17,000×g for 10 min (Model J2-21 Centrifuge; Beckman Instruments, Inc., Palo Alto, CA, USA). The supernatant (considered the extract) was then collected to determine the amylase and protease activities (17).

To measure α-amylase activity (18), 1.0 mL of the supernatant was added to a mixture of 1.0 mL of 1% soluble starch (pH 5.0) and 1.0 mL of acetate buffer, and heated at 40°C for 30 min. Ten mL of 0.5 M acetic acid was then added to stop the reaction, after which 10 mL of 3.33×10^{-4} N iodine solution was added. The activity of the reaction mixture was measured as its absorbance at 660 nm using a UV-spectrophotometer (Shimadzu UV-1201, Kyoto, Japan) and expressed as units per 1.0 mL of the supernatant.

β-Amylase activity was determined by the dinitrosalicylic acid method (19), in where a mixture of 1.0 mL of 1% soluble starch and 1.0 mL of acetate buffer was

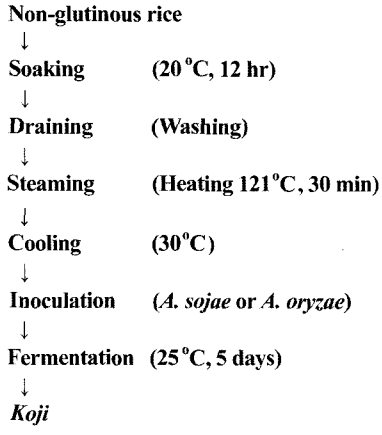

Fig. 1. Procedure of *koji* preparation.

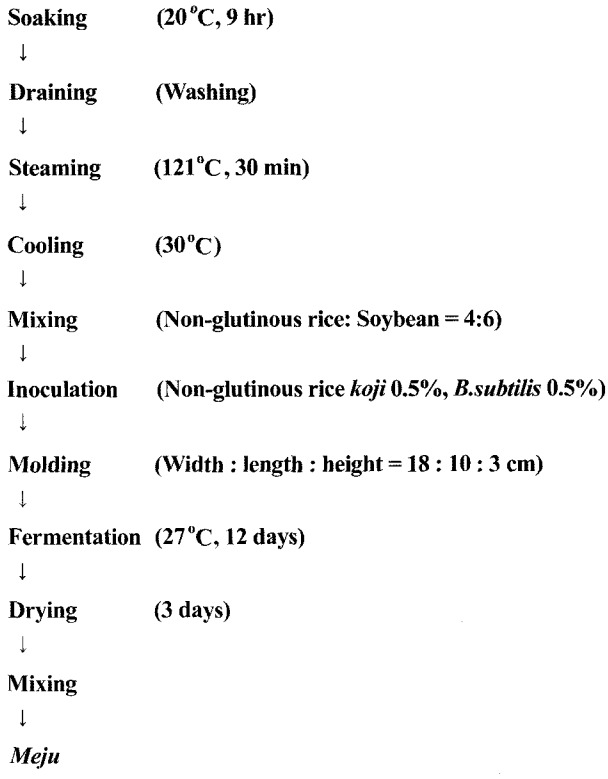

Fig. 2. Procedure of *meju* preparation.

added to 1.0 mL of the supernatant and warmed for 10 min at 30°C. Dinitrosalycylic acid (3 mL) was added to the reaction mixture, and the activity level was determined as the absorbance at 550 nm using the UV-spectrophotometer. Using maltose as the standard, a unit of enzyme activity was expressed as the liberation of 1.0 mg maltose per 1.0 mL of the supernatant.

Aliquots of 1.0 mL of the supernatant were used to measure protease activity (20); the pH was adjusted to 3.0 (acidic) or 7.0 (neutral). The pH of 2 mL of 0.6% casein was adjusted to 3.0 or 7.0, and warmed for 2 min at 30°C. The supernatant (0.5 mL) was then added to 2 mL of 0.6% casein with the corresponding pH and reacted at 30°C for 20 min. The reaction was stopped by adding 5 mL of 0.4 M trichloroacetic acid, and filtered. Five mL of 0.4 M Na_2CO_3 and 1.0 mL of diluted Folin reagent were added to 1.0 mL of the filtrate and reacted for 30 min at 30°C. The protease activity was then measured as the absorbance at 660 nm by a UV-spectrophotometer, and the units were expressed as the liberation of 1 mM of tyrosine per 1.0 mL of the supernatant.

Amino type nitrogen The *meju* powder (5 g) was mixed with 25 mL of distilled water and shaken for 1 hr; the pH was then adjusted to 8.4 with 0.1 N NaOH. Twenty mL of neutral formalin (pH 8.3) was added to the mixture as above, and the pH 8.4 was again adjusted to 8.4 with 0.1 N NaOH. The final titrated volume was used to calculate the amino type nitrogen content; distilled water was used as the blank test (21).

Statistical analysis All statistical analysis was performed with SAS (Statistical Analysis System) (22). Mean and standard deviations were calculated, and Duncan's multiple range tests were applied. Evaluations were performed in triplicate. A probability level of less than 0.05 ($p<0.05$) was taken to indicate statistical significance.

Results and Discussion

Moisture content Changes in the moisture content of the *meju* during fermentation are shown in Table 2. Moisture contents in the brick-shaped and grain-shaped *meju* gradually decreased from 50.47 to 54.89% in the initial stage to 12.91 to 16.25% after a 12-day of fermentation period. The moisture content of the *meju* had decreased considerably by day 4 of fermentation. The moisture contents of the brick-shaped *meju* were slightly higher than those measured for the grain-shaped *meju,* primarily because the brick-shaped *meju* were larger in size and more solid. In support of this notion, Yoo and Kim (23) have reported that the moisture content inside traditional *meju* is higher (38.13±14.80%) than that on the outside (22.83±9.65%). Similarly, Choi *et al.* (24), in their recent report, noted that the moisture content of *meju* varies widely (7.2±0.1 to 28.8±0.2%) and that the differences are due to the temperature during aging, as well as the shapes and thickness of the *meju*, which reflect the region of origin. The moisture content of our *meju* was relatively low compared to those cited, most likely because our *meju* were prepared with a limit of 3 cm of thickness.

pH and titratable acidity Changes in pH of the *meju* prepared with different shapes and various strains of microorganisms are shown in Table 3. The pH for the brick-shaped and grain-shaped *meju* decreased slightly from 6.78 to 6.86 during the initial stage to 6.48 to 6.66 on day 12 of fermentation. It is of interest that the pH was lowest (4.88 to 5.07) on day 2 of fermentation regardless of treatments, and thereafter increased. We speculate that hydrolysis and the release of ammonia-type nitrous compound from the soybean proteins were produced by enzymatic action of microorganisms, causing the medium to become increasingly acidic (8, 25). Our results differed from those resulted by Kwon *et al.* (26) who indicated that the pH of a barley grain *meju* during fermentation was 5.6 to 5.2. Our results, however, were similar to the results of Chung *et al.* (27), who reported that the pH of commercial *meju* and *sigumjang*, a traditional Korean fermented food made with barley bran flour, collected from various regions in Korea were 6.0±0.5. Also, the results of Yoo and Kim (23), who reported that the pH of 123 kinds of traditional *meju* collected from various regions in Korea measured 7.0±0.8 on the inside and 6.9±0.5 on the outside, were similar to ours.

Table 4 shows the changes in titratable acidity (in mL of NaOH) in the different *meju* types that were prepared with various microorganism strains during fermentation at

Table 1. Raw materials used (%, w/w) for the preparation of *meju*

Shape	Groups[1)]	Raw materials				
		Non-glutinous rice	Soybean	*A. sojae koji*	*A. oryzae koji*	*B. subtilis*
Brick	A.o	40	60	0	0.5	0
	A.s	40	60	0.5	0	0
	A.o+B.s	40	60	0	0.5	0.5
	A.s+B.s	40	60	0.5	0	0.5
Grain	A.o	40	60	0	0.5	0
	A.s	40	60	0.5	0	0
	A.o+B.s	40	60	0	0.5	0.5
	A.s+B.s	40	60	0.5	0	0.5

[1)]A.o, *A. oryzae*; A.s, *A. sojae*; A.o+B.s, *A. oryzae* + *B. subtilis*; A.s+B.s, *A. sojae* + *B. subtilis*.

Table 2. Changes in moisture contents of *meju* during fermentation (unit: %, wet basis)

Shape	Strains used[1)]	Fermentation time (day)				
		0	2	4	8	12
Brick	A.o	54.89$^{aA2)}$	53.11^{aA}	20.26^{bA}	20.06^{bB}	15.03^{cB}
	A.s	54.49^{aA}	52.97^{aA}	19.77^{bA}	18.58^{bC}	13.39^{cC}
	A.o+B.s	54.45^{aA}	52.33^{aAB}	21.25^{bA}	21.59^{bcA}	16.25^{cA}
	A.s+B.s	54.12^{aA}	52.79^{abA}	20.10^{bA}	20.46^{bB}	15.83^{cA}
Grain	A.o	53.58^{aA}	47.77^{bC}	19.72^{cB}	17.68^{cdC}	13.41^{dB}
	A.s	50.47^{aC}	50.69^{aB}	23.35^{bA}	19.75^{cB}	13.64^{dAB}
	A.o+B.s	51.68^{aB}	50.71^{aA}	20.24^{bB}	20.49^{bA}	14.02^{cA}
	A.s+B.s	53.87^{aA}	47.55^{bD}	18.53^{cdB}	19.98^{cB}	12.91^{dC}

[1)]A.o, *A. oryzae*; A.s, *A. sojae*; A.o+B.s , *A. oryzae* + *B. subtilis*; A.s+B.s , *A. sojae* + *B. subtilis*.
[2)]Means with the same lowercase letters and capital letters within rows and columns, respectively, were not significantly different at $p<0.05$.

Table 4. Changes in titratable acidity of *meju* during fermentation (unit: mL, wet basis)

Shape	Strains used[1)]	Fermentation time (day)				
		0	2	4	8	12
Brick	A.o	0.13$^{eA2)}$	1.10^{aAB}	0.50^{dB}	0.70^{cAB}	0.80^{bC}
	A.s	0.13^{bA}	1.05^{aB}	0.50^{aB}	0.80^{aA}	0.87^{aB}
	A.o+B.s	0.10^{bA}	1.15^{abA}	0.67^{abA}	0.67^{abC}	1.10^{aA}
	A.s+B.s	0.10^{eA}	1.16^{aA}	0.33^{dC}	0.70^{cAB}	0.93^{bB}
Grain	A.o	0.10^{eA}	1.20^{aA}	0.50^{dA}	0.73^{cA}	1.00^{bA}
	A.s	0.10^{eA}	1.20^{aA}	0.40^{dB}	0.60^{cB}	0.80^{bC}
	A.o+B.s	0.10^{eA}	1.20^{aA}	0.50^{dA}	0.67^{cAB}	0.90^{bB}
	A.s+B.s	0.10^{eA}	1.17^{aAB}	0.40^{dB}	0.60^{cB}	0.90^{bB}

[1)]A.o, *A. oryzae*; A.s, *A. sojae*; A.o+B.s , *A. oryzae* + *B. subtilis*; A.s+B.s , *A. sojae* + *B. subtilis*.
[2)]Means with the same lowercase letters and capital letters within rows and columns, respectively, were not significantly different at $p<0.05$.

28°C. The initial titratable acidity of the brick-shaped and the grain-shaped *meju* was 0.10 to 0.13 mL, which increased to 0.80 to 1.10 mL by day 12 of fermentation. The titratable acidity volume on day 2 of fermentation, however, was higher for all treatments (1.05 to 1.20) than on all other days of fermentation. These results were consistent with the pH changes discussed earlier. The final titratable acidities ranged from 0.80 to 1.10 mL, and this was not influenced by the shape of the *meju* or the microbial strains used during fermentation. Our titratable acidity volume results were lower than those of Yoo and Kim (23), who reported that the titratable acidities for 123 traditional *meju* collected from various region in Korea were 1.54±0.69 mL in the interior and 1.98±0.63 mL for the outside. These differences are likely caused by variations in the fermentation; the traditional *meju* studied by Yoo and Kim (23) were fermented for 3 months, in contrast to our *meju* that were fermented for only 12 days.

Protease activity The acidic and neutral protease activities in *meju* of different shape, and prepared with various microflora strains (after 12 days of fermentation), are shown in Fig. 3 and 4. Acidic protease activities (Fig. 3) of the brick-shaped *meju* ranged from 2.36±0.24 to 2.56±0.24 unit/mL. On the other hand, with the exception of the A.o+B.s treatment (2.14±0.08 unit/mL), the grain-shaped *meju* ranged from 0.44±0.04 to 0.76±0.21 unit/mL, much lower than those of any brick-shaped *meju*.

Neutral protease activities (Fig. 4) of the brick-shaped *meju* ranged from 1.19±0.12 to 1.25±0.28 unit/mL, and were similar for all treatments. Neutral protease activity of the A.s grain-shaped *meju*, however, was the lowest (0.07±0.01 unit/mL), while the A.s+B.s grain-shaped *meju* was the highest (1.93±0.11 unit/mL) among the treatments.

Pack and Oh (17) have reported that, during a fermentation period of 60 days, the acidic protease activity of traditional *kochujang meju* was the highest when the pH level was the lowest during fermentation, whereas the neutral protease activity was the highest when the pH was neutral and was even higher than the acidic protease activity. However, our results indicate that the acidic protease activities of brick-shaped *meju* were approximately twice that of neutral protease by day 12 of fermentation when the pH ranged from 6.55 to 6.57; no consistent tendencies were observed for the grain-shaped *meju*.

Table 3. Changes in pH of *meju* during fermentation

Shape	Strains used[1)]	Fermentation time (day)				
		0	2	4	8	12
Brick	A.o	6.86$^{aA2)}$	4.99^{dA}	6.11^{cD}	6.53^{cB}	6.55^{cC}
	A.s	6.81^{aA}	4.90^{dB}	6.39^{cA}	6.60^{bA}	6.56^{bAB}
	A.o+B.s	6.78^{aA}	4.89^{eB}	6.22^{dC}	6.35^{cD}	6.55^{bBC}
	A.s+B.s	6.74^{aA}	4.89^{eB}	6.32^{dB}	6.44^{cC}	6.57^{bA}
Grain	A.o	6.79^{aA}	4.93^{dC}	6.46^{cD}	6.57^{bD}	6.48^{cC}
	A.s	6.83^{aA}	5.07^{dA}	6.64^{cB}	6.69^{bA}	6.66^{bcA}
	A.o+B.s	6.81^{aA}	4.99^{dB}	6.56^{cC}	6.58^{bC}	6.60^{bB}
	A.s+B.s	6.78^{aA}	4.88^{dD}	6.71^{cA}	6.66^{bB}	6.63^{bB}

[1)]A.o, *A. oryzae*; A.s, *A. sojae*; A.o+B.s , *A. oryzae* + *B. subtilis*; A.s+B.s , *A. sojae* + *B. subtilis*.
[2)]Means with the same lowercase letters and capital letters within rows and columns, respectively, were not significantly different at $p<0.05$.

Amylase activity The α-amylase and β-amylase activities in the *meju* of different shape prepared with various strains of microflora, after 12 days of fermentation, are shown in Fig. 5 and 6. α-Amylase activities (Fig. 5) in the A.s+B.s treatment of brick-shaped and grain-shaped *meju* were the highest at 11±0.6 and 9±0.7 unit/mL, respectively, while the A.s treatment of both shapes were the lowest at 2±0.7 and 2±0.8 unit/mL, respectively.

β-Amylase activities (Fig. 6) ranged from 1.53±0.01 to 1.56±0.02 unit/mL and were similar in all treatments. Lee *et al.* (28) reported that, among the different starch sources, the α-amylase activity of glutinous rice *koji* peaked (38 unit/g) at 96 hr of fermentation and then decreased.

Amino-type nitrogen contents Amino-type nitrogen

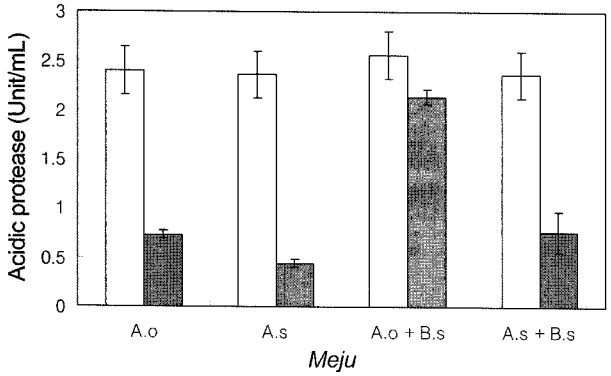

Fig. 3. Acidic protease activities in 8 varieties of *meju* prepared with various microorganisms. A.o, *A. oryzae;* A.s, *A. sojae;* A.o+B.s, *A. oryzae+B. subtilis;* A.s+B.s, *A. sojae+B. subtilis.* □, Brick-shaped *meju;* ■, Grain-shaped *meju.*

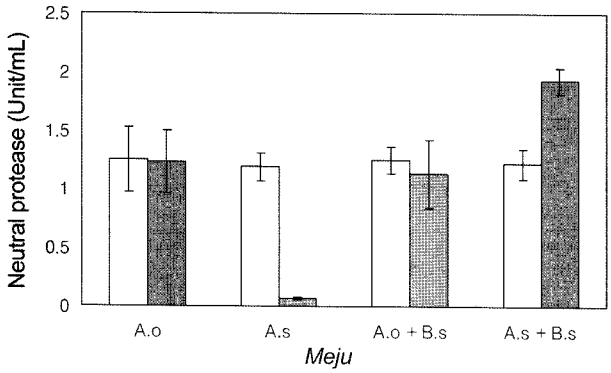

Fig. 4. Neutral protease activities in 8 varieties of *meju* prepared with various microorganisms. A.o, *A. oryzae;* A.s, *A. sojae;* A.o+B.s, *A. oryzae+B. subtilis;* A.s+B.s, *A. sojae+B. subtilis.* □, Brick-shaped *meju;* ■, Grain-shaped *meju.*

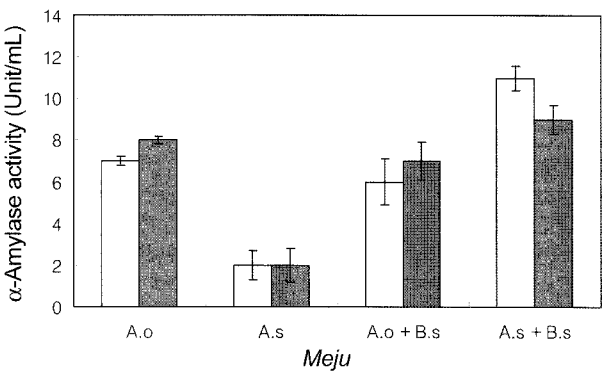

Fig. 5. α-Amylase activities in 8 varieties of *meju* prepared with various microorganisms. A.o, *A. oryzae;* A.s, *A. sojae;* A.o + B.s, *A. oryzae+B. subtilis;* A.s+B.s, *A. sojae+B. subtilis.* □, Brick-shaped *meju;* ■, Grain-shaped *meju.*

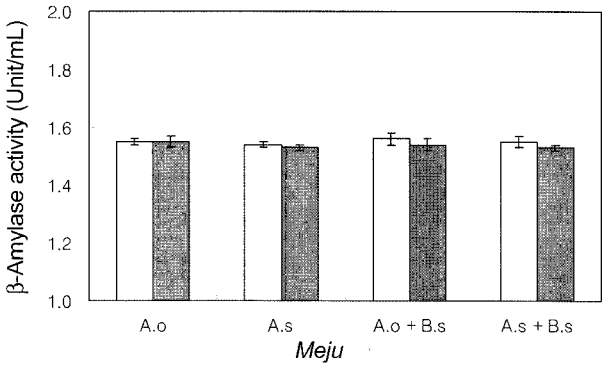

Fig. 6. β-Amylase activities in 8 varieties of *meju* prepared with various microorganisms. A.o, *A. oryzae;* A.s, *A. sojae;* A.o +B.s, *A. oryzae+B. subtilis;* A.s+B.s, *A. sojae+B. subtilis.* □, Brick-shaped *meju;* ■, Grain-shaped *meju.*

content represents the degree of soy protein hydrolysis by microorganisms and also reflects the free amino acid content that affects the taste of soy products. After 12 days of fermentation, the amino-type nitrogen contents in *meju* of different shapes prepared with various strains of microflora are shown in Fig. 7. Of the brick-shaped *meju*, the amino-type nitrogen content of the A.o+B.s brick-shaped *meju* was the highest at 0.39±0.03%, while the A.s+B.s brick-shaped *meju* was the lowest at 0.17±0.01%. Amino-type nitrogen content of the brick-shaped *meju* was higher than that of the grain-shaped *meju* for all treatments, except for the A.s+B.s treatment. Our results indicate that an amino-type nitrogen content of *meju* prepared with *A. oryzae*, which showed strong protease activity in the brick-shaped *meju*, was higher than that of *A. sojae*.

Lee and Chung (29) reported that the amino-type nitrogen content of a *doenjang* prepared with *Bacillus natto* was much higher than that prepared with *A. oryzae*. However, Seo *et al.* (30) reported that the amino-type nitrogen content of a *doenjang* prepared with *A. oryzae meju* was the highest during aging, followed by conventional *meju* prepared with *B. natto* or *B. subtilis*. We presume that the difference in these results may be due to the physiological characteristics and the enzyme activities of the various

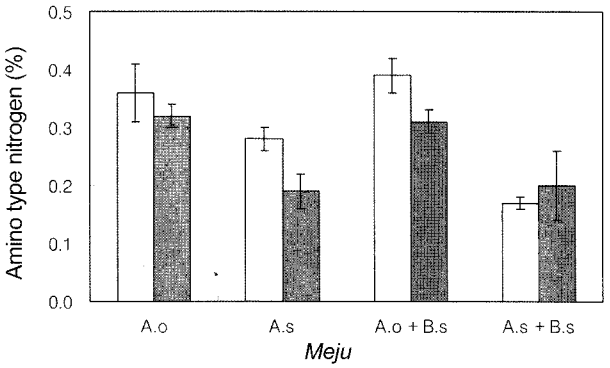

Fig. 7. Amino-type nitrogen in 8 varieties of *meju* prepared with various microorganisms. A.o, *A. oryzae;* A.s, *A. sojae;* A.o +B.s, *A. oryzae+B. subtilis;* A.s+B.s, *A. sojae+B. subtilis.* □, Brick-shaped *meju;* ■, Grain-shaped *meju.*

microorganisms.

Acidic protease activity, β-amylase activity, and amino-type nitrogen content were the highest in the brick-shaped *meju* prepared with *A. oryzae* and *B. subtilis*. In addition, the acidic protease activity and amino-type nitrogen content in the brick-shaped *meju* were generally higher

than those of the grain-shape *meju*. We conclude that the brick-shaped *meju* prepared with *A. oryzae* and *B. subtilis* could be used in the preparation of traditional *kochujang* to yield a product with improved quality.

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