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Meju Fermentation for a Raw Material of Korean Traditional Soy Products

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ABSTRACT: ***Meju*** **is a raw material used to make Korean soy sauce (*Kanjang*) and soybean paste (*Doenjang*), both of them rich in plant protein. The twenty-nine fungal and a bacterial species were identified from twenty-three traditionally homemade *meju* cakes. Out of them, only a few species were found to be involved in the actual fermentation process of *meju*; The other species were contaminants during the improper subprocesses of *meju* fermentation. The fungal floral successions were observed to be related to two physical and biochemical changes of *meju* cakes during *meju* fermentation: drying and heat releasing processes. The zygomycetous fungi were first observed to exist mainly during the first stage. *Scopulariopsis brevicaulis* was observed to grow on the surface of *meju* cakes and then to coexist with *Bacillus megatrium* in the inner part of *meju* cakes during the second stage. Based on the biochemical tests, the proteases secreted by the different microorganisms were involved in the degradation of soybean proteins with a mutual relationship. Also, zygomycetous fungi were speculated to be important microorganisms for inducing the second stage in the traditional Korean homemade *meju*.**

KEYWORDS: Korean homemade *meju*, zygomycetous fungi, successions, mutual relationship

Soy sauce and soybean paste are important foods, made from soybeans through the process of traditional fungal fermentation in Eastern Asia. Two kinds of processes for making maeju exist in Korea; One is the Korean home-made way, known as a traditional way, and the other is the Japanese refined way, known as a modern and refined way; both lead to the production of soy sauces, though different in brand names and specific in tastes. During the last three decades the process of making Japanese refined *meju* has been upgraded to a method in which inocula of a single species of *Aspergillus* lead to the commercial productions (Hahn & Park, 1957; 1958). The process of making Korean homemade *meju* has been traditionally passed down from the older to the younger. Korean soy sauce and soybean paste recently aroused public interest, because of the general preference for their excellent taste and nutritional value over Korean. However, little has been studied about the fungal flora and their ecological roles of them in the traditional Korean *meju* cakes.

Traditional Korean homemade *meju* (*meju*) cake is a rectangular cake of boiled and mashed soybeans pressurized by bare hands. As a raw material for both soy products, *meju* cakes are stored at room temperature for about two or three months during which the process of natural fungal fermentation takes place. The fermentation proce-

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sses of *meju* cake are similar to those of cheese in Western cultures; the fermentation process of *meju* is known as "koji" in Japanese throughout the world (Kim *et al.*, 1961). Thus, *meju* or *meju* cake is considered to be very important in the industries of soy-foodstuffs The indigenous taste of Korean soy products. are considered to be very important especially for making *meju* cakes in Korea.

Zygomycetous fungi (*Mucor* or its related species, Hahn & Kim, 1962; Hahn *et al.*, 1962) and hyphomycetous fungi (*Aspergillus, Penicillium*, and their related species, Kim & Kim, 1986; Park *et al.*, 1977; Hahn & Park, 1957; 1958) were reported to be the fungal species involved in fermentation of *meju.* Some species of *Aspergillus*, an important fungus in making of *meju*, were reported to inhabit *meju*, and known to be the organisms that causes fermentation in Japanese *mejus* (Hahn & Park, 1957; 1958). Some species of *Penicillium* were also reported to inhabit in the surfaces of *meju* cakes but unlike *Aspergillus*, they are known to be contaminants (Koh *et al.*, 1974; Lee, 1976). Several species of *Mucor* and *Rhizopus* were reported to inhabit *meju* cakes, but these were not considered to be important to the fermentation of *meju* cakes (Yihn & Lee, 1968; Cho & Lee, 1970; Park *et al.*, 1977; Chung, 1977). *Bacillus subtilis* and its related bacteria were reported to be involved in the inner parts of *meju* cake, and to produce both amylase and protease which work toward the fermentation of *meju* cake (Kim & Kim, 1989; Kwon *et al.*, 1986; Cho & Lee, 1970; Lee & Koh, 1976). The results of the works cited above have led to the development of a soy sauce with a taste similar to that of Japanese soy sauce. Ironically, Korean food industries had geared the studies toward the making of a soy sauce with the "Chosun" or Korean taste in reality, at this moment.

Mycotoxins were reported to be identified in a culture of *A. flavus*, a fungus in the Oriental *meju* (Arnold & Peterson, 1974). The 150 isolates of *A. flavus* or its related species were reported to produce aflatoxin or its related compounds in fermented foods; aflatoxin was first isolated from peanuts comtaminated with *A. flavus* in Africa (Hesseltine *et al.*, 1976; Pederson, 1980; Arnold & Peterson, 1974). The mycotoxins have been studied for last three decades in Korea (Joo & Woo, 1980; Koh *et al.*, 1974). Ochrotoxin A was also reported to be produced by several species of *Penicillium*, and was also reported to be found in traditional Korean *meju* cakes (Kang *et al.*, 1991). The two mycotoxins mentioned above, were produced during the fermentation of *meju.* Thus, the fungi involved in the fermentation of *meju* were considered to be very important for human health for toxin productions, and also for food industries in fermentation.

Taking these givens, the purposes of the following experiments are subjected to i) isolate and identify the fungi involved in the fermentation of *meju* and ii) understand the role (s) of fungus in the making of soy products with a single isolate. Studies toward these goals have been taken in this regard for five years with many observations of making *meju* cakes in Korean rural homes. Different kinds of *meju* cakes fermented by various combination of fungal isolates were prepared, and soy products were made from those kinds of *meju* under natural conditions.

Materials and Methods

Meju cakes

The twenty three Korean homemade *meju* cakes were collected nationwide from March, 1990 to March, 1993, during which the collected samples were stored at 0℃. Each state of *meju* cakes collected were observed with naked eyes as well as under light microscope. The fungi and bacteria inhabiting the inner and outer parts of *meju* cakes were isolated from a few grams of *meju* cake for the plating on Potato Dextrose agar (PDA) or Complete agar (CM) (Raper & Raper, 1972). A colony of bacterium appeared early within the 24 hours of incubation; on the other hand, the colonies of the fungi and yeasts appeared slowly after 3 to 5 days of incubation at 28℃. The resulting isolates were then reselected for further experiments, based on their shapes, colors, and growth rates of the colonies on PDA. These isolates were obser-

ved under light microscopes, transferred to PDA, and then stored at 0℃ for later upcoming experiments.

Identification

The slide culture technique was employed for observing ontogeny of conidium of hyphomycetous fungi (Barron, 1977; Raper & Fennell, 1973; Pitt, 1979), and also for observing the shape of vesicle or sporanganium of zygomycetous fungus (Gilman, 1968). The fungal cultures on CMA, PDA, Czapek' s agar, Malt extract agar, and Czapek yeast-extract agar were used for their physiological characteristics (Ramirez & Martinez, 1982; Domsch *et al.*, 1980; Barron, 1977). The species of zygomycetous fungi were identified as the morphologies of sporangium and sporangiophores with the use of the detailed descriptions made by Zycha & Siepmann (1969). Gram staining and following observations under light microscope were also made using the Luria Bertari (LB, Atlas & Parks, 1993), placed on the broth for the identification of a heat resistent bacterium, *Bacillus*. This bacterial isolate (W-1) was sent to Analytical Services in the U.S. (P.O. Box 626, Essex Junction, VT 05453) and identified through the 95 biochemical tests (Biolog).

Making *Meju* and Soy sauce

Soybeans, ***Glycine max* (L.) MERR commercially available in Korea were boiled and fermented into *mejus***, and then made into soy sauce with saline solution. First, 200 grams of soybean cereals was poured into a glass bottle (9×9×17 cm, MAXIM coffee bottle, which is also commercially available, with 200 m*l* of tap-water, autoclaved at 126℃ for 1 h. A single isolate of the fungus or bacterium selected was then inoculated to the bottle and then incubated at 28℃ for two weeks as the artificial "*meju* fermentation setting" (Kim & Kim, 1963; Hahn & Kim, 1962). This artificial *meju* fermentation is commonly employed in the food industries, known as the Japanese method (Kim & Kim, 1963; Lee & Koh, 1976) in Korea. The resulting *meju* was evaluated by their smell and colour by three selected experts (see the below).

Since *mejus* can not be directly tasted or evaluated, the *meju* was made into soy sauce and then tasted by the experts. *Mejus* were then dried under natural sunlight for two weeks; afterwards the saline solution was added to adjust the salt level of *meju* cakes to 25% (w/v). The formation of soy sauce was completed for eight weeks later. The dark colored liquid part of *meju* fermented in the saline solutions, so-called "soy sauce", was collected for the panel tests. The panel tests, consisted of drinking, smelling and observing the color of soy sauce samples, was carried out by the special experts having more than 20 years of experiences in Korea traditional homemade *meju* and working as cooks at the Korea National University of Education (Cheong Won Kun, Chung-Puk 363-791, Republic of Korea).

Activities of Protease and Amylase

A gram of *meju* cake was first mixed with 30 m*l* of tris [hydroxymethyl] aminomethane buffer (pH 8.1), was liquified in blender for two min, and then centrifuged for 10 min at 12,500×g. The supernatant was collected for measuring protease and amylase activities. The activity of protease was determined by the various substrates (Bergmeyer, 1974): 10 mM of n-ρ-tosyl-L-arginine methyl ester (TAME, Sigma), n-benzoyl- L-arginine ethyl ester (BAEE, Sigma), L-tyrosine ethyl ester (TEE, Sigma), and n-acetyl L-tyrosine ethyl ester (ATEE, Sigma). The activity of amylase in 0.1 M acetate buffer (pH 5.5) was also measured by using soluble starch (Lee & Koh, 1976; Kim & Kim, 1989).

Fungal and bacterial growth

The fungal and bacterial growth were hourly checked on basis of evolution of carbon dioxide (CO_2), measured Gas Chromatography (HEWLETT PACKARD 5800, Eom *et al.*, 1994). Fifty grams of soybean cereals was poured into a 250 m*l* Erlenmeyer flask, mixed with various amounts of tap water, and then autoclaved at 125℃ for 15 min. The different amounts of 20, 30, 40, and 50 m*l* of tap-water for the 50 g of soybean cereals in the 250 m*l* flasks were represented to the different water activities of a_w=0.95, 0.96, 0.97, and

0.98, respectively. However, the lowest water activity level (a_w=0.95) was adjusted with the addition of a 5% (w/v) saline solution for further experiments. The water activity was measured by NOVASINA-Electronic Hygrometer (EEJA-3/Zurich). The microorganism selected was inoculated and incubated at 28℃ for two weeks.

Results

Traditional Korean homemade *meju* cakes

Soybeans (*Glycine max* (L.) Merr.) commercially available were first cleaned, moistured for half a day, and boiled with water in a big iron container for half a day. After then the soybean cereals were cooled down to approximately 35 to 40℃, mashed, and shaped into round (10 to 12 cm diam) or rectangular cakes (20×20×7 to 9 cm). The size and shape of *meju* cakes were as various as those made by local people or family customs. *Meju* cakes were dried in special room at 25 to 30℃ for awhile (usually a week) until it held its shape firmly, and after that, it was hung up with strings made of rice straw. They were stored for two or three months during Winter, usually November to February. The fermented *meju* cakes showed some colour, indicating the spores of hyphomycetes, during the storage. In the early Spring, the *meju* cakes sold were rewashed, and stored in warm rooms for three to four days for a week; afterwards, saline solution was added to upgrade the salt level of *meju* cakes to 25% (w/v) in big clay containers. *Meju* cakes were stored in the containers for two months or longer, and some additives were also added to the containers, according to the familys' customs. With different concentrations of salt, different variables affected the smell, colour, and taste of the soy products later made in these different regions from *maejus*. After this procdure, the liquid part of the resulting substances was the soy sauce (called 'kanjang') while the solid part became the soy paste (called 'Doenjang', whic is simiar to "Miso" in Japan).

Observations of *Meju* Cake

Hyphae and conidia were first found on the surfaces of *meju* cakes and the colour of fungal spores mostly became those of *meju* cakes, finally to become the dustlike on the surfaces of *meju* cake. Most fungal growths occurred on the surfaces, not in the inner parts of *meju* cakes. The fungal growth on *meju* cakes was developed into mats of several distinct colours. White hyphae, white hyphae & blue spores, blue spores, yellow spores, yellowish green spores, grey spores, brown spores, or white spores were observed on the surfaces of *meju* cakes collected from various areas in Korea. *Meju* cakes were, sometimes, covered with the colours of spores and the fungal spores partially or totally changed into a powdery substance. The spores, representing the colour of *meju* cakes, were directly isolated, and identifiied with various agars: The white hyphae on the surfaces of *meju* cakes were mostly recognized as *Penicillium miczynski* or *P. citrinum* or *P. gorlenkoanum* or *P. godlewskii* or *P. funiculosum*; The white hyphae & blue spores as *P. griseo-purpureum* or *P. graditanum* or *P. gorlenkoanum* or *P. godlewskii* or *P. funiculosum*; the blue spores as *P. botryosum* or *P. turolense* or *P. rubicundum* or *P. jensenii* or *P. volgaens* or *P. verrucosum*; the yellow spores as *P. roqueforti* or *A. oryzae* or *A. flavus* var. *columnaris*; yellowish green spores as *A. oryzae* or *A. flavus* var. *columnaris*, the grey or brown spores as *A. terreus*, white and grey spores as *Scopulariopsis brevicaulis* (Table 1).

The white fungal hyphae first grew on the surfaces, then reached into the inner part of *meju* cakes in a depth of about 1 to 1.5 cm, but did not grow in the inner parts of *meju* cakes further. It was found under dissection microscope that most fungi found in *meju* cakes belonged to Hyphomyctes, but the variety of the fungi were not identified as a species of zygomycetous fungi after storage. The green spots were contamination caused by the species of *Penicillum*. The white areas mostly indicated the hyphal growths of hyphomycetous fungi and later changed to colour of blue, in most cases. These blue or green colours on the surfaces were not observed in the well-done *meju* cakes, recognized by the experts. Also, well-done *meju* cakes were observed to have dark

Table 1. The fungi and the bacterium identified from the traditional Korean homemade *Meju* cakes.

Marks of isolates	Areas collected[a]	Fungi and Bacteria isolated		
		Zygomycetes[b]	Hyphomycetes[c]	Bacterium[d]
A	Seoul (rm)	ND[e]	ND	*Bacillus* sp.
B	Woltan, Chungpuk	*Mr. isabellina*	*P. botryosum*	ND
C	Angayng, Kyunggi	ND	*P. gorrlenkoanum* *P. griseo-purpreum* *P. citrinum* *P. miczynskii*	ND
D	Seosan, Chungnam	*M. circinelloides* f. *griseo-cyanus*	*S. brevicaulis*	*Bacillus* sp.
E	Dalseoku, Daegu	*M. circinelloides* f. *griseo-cyanus* *M. hiemalis* f. *hiemalis*	*P. gaditanum* *S. brevicaulis*	*Bacillus* sp.
F	Zungpyong, Chungbuk	ND	ND	*Bacillus* sp.
G	Daeshindong, Pusan	*Mr. isabellina*	*P. turolense*	*Bacillus* sp.
H	Pyeuyeo, Chungnam	*M. circinelloides* f. *griseo-cyanus*	ND	*Bacillus* sp.
I	Namwon, Chunbuk	ND	*P. funiculosum* *P. rubicundum*	*Bacillus* sp.
J	Yeongdoku, Pusan	ND	*A. oryzae* *S. brevicaulis*	*Bacillus* sp.
K	Moonkeong, Kyoungbuk	*M. jansseni*	*P. godlewskii* *P. jensenii*	*Bacillus* sp.
L	Ockcheon, Chungbuk	*M. circinelloides* f. *griseo-cyanus*	ND	*Bacillus* sp.
M	Namwon, Chunbuk	*M. circinelloides* f. *griseo-cyanus*	ND	*Bacillus* sp.
N	Seosan, Chungnam	*M. hiemalis* f. *hiemalis*	ND	*Bacillus* sp.
O	Ichun, Kyunggi	*M. circinelloides* f. *circinelloides*	*A. flavus*	*Bacillus* sp.
P	Pyeuyeo, Chungnam	ND	*A. flavus* *S. brevicaulis*	*Bacillus* sp.
Q	Andong, Kyoyngbuk	ND	*A. flavus*	*Bacillus* sp.
R	Andong, Kyoyngbuk	*M. racemosus* f. *racemosus*	*P. rogyenforti* *P. verrucosum* v. *corymbiferum*	*Bacillus* sp.
S	Daejeon	*Ab. corymbifera* *M. hiemalis* f. *hiemalis*	*P. volgaense* *A. oryzae*	*Bacillus* sp.
T	Sungnam, Kyunggi	*Ab. corymbifera* *R. stolonifer* *R. oryzae*	*A. oryzae* v. *effusus*	*Bacillus* sp.
U	Kochang, Kyounnam	*R. stolonifer* *Ab. corymbifera*	*P. verrucosum* v. *corymbiferum*	*Bacillus* sp.
V	Bucheon, Kyunggi	ND	*A. terrus* *A. flavus* v. *column*	*Bacillus* sp.
W	Chungju, Chungbuk		*S. brevicaulis*	*B. megaterium*

[a] The home made *meju* cakes were collected nation wide from individual houses at different areas in Korea. The rm indicated the *meju* made by the modified methods, otherwise all other *meju* cakes made by Korean traditional methods.

[b] Microscopic observation for identifications by Zycha and Siepmann's descriptions.

[c] Microscopic observationfor identifications by Raper and Fennell's and Ramirez and Martinez's descriptions.

[d] The bacterium isolated from each maeju were determined by Biolog test (see Materials and Method).

[e] Any microorganisms were not found at the intact *meju* as based on our methods.

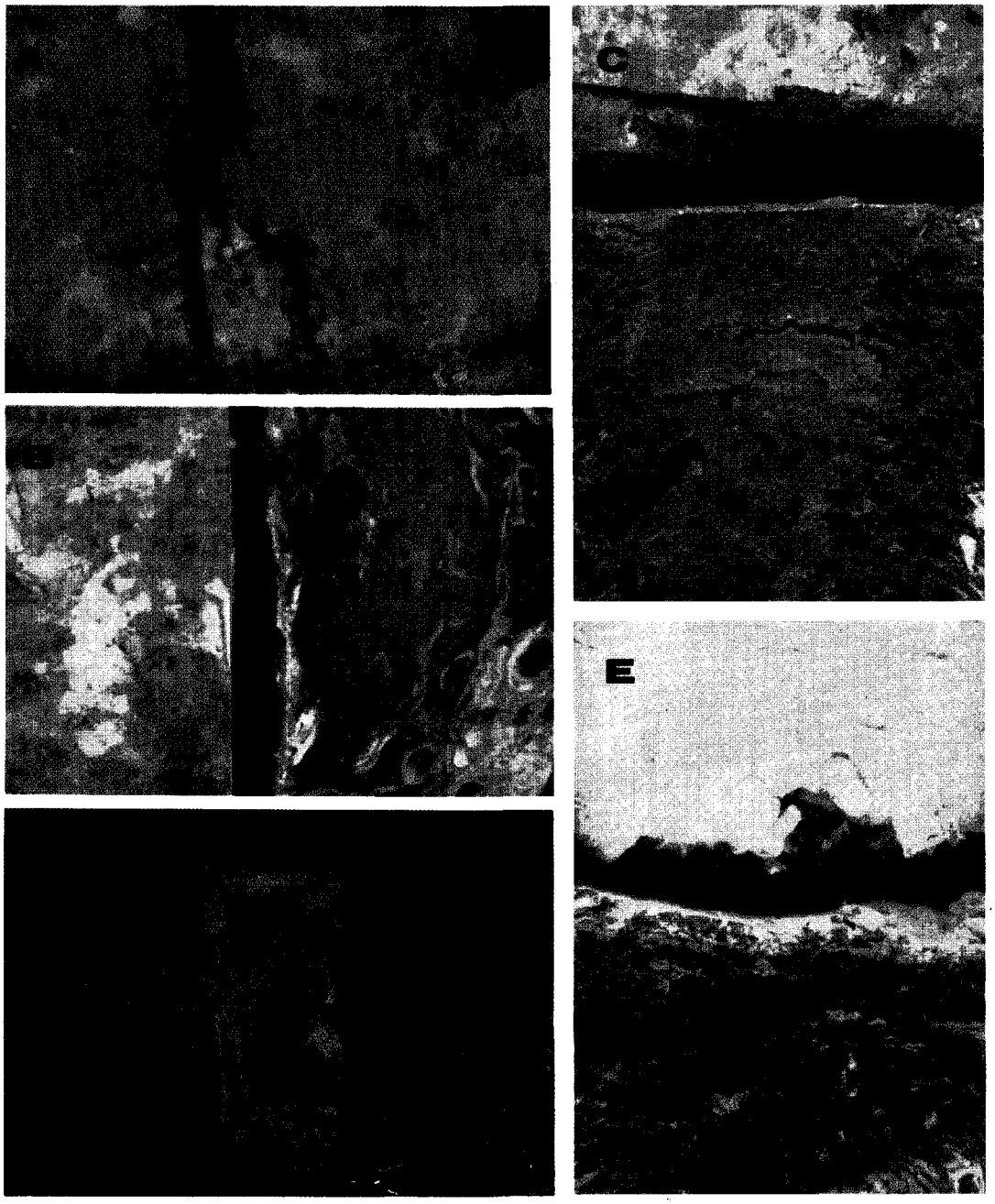

Fig. 1. Photographs of *meju* cakes inoculated with the five species; Green spores (dusts) and white mycelia covered on the surfaces (A), but only a little bacteria growth found in the inner partsf maeju cakes (B) for a month's fermentation or for two months' fermentation (C). The other photographs of *Meju* cakes inoculated with the four species except for *A. oryzae* (D and E) The white mycelia and dusts originated from the growths of *Scopulariopsis brevicaulis* and the pale white mycelia from zygomycetous fungi (mostly considered to be *Mucor circinelloides* f. *griseo-cyanus*) on the surfaces of *meju.* The dark brwon spots indicated the groths of *Bacillus megaterium* in the inner parts of *meju* cakes for both a (D) and two (E) months' fermentation.

brown rings on the sufrace or in the inner parts of *meju* cakes when cut (Fig. 1-DE). The dark brown spots or areas were quite often observed from most *meju* cakes collected. A bacterium were isolated from the dark brown spots of *meju* cake and tested for a heat resistance at 90℃ for 10 mins. All isolates of bacterium were found to be heat resistant up to the temperature of 90℃ for 10 mins and were in bacterial genus of *Bacillus*, all throughout the different isolates (Table 1).

Microflora in maeju cake

The isolates of the fungi or bacteria were collected from *meju* cakes purchased from various local areas during three years. The twenty nine fungal species were finally identified from various *meju* cakes, based on the morphological features. The morphological or taxomonical features of the fungi isolated were detailed in other publications (Lee *et al.*, 1994a; 1994b). Different species of zygomycetous fungi were found in the different *meju* cakes, that were, *M. circinelloides* f. *griseo-cyanus* (*M. circinelloides*, found with 5 incidences for the local *meju* cakes), *M. hiemalis* f. *hiemalis* (*M. hiemalis*, 3), and *Absidia corymbifera* (3). Under microscope, the species of zygomycetous fungi were not frequently found especially in the old *meju* cakes, but the incidences were all different at the early stage of *meju* fermentation. The fungi, *A. flavus* (2), *A. oryzae* (2), and *S. brevicaulis* (5), were found on the different *maeju* cakes with two to five incidences in the species of hyphomycetous fungi (Table 1). With many incidences among twenty nine fungi, *S. brevicaulis* were evenly distributed from the widely collected *meju* cakes. The bacterium with high incidences was isolated from each *meju* cake. It was found that most fungi found in one specific regional *meju* cakes was not found in other *meju* cakes, with the exception of a few fungi and a bacterium. The fungal species were just overlapped within *meju* cakes collected locally; *M. circinelloides* and *M. hiemalis* isolated from the areas of Chung Cheong Do and *S. brevicaulis* from the areas of Kyoung Sang Do. The common differences of diversity for the fungal species were not shown in individual *meju* cake; all fungal flora were different for the meju cakes collected from the different areas.

***Meju* and Kanjang**

The thirty species of microorganisms isolated were employed for *meju* fermentation with a single inoculumn. None of the *mejus* made from the single inoculumn were similar to a traditional homemade *maeju*, but several of *mejus* made of the single inoculumn were similar to the Japanese type *meju*. The mild smells were given off from the *mejus* made with an inoculumn of each zygomycetous fungi, but the bad smell were given off from the *mejus* made with an inoculumn of most species of *Penicillium* (Table 2). The isolates of *Rhizopus. oryzae, P. citrinum, P. miczynskii, P. funiculosum* and *B. megaterium* produced the protease at high levels of activity while the *meju* was fermenting. The flavors of the soy sauce made from *meju* containing a single inoculumn was not similar to those of home-made Korean soy sauces, but were similar to those of commercial soy sauces, especially, sweety soy sauces shown in Table 2, when good quality *meju* were used to make the soy sauce. The taste of soy sauce made from the *meju* containing a single inoculumn was not related to the activities of the protease produced by the isolates of the fungi. Thus, the flavor of soy sauce from the fermenting *meju* containing a single inoculumn was also not related to result in which the activities of protease measured were determined. The isolates also produced a sweet taste in the soy sauce made with the *meju* were correlated to the the protease productions of the isolates with the exception of *B. megaterium* (see Table 2).

Selection

The five species mentioned above were actually selected on basis of the different genera and the incidences showing the *meju* cakes collected from the different regions (Table 2). The species of *Penicillium* were not selected for this study because of the general dislike in Korean for the blue spores on *meju* cakes contaminated with the above fungus and the general allergic reaction to it.

Table 2. States of *Mejus* made with a single inoculumn of fungal or bacterial isolate and the taste of soy sauces made from the above *meju.*

Microorganisms isolated[a]	*Maeju* made with the single isolate[b]		Kangjang[c]	Mark of isolates
	Colors or Smells	Protease activity, (U)		
Zygomycetes				
Mr. isabellina	black-rotten	389	soysauce	B-1, G-1
M. hiemalis f. *hiemalis*	black-savory	6,780	sweety soysauce	E-1, S-1, N-1
M. circinelloides f. *griseo-cyanus*	black-savory	7,640	soysauce	D-1, D-2, E-2, M-1
M. circinelloides f. *circinelloides*	white-acidic	300	salty	H-2, N-1, L-1
M. jansseni	black-acidic	400	salty	K-4
M. racemosus f. *racemosus*	gray-brown	1,929	salty	R-1
R. stolonifer	dark brown-acidic	5,504	bitter	T-1, U-1
R. oryzae.	black-savory	14,313	salty	T-2
Ab. corymbifera.	black	1,223	soysauce	S-2, T-3, U-2
Hyphomycetes				
A. flavus	gray-rotten	722	soysauce	O-2, Q-3
A. oryzae	green-rotten	5,990	sweety soysauce	J-2, S-3
A. oryzae v *effus*	gray-green	7,830	soysauce	T-4
A. terreus	cinnamon	375	soysauce	V-1
A. flaus v. *colum.*	dark-green	111	soysauce	V-2
P. botryosoum	green-rotten	111	soysauce	B-2
P. gorlenkoanum	white-green	nd	rotten	C-1
P. griseo-purpureum	green-rotten	202	salty	C-2, C-6
P. citrinum	green	12,621	soysauce	C-4
P. miczynskii	green-rotten	11,175	salty	C-5, C-7
P. gaditanum	green-rotten	4,948	salty	E-3
P. turolense	green	8,340	sweety soysauce	G-2
P. funiculosum	green	10,452	sweety soysauce	I-1
P. rubicundum	green-rotten	2,780	salty	I-2
P. godlewskii	green-rotten	112	salty	K-2
P. jensenii	green-rotten	722	salty	K-3
P. roqueforti	green-rotten	8,173	salty	R-2
P. volgaense	green-rotten	4,670	salty	S-4
P. verrucosum v. *corymbiferum*	gray-rotten	3,447	salty	R-3, U-3
S. brevicaulis	cinnamon, savory	8,775	sweety soysauce	D-4, E-4, J-1, P-2, W-2
Bacterium				
B. megaterium	brown	12,754	tasteless	W-1

[a] Most microorganisms directly isolated from the traditional Korean homemade *meju* cakes.

[b] The meju made from the autoclaved soybeans with the inoculumn of a single isolate and culture at 28℃ for two weeks; The colour and smell of *meju* made by native eyes.
Relative protease activities of *meju* cakes (see Materials and Method in detail).

[c] Soy sauces made with the saline solution for two months and evaluated by the experts, having more than 20 years' experiences for Korean traditional homemade *maejus.*

Table 3. Activities of protease and amylase produced by the isolates selected, states of *meju* made with a single inoculamn of the isolate, and taste of soy sauces made from the *mejus*.

Microorganism[a]	*Meju* fermentation for eight days			Taste of Soy sauces
	Protease activity[b]	Amylase activity[c]	Smell	
M. circinelloides f. *griseo-cyanus* (D-1)	7,450	422	Savory	Soysauce
M. himalis f. *hiemalis* (E-1)	6,672	350	Savory, slightly acidic	Soysauce
S. brevicaulis (E-4)	8,451	515	Savory	Sweet soysauce
A. oryzae (S-3)	5,893	523	Odorless	Sweet soysauce
Bacillus megaterium (W-1)	12,287	400	Savory, slightly acidic	Tasteless

[a] The mark of the isolate used for this work.
[b, c] Relative protease activities (U) of each isolates fermented on the autoclaved soybeans for eight days, and see Materials and Method in detail.

These organisms, *M. circinelloides, M. hiemalis, S. brevicaulis, A. oryzae* and *B. megaterium* were selected based on the smell and taste of soy sauce, even though not quite satisfactory for the traditional Korean *meju* cakes. The five species were re-evaluated to produce the protease activities, the smell of the *meju*, and the taste of the soy sauce made from the *meju* above (Table 3). All isolates selected were capable of utilizing soluble starch with different numbers of their activities. The smells of *meju* made with a single inoculumn appeared similar to those results listed in Table 2, but those with *A. oryzae* appeared odorless. Also, the taste of the soy sauce made from the above *meju* showed results similar to the previous work shown in Table 2. The results made in Table 3 were consistent with those shown in Table 2. The isolated bacterium just from meju cakes of W-1 collected from Chung-Ju, was identified to *Bacillus megateriumas*, as based on the Biolog tests.

Making *Meju* cakes

Five isolates previously selected were employed for homemade *meju* cakes. All cultures of five isolates on soybeans, as the inocula, were directly added and mixed with the boiled soybean cereals stated. Afterwards, *meju* cakes were made by bare hand pressures and processed for two months, as previously mentioned. The species of zygomycetes were observed to appear at the early stage of meju fermentation (two to three days later after inoculation) or at drying process. The greenish yellow spores appeared on the surfaces of *meju* cakes during the mid-final stage (Fig. 1A). A little bacterial growths were found in the inner parts of *meju* cakes. The smell of these *meju* cakes would not be the those of good quality *meju* cakes and, therefore, the *meju* cakes would be finally spoiled or become a bad quality *meju* cake (Fig. 1BC). The *meju* cakes were covered with a greenish yellow spores, easily recognized as the spores of *A. oryzae* under microscope. However, the soy sauce made from these *meju* cakes tasted similar to Japanese type soy sauces, but not to traditional Korean homemade soy sauce.

The states (colours and smells) of *meju* cakes made with five inocula were quite different from those of the good quality *meju* cakes collected. Also, the soy sauce made of *meju* cakes above were different from those of a traditional Korean homemade one. Thus, the inoculumn of *A. oryzae* was excluded to the next experiment. Four isolates previously selected were employed for the traditional *meju* cakes, again. The species of zygomycetes inoculated appeared along with the transparent hyphae on the surfaces of *meju* cakes during

the early stage of the meju fermentation process (two or three days after making *meju* cakes). An acidic smell was strongly detected after two to three days of incubation. The white hyphae, observed under 20 or 30 magnifications, grew at the inner parts (Fig. 1E) while white spores from *S. brevicaulis* appeared on the surface of *meju* cakes during the mid-final stages (which was two weeks later, Fig. 1D)). Bacterial cells of *B. megaterium* were found to grow and make the colour changes in the inner parts of *meju* cakes. Exothermic reactions, realized by touching with hands, occurred in fermentations of *meju* cake two weeks, after inoculations. The odor of this *meju* cake would be similar to that given off from good quality *meju* cakes. Dark brown spots were observed to occur at the center of *meju* cakes when cut and, therefore, the quality of these *meju* cakes was acknowledged as "good" by the experts. The dark brown spots were observed to take place with the hyphal growth in the inner part and with the white powder surrounding the dark spots on the surfaces of *meju* cakes (Fig. 2DE). It was confirmed by the experts, before this work was done, that these signs mentioned above would be a good indicator for good quality-Korean style *meju* cakes.

Growth and Protease

The five microorganisms selected above were individually inoculated on/in the autoclaved soybeans and incubated at 28℃ for two weeks. The growth of the microorganism was measured daily with the production of carbon dioxide ml/hr (Table 5). The isolates of *A. oryzae* and *B. megaterium* grew well under the conditions of lower water activities or in a broad range of water activities, while the zygomycetous fungi, *M. circinelloides* and *M. hiemalis*, grew well only under the conditons of high water activities. The fungus *S. brevicaulis* grew well only at the water activity level of a_w= 0.96, but *S. brevicaulis* did not grow as well as the other isolates, *B. megaterium* and *A. oryzae*. The species of zygomycetes (*M. circinelloides* and *M. hiemalis*), were observed to grow well at the early stage of meju fermentation (state of high water activity), whereas the species of hyphomycetes (*S. brevicaulis* and *A. oryzae*) grew well during the middle to the final stages of meju fermentation (a lower stage of water activity than that of the earlier stage).

The activity of protease produced by the five isolates was measured with four different substrates generally reported (Table 6). The proteases produced by the isolates of *M. circinelloides* and *B. megaterium* reacted with the substrate of n-benzoyl-L-arginine ethyl ester (BAEE) and showed the highest activities, as compared with other three substrates, whereas the other isolates reacted

Table 4. Carbon dioxide prodcutions of the microorganisms collected when they were cultured at the at the different conditons of water activities (α_w) for four days' incubations at 30℃.

Microorganisms[a]	Carbon dioxide prodcutions (ml/hr) at the different conditons of water activities (α_w)[b]				
	0.92	0.95	0.96	0.97	0.98
Bacillus megaterium	2.38	2.81	2.84	2.94	3.42
M. circinelloides f. *griseo-cyanus*	0.01	0.14	1.78	2.70	3.32
M. hiemalis f. *hiemalis*	0.02	0.08	1.59	2.78	2.98
S. brevicaulis	0.02	1.32	2.75	2.59	2.50
A. oryzae	1.34	2.93	3.03	2.30	2.20

[a] The microorganisms were collected from the *meju*'s of Korean traditional foodstuffs (see Materials and Methods).

[b] Average values of carbon dioxide productions (ml/hr) measured by Gas-chromatograph (HP-5890) from four days-incubations on the boiled soybeans in 250 ml erylenmyer flasks, duplicated. The different water activities (α_w) adjusted with the amount of the tap water, and with salts at the level of water activities (α_w)=0.92.

Table 5. Relative Protease Activities of the different isolates on the synthetic ester substrates (U/L) and the relative values[a].

Microorganism	TAME[b]	BAEE[b]	TEE[b]	ATEE[b]
M. circinelloides f. *griseo-cyanus*	7,450 (0.20)[c]	36,696 (1)	3,725 (0.12)	2,168 (0.06)
M. himalis f. *hiemalis*	6,672 (1)	1,278 (0.19)	722 (0.11)	1.223 (0.18)
S. brevicaulis	8,451 (1)	3,336 (0.40)	4,336 (0.51)	4,392 (0.52)
A. oryzae	5,893 (1)	5,226 (0.89)	945 (0.16)	3,224 (0.54)
B. megaterium	12,287 (0.91)	13,455 (1)	611 (0.06)	6,227 (0.46)

[a]Relative protease activities of isolates fermented the autoclaved soybean for eight days.
[b]N-p-Tosyl-L-Arginine Methyl Ester (TAME), N-Benzoyl-L-Arginine Ethyl Ester (BAEE), L-Tyrosine Ethyl Ester (TEE), N-Acetyl-L-Tyrosine Ethyl Ester (ATEE) indicated, respectively.
[c]The values were relative values (the protease activity divided by maximal protease activity) using the different substrate at 40℃ for 10 min. It was speculated that the protease produced by *M. circinelloides* f. *griseo-cyanus would be similar to that by Bacillus megaterium* in its reactions of protein.

with TAME and showed the highest activities. Between the two protease produced by the isolates of *M. circinelloides* and *B. megaterium*, they reacted with the three substrates of BAEE, TEE and ATEE and showed different activities in the specific responses of protease enzyme. In other words, the abilities of proteases produced by three isolates reacted with TAME were similar for three isolates of *M. hiemalis, S. brevicaulis,* and *A. oryzae*, but those reacted with other three substrates (BAEE, TEE and ATEE) showed different responses of degradations of proteins. At least, the proteases produced by the five different isolates appeared disimilar to one another for degradations of soybean proteins.

Discussions

Fungal diversity

The fungal species isolated from different *meju* cakes were different and were not found to be overlapped in one another, the exception being a small number of species. It was predicted, at least before this scheduled work, that the few common fungi should be overlapped in the *meju* fermentation for the traditional Korean taste soy sauce. However, twenty-nine fungal species and a bacterium were identified here from the twenty three *meju* cakes; more than a fungal species were conclusively isolated and identified per their/its own *meju* cake (Tables 1 & 2). In other words, only a few fungal species were commonly found to coexist in *meju* cakes collected from the areas similar to each other. Otherwise, the other different fungi were observed to be on each *meju* cake. Two questions regarding the fungal species thought to be involved in *meju* fermentation were posed here: Was the role of the fungal isolates not important in the process of making *meju* cakes ? or were our identifications of the fungal isolates wrong, at all ?

Several keys for the fungi of order Mucorales are originated from Zycha's works (written by Germany in the year of 1931). The descriptions of them revised with Zycha and Siepman (1963) were employed for this study of ecological purpose for *meju* fermentation. In the present study the fungi were identified only on the basis of cryptomorphology therefore, it would need futher review dealing with some physiological aspects, Most hyphomycetous fungus identified here was a species of *Aspergillus* or *Penicillium* (Hahn & Park, 1958; Park *et al.*, 1977). The tendencies of the fungal morphological differences were matched with those shown from the physiological responses evaulated by the protease activity, smell of *meju* and taste of soy sauces, of which the characteristics are usually not employed for the classic taxonomy.

Mostly, various diversities of fungal species iso-

lated from *meju* cakes were considered to be similar to those from the different areas. The same fungal species, except for a few species, were hardly found in other *meju* cakes collected. Most fungi isolated were known to be air borne fungi, so that they could be involved in fermentations of *meju* cake under natural conditions. The fungal species identified did not coexist in *meju* cakes collected nationwide, but overlapping ecological niches within *meju* cakes collected locally. The observations for *meju* cakes were convinced to be done in their environments, such as rainfall, temperature, plant flora and animal fauna, from which *meju* cakes were made and collected from (Pitts, 1973; 1979; Domsh *et al.*, 1980). On the other hand, the different tastes of soy sauce made up of *meju* cakes were, perhaps, recognized to "localities of their soy sauces". If the process of making soy sauces was assumed to be similar at the different areas in Korea, it could be asked why such different diversities in tastes exist for the soy sauce; it was speculated, as based on this fungal diversity resulting from our work, that different fungi would be involved in meju fermentation and would cause the different tastes in traditional Korean homemade soy sauces. Thus, different fungal species isolated here were speculated to be distinct because of thier biological and physical environments.

Korean *Meju* Cakes

A traditional Korean *meju* cake could be not made with a single inoculumn in Table 2, but the taste of soy sauce made was slightly similar or close to that of Japanese soy sauce. A single inoculumn of *A. oryzae* or its related species was known to be employed in the refined method of making commercial soy sauces (Hahn & Park, 1957; 1958), Our result shown in Table 2 was consistent with Japanese style refined method of making *mejus.* However, the questions regarding the fungal roles in *meju* cakes were posed on traditional Korean homemade *meju*: would the species of zygomycetous fungi, *S. brevicaulis*, and *B. megaterium* not play an important role in the meju fermentation ? Nevertheless, why were the microorganisms mentioned above still isolated from traditional Korean homemade *meju* cakes ?

From this work, it could be recognized that a traditional homemade *meju* was not made with an inoculumn of single isolate, but with inocula of multiple isolates. Generally, *meju* cakes showing the blue (abundance of spores of species of *Penicillium*) or the greenish yellow (*Aspergillus* species) spores on their surfaces were not acceptable because they produced the smell and taste of soil or bitterness in the taste of soy sauces. It was commonly speculated that some unknown or known antibiotics produced by *A. oryzae* or species of *Penicillium* hindered the growth of *Bacillus* species and other fungi in *meju* fermentation process (Wang *et al.*, 1972). In detail, the commercial *meju* cakes were easily recognized to be fermented by the species of fungi involved as the colour on their surface. Thus, this was considered to result from long experiences of Koreans thoughout generations without any scientific knowledge.

Mucor species grew during the early stage of *meju* process, during which a high moisture level was maintained, and ceased to grow on *meju* cakes within a week. When the hyphae of *S. brevicaulis* grew with the cells of *B. megaterium*, the heat was released. The *meju* fermented by a single inoculumn of the zygomycetous species resulted in a weak taste of soy sauces, which was similar to the characteristics of Japanese *meju.* The proteases produced by the five isolates selected were considered to be different in enzymatic responses, kinds or structures, because they reacted differently with four substrates (Table 5). Thus, some roles of different protease were also expected to be played in degradation of soybean protein with mutual relationships. The different kinds of proteases should be originated from the different isolates of fungi or bacteria and degrade the soybean protein with conservational and mutual ease, if the hypothesis was correct that homemade *meju* cakes were needed for the mixed flora of the microoganisms. It was concluded that the zygomycetous species were important during the early stage, at least, for three days after inoculation. *S. brevicaulis* and *B. megaterium* grew during

the mid-final stages for two to three weeks after the zygomycetous species stopped growing.

This result indicated that fungal growths were related to the water activities in the boiled soybeans. Further speculations were made based on the given information: the species of zygomycetous fungi, known as water molds (*M. circinelloides* and *M. hiemalis*), grew well during the early stage of *meju* fermentation (state of high water activity), whereas the species of hyphomycetes, known as air-borne molds (*S. brevicaulis* and *A. oryzae*), grew well during the middle stage of *meju* fermentation (a lower stage of water activity than that of the early stage). However, some importances of zygomycetous fungi were recognized for traditonal *meju* cakes, at least, for the taste of Korean soy sauce within some localities for smell of *meju* or taste of traditonal Korean soy sauces. A theorum that the bacterium, *B. megaterium*, would grow well in the inner parts of *meju* can be made on the basis of this work and other previous works (Kim & Kim, 1989; Cho & Lee, 1970; Lee, 1986). Therefore, *B. megaterium* revealed that soy sauces made from the inoculated *meju* cake were tasteless (Table 2). The speculations are similar to the observations made about the traditional homemade *meju* cakes. Thus, it was logically concluded that some fungi of the order Mucorales play an important role in the fermentations of traditonal homemade *meju* cakes.

Conclusively, Korean soy sauce (kanjang) was, in some ways, different from commercial or other soy sauces because it was made from a traditional Korean homemade *meju*. Mostly, traditional Korean homemade *meju* cakes were, even though their taste showed a great range of diversity, defined to the cakes of the aggregated soybean cereals fermented with several fungi and a bacterium during two or three months' storage. Under natural conditions, the fungal successions seemed to occur during two physical stages (the borderline between two stages was not clearly cut); drying process taking place during the week after *meju* cakes were made afterward and heat releasing process fermented by the bacterium during the mid-final stage). The zygomycetous fungi, as a water mold, were involved in the early stage of *meju* fermentations and other hyphomycetous fungi, as mostly contaminant fungi, on the surfaces of *meju* cakes during the mid-final stage after the drying process. Also, the bacterium, identified as *B. megaterium* here, continued to grow, release the heat, and cause the brown spots or rings in the inner part of *meju* cakes during the mid-final stage. This fungal floral succession seemed to be caused by the water activities of *meju* cakes during fermentation processes. The zygomycetous fungi were speculated to aid other microorganisms to grow during the mid-final stage of *meju* fermentation process within some localities of their smells or soy sauces.

적 요

메주는 간장과 된장의 원료로 우리나라의 식물성 단백질인 조선 전통식품으로 중요하다. 조선전통식으로 농가에서 만들어진 23종류의 메주덩어리를 수거하여, 29종의 균과 한 종의 세균을 채집하여 동정하였다. 이러한 중에서 다만 몇개의 미생물만이 메주발효과정에 작용하는 것을 발견하였고, 나머지 미생물은 메주발효에서 잘못 발효로 일어나는 오염미생물로 밝혀졌다. 메주발효과정에서는 물리적인 변화로 건조과정(수분활성도)과 열방생과정이 메주발효 중에 일어나며, 이러한 과정이 미생물(대부분의 경우 메주 표면에서는 균)의 천이를 유도시켜 메주의 생화학적인 변화를 유도하는 것으로 나타났다. 균의 천이 과정에서 수분이 많았을 때는 물곰팡이인 접합균이 첨 서식한 것이 발견되었으며, 메주발효를 유도하는 것으로 나타났다. 흰포자를 방출하는 *Scopulariopsis brevicaulis*는 메주표면에서 고초세균인 *Bacillus megatrium*와 함께 후기 발열반응과정에서 발견되고 있었다. 미생물들이 분비하는 단백질 분해효소에 관한 조사에서, 각각의 미생물들은 콩단백질 분해에 서로 상호보안적인 역할을 하였다. 또한, 접합균들은 조선 전통의 메주발효과정에서 후기 메주발효를 유도하는 중요한 균으로 관찰되었다.

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