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Effects of Raw Materials and Various Molds on the Production of Koji

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Abstract

Alpha-amylase and glucoamylase activities were higher in koji with 40% water than that with 30 and 50% water, and *A. oryzae* exhibited very high alpha-amylase and glucoamylase activities compared to *A. sojae* and *A. niger*. Acidic, neutral and alkaline protease activities also showed higher activities in koji prepared with flour, Korean wheat powder and soybean powder with 40% water based on the weight of the sample. Alpha-amylase, glucoamylase, acidic, neutral and alkaline protease activities of all the koji samples according to incubation periods increased until 3~4 days of incubation and maintained nearly the same level or slightly decreased after 5 days of incubation. The protease activities of *A. oryzae* and *A. sojae* showed nearly the same trend regardless of differences in substrate conditions and koji materials, but those of *A. niger* showed a lower activity than those of *A. oryzae* and *A. sojae*. These results suggest that the preparation of koji is possible with Korean wheat powder and soybean powder and *A. sojae* can be utilized as a new strain for fermented foods using soybean as the main materials to increase functional properties and produce products having a new taste and flavor.

Key words: koji, enzyme activity, *Asp.* spp.

INTRODUCTION

Koji, known as "qu" in China, is made by growing microorganisms on steam-cooked starch ingredients such as rice, barley, wheat, and soybean under suitable moisture content and temperature (1,2). In fermented foods, koji utilized as a starter for fermentation plays an important role in the formation of their unique quality (3-8). Koji contains various enzymes (alpha-amylase, glucoamylase and protease) produced by inoculated microorganisms during an incubation period, and the enzymes contained in koji are related to the production of amino acids, protein, flavor, organic acids and alcohol in fermented foods such as soy sauce (8), soybean paste (9-14), red pepper paste (15-22) and alcoholic beverages (4).

In commercially fermented foods, the high activities of enzymes are very important because the enzymes can reduce the fermentation period and improve food quality. The enzymes produced from various microorganisms have distinctive properties to break down protein, carbohydrate, and lipids in raw materials and produce products including various tastes and flavors according to the fermentation procedure (9-14, 17,18). Hence, koji prepared from different materials and strains will show different properties in amylase and protease activity, and eventually, produce unique fermented foods having various tastes and flavors.

Recently since it can be purchased more easily and inexpensive than any starch ingredient, koji is mainly prepared with flour. However, if preparation of koji is possible by Korean wheat powder and soybean powder, the consumption of Korean wheat powder can be promoted. Additionally, soybean powder could have many benefits because soybean is an antioxidant (23) and is known to aid in the prevention of cancer and bone loss (24,25).

Based on this background, the aims of this research on koji were to use as the basic data in the preparation of various fermented foods, and to compare differences in amylase and protease activities according to prepared materials (flour, soybean powder and Korean wheat flour) and inoculation molds (*Aspergillus oryzae, Aspergillus sojae* and *Aspergillus niger*) for koji. Finally soybean powder, Korean wheat flour, *A. sojae* and *A. niger* were investigated in order to determine whether these can be used as koji materials and strains.

MATERIALS AND METHODS

Materials and preparation of koji

Flour (medium flour, CheilJedang Co., Seoul, Korea), Korean wheat powder (Worimil Co., Tacjon, Korea) and soybean powder (Gangwonnongsan Co., Gangwon do, Korea) used in this research were purchased from a local market. Samples were mixed with 30, 40 and 50% of water on the weight of the sample, put into 250 mL flask (20 g), and then steamcooked at 121°C for 15 min. After the steamcooked samples were cooled to approximately 30°C, the samples were inoculated with wheat bran starter (0.1 g) incubated for 4 days with *Aspergillus oryzae* (KCTC 2114), *Aspergillus sojae* (KCTC 6376) *and Aspergillus niger* (ATCC 46951) obtained from

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Jinmi Co. Ltd. (Taejon, Korea). The inoculated samples were incubated at 30°C for 5 days and used for preparing crude enzyme solution.

Measurement of alpha-amylase and glucoamylase activities

Alpha-amylase activity was assayed as follows; 20 g of the koji was suspended in 100 mL of distilled water. After shaking for 2 h at 30°C, the suspension was filtered with Whatman No. 2 filter paper (Whatman International Ltd., England). The filtrate was used as a crude enzyme solution. Absorbance of blank was determined with inactivated crude enzyme solution heated for 30 min in a boiling water bath instead of the crude enzyme solution. One milliliter of the crude enzyme solution and 1 mL of 2% starch solution, together with 1 mM parachloromercuribenzoic acid (PCMB) to inhibit glucoamylase activity, were mixed in a glass test tube and reacted at 30°C for 30 min. Then, the reaction mixture was cooled in an ice bath. The aliquot of reaction mixture (0.5 mL) was taken to a new glass test tube. The enzyme reaction was stopped by adding 0.5 M acetic acid (10 mL). After 1/300 N I_2 solution (1 mL) was added, 5 mL of distilled water was added, and then the absorbance was measured at 620 nm by a spectrophotometer (Spectronic genesys 5, Milton roy Co., USA). The α - amylase activity was determined by the absorbance difference between blank and sample. The amount of decreased starch was calculated from the standard curve constructed by the soluble starch (purity; 95%, Duksan Pure Chemical Co. Ltd, Ansan, Korea). The alpha-amylase activity was calculated as the activity hydrolyzing 1 mg of starch for 30 min at 30°C by 1 mL of crude enzyme solution and expressed as a unit per 1 g koji (2,26,27).

Glucoamylase activity was assayed as follows; 0.5 mL of crude enzyme solution was added to the 0.5 mL of 1% starch solution. It was then reacted at 30°C for 30 min in a water bath. After the reaction, the mixture was cooled in an ice bath. The 0.5 mL reaction mixture was taken to a new glass test tube. Five milliliter of DNS solution (28) in which 0.25 g of 3,5-dinitrosalicylic acid and 75 g sodium potassium tartrate (Rochelle salt) was dissolved in 50 mL 2 M sodium hydroxide (made by dissolving 4 g NaOH in 50 mL water) and diluted to 250 mL with water, was added to the reaction mixture and heated in a boiling water bath for 10 min, then cooled for 10 min. The absorbance was measured at 570 nm by a spectrophotometer. The amount of glucose produced was calculated from the standard curve constructed by using glucose. One unit of glucoamylase activity was calculated as the activity producing 1 mg of glucose by 1 mL of crude enzyme solution and expressed as a unit per 1 g koji (2,26,27).

Measurement of acidic, neutral and alkaline protease activities

The substrate mixture of 0.6% milk casein (0.5 mL) and 4.5 mL of McIlvaine buffer (0.2 M $Na_2HPO_4 \cdot 12H_2O$+0.1 M citric acid, acidic protease : pH 3.0, neutral protease : pH 6.0) were allowed for 10 min at 30°C in a water bath to adjust temperature. One milliliter of crude enzyme solution was added to a glass test tube containing the substrate mixture and reacted at 30°C for 10 min. The enzyme reaction was stopped by adding 5 mL of TCA mixture including 0.1 M trichloroacetic acid, 0.2 M sodium acetate and 0.3 M acetic acid. After the reaction mixture was filtered with Whatman No. 2 filter paper, 1 mL of filtrate was taken to a new glass test tube. Five milliliter of 0.5 M Na_2CO_3 solution and 1 mL of diluted (×3) folin reagent were added in the 1 mL of filtrate and allowed at 30°C for 30 min for the reaction. The absorbance at 660 nm was measured by a spectrophotometer. One unit of protease activity was calculated as the activity producing tyrosine of 1 μg per minute and expressed as a unit per 1 g koji . Absorbance of blank was determined with inactivated crude enzyme solution by heating for 30 min in boiling water bath (2,27). Alkaline protease activity was measured with sodium borate, boric acid buffer (0.025 M $Na_2B_4O_7$+0.1 M H_3BO_3, pH 9.0) instead of the McIlvaine buffer (29).

RESULTS AND DISCUSSION

Alpha-amylase and glucoamylase activities according to added water amount and materials

Activities of alpha-amylase and glucoamylase according to the amount of water added on flour, Korean wheat powder and soybean powder koji inoculated with *A. oryzae, A. sojae* and *A. niger* and incubated at 30°C for 4 days are shown in Fig. 1. Alpha-amylase and glucoamylase activities of flour, Korean wheat powder and soybean powder koji with added water at 40% for weight of the materials, exhibited a very high level compared to those with 30% added water, and in the case of 50% added water, nearly the same or slightly low activity except for alpha-amylase and glucoamylase activities of koji prepared with soybean powder. In all the samples, alpha-amylase and glucoamylase activities showed higher activities in koji with added water of 40%, than that of added water of 30 and 50% and *A. oryzae* exhibited a very high alpha-amylase and glucoamylase activities compared to *A. sojae* and *A. niger*. Alpha-amylase and glucoamylase activities showed nearly the same activities in koji prepared from flour and Korean wheat powder but koji prepared from soybean powder showed lower activities than those in koji prepared from flour and Korean wheat powder. Compared to *A. sojae* and *A. niger*, the higher alpha-amylase and glucoamylase activities in *A. oryzae* seem to be caused by difference of ability in enzyme secretion according to differences of composition and sequence in genes and the lower alpha-amylase and glucoamylase activities in soybean powder koji were apparently due to a lower starch amount than that of the flour and Korean wheat powder kojis.

Acidic, neutral and alkaline protease activities according to added water amount and koji materials

Acidic, neutral and alkaline protease activities of flour, Korean wheat powder and soybean powder koji according

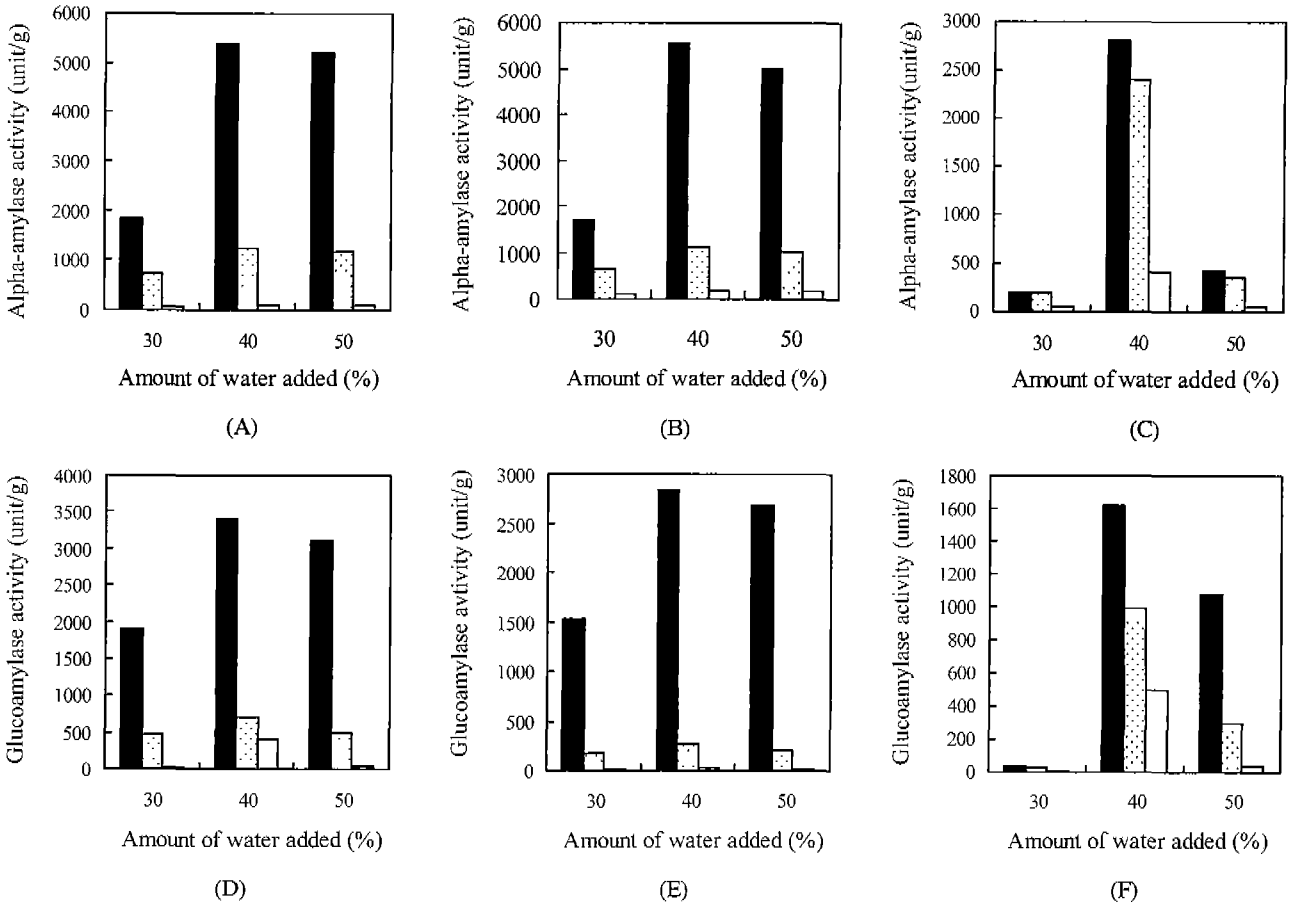

Fig. 1. Difference of alpha-amylase and glucoamylase activities according to added water amount of flour, Korean wheat powder and soybean powder koji inoculated with *A. oryzae, A. sojae* and *A. niger* and incubated at 30°C for 4 days. A, D) Flour koji ; B, E) Korean wheat powder koji ; C, F) Soybean powder koji. ■, Koji incubated with *A. oryzae* ; □, Koji incubated with *A. sojae* ; □, Koji incubated with *A. niger.*

to added water amount and molds are shown in Fig. 2. As seen in Fig. 2, acidic, neutral and alkaline protease activities showed relatively high activities in flour, Korean wheat powder and soybean powder koji with added water at 40% for weight of the materials. The activities of *A. oryzae* and *A. sojae* showed nearly the same trend regardless of difference in substrate conditions and koji materials but that of *A. niger* showed a lower activity than that of *A. oryzae* and *A. sojae*. Difference in acidic protease activity according to koji materials was not observed. However, neutral and alkaline protease activities showed higher activities in koji prepared from soybean powder than those in koji prepared from flour and Korean wheat powder. The relatively low enzyme activities in kojis with 30% added water were possibly due to lack of water needed for the growth of microorganisms. In the case of kojis with 50% added water, relatively low enzyme activities compare to the kojis with 40% added water seems to be caused by the larger particle size in koji materials than those of 40% made by adding too much water to koji as the larger particle size of koji materials bring about less growth surface for the growth of strains.

According to Lee et al. (2), when the moisture content of rice was adjusted by the addition of water from 10 to 100% of the weight of soaked rice and then cooked 120°C for 30 min, the degree of gelatinization (DG) in rice showed an increase as water increased and alpha-amylase, beta-amylase and neutral protease activities were the highest in 40% DG, but slightly decreased in 50% DG. These results agreed with our results.

Changes of alpha-amylase and glucoamylase activities according to incubation periods and koji materials

Changes in alpha-amylase and glucoamylase activities according to incubation periods of koji prepared with flour, Korean wheat powder and soybean powder are shown in Table 1. Alpha-amylase activity of flour and Korean wheat powder koji inoculated with *A. oryzae* increased until 5 days of incubation and that of soybean powder koji increased until 4 days of incubation but maintained nearly the same level on the fifth day of incubation. Alpha-amylase activity of flour, Korean wheat powder and soybean powder koji inoculated with *A. sojae* and *A. niger* increased until 4 days of incubation and maintained nearly the same level on the fifth day of incubation. Differences in alpha-amylase activity in flour and Korean wheat powder koji were not observed and

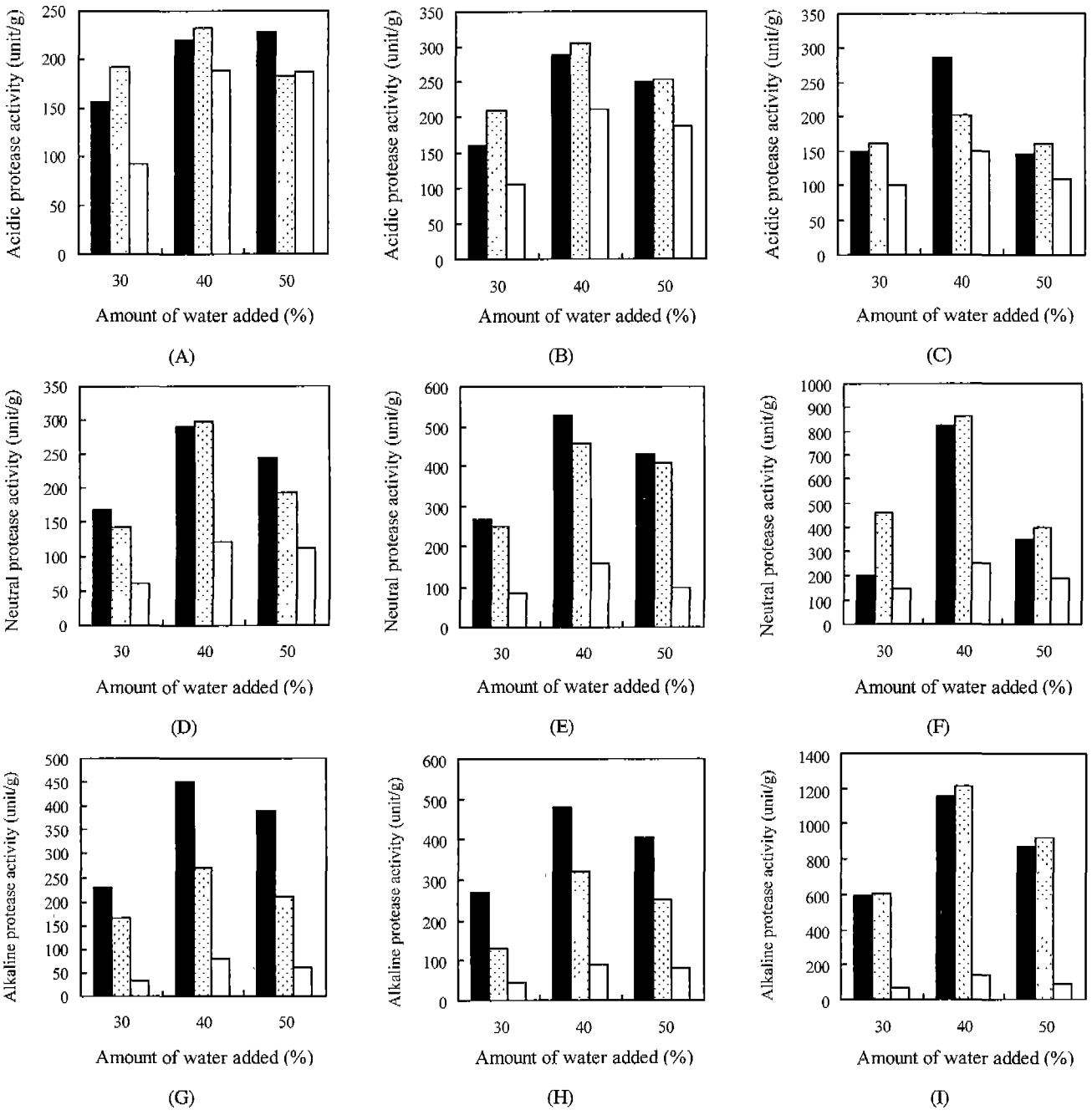

Fig. 2. Difference of acidic, neutral and alkaline protease activities according to added water amount of flour, Korean wheat powder and soybean powder koji inoculated with *A. oryzae*, *A. sojae* and *A. niger* and incubated at 30°C for 4 days. A, D, G) Flour koji; B, E, H) Korean wheat powder koji; C, F, I) Soybean powder koji. ■, Koji incubated with *A. oryzae*; □, Koji incubated with *A. sojae*; □, Koji incubated with *A. niger*.

alpha-amylase activity in soybean powder koji showed a comparably very low activity. In all the samples, alpha-amylase activity was the highest in order of *A. oryzae*>*A. sojae*>*A. niger*.

Glucoamylase activity of flour and Korean wheat powder koji increased until 4 days of incubation and exhibited nearly the same activity on the 5 days of incubation. Glucoamylase activities according to koji materials showed higher activities in koji prepared from flour and Korean wheat powder than those in koji prepared with soybean powder. Glucoamylase activity was the highest in order of *A. oryzae*>*A. sojae*>*A. niger*.

Changes of acidic, neutral and alkaline protease activities according to incubation periods and koji materials

Table 2 shows the acidic, neutral and alkaline protease activities according to incubation periods of koji prepared from flour, Korean wheat powder and soybean powder inoculated

Table 1. Changes of alpha-amylase and glucoamylase activities according to incubation periods of koji prepared with flour, Korean wheat powder and soybean powder inoculated with *A. oryzae*, *A. sojae* and *A. niger* and incubated at 30°C (Unit/g)

Enzyme activity	Koji materials	Strains	Incubation periods (day)			
			2	3	4	5
Alpha-amylase	Flour	*A. oryzae*	2,360	2,800	4,500	5,010
		A. sojae	860	930	1,128	880
		A. niger	80	132	178	80
	Korean wheat powder	*A. oryzae*	2,520	3,080	4,800	6,680
		A. sojae	800	1,100	1,250	1,150
		A. niger	88	108	132	54
	Soybean powder	*A. oryzae*	1,100	2,320	2,900	2,800
		A. sojae	900	2,000	2,400	2,200
		A. niger	100	350	400	410
Glucoamylase	Flour	*A. oryzae*	1,570	2,580	2,840	2,800
		A. sojae	300	505	620	340
		A. niger	11	25	26	34
	Korean wheat powder	*A. oryzae*	1,610	2,600	2,800	3,300
		A. sojae	284	510	320	236
		A. niger	10	21	26	30
	Soybean powder	*A. oryzae*	25	1,500	1,620	1,630
		A. sojae	24	850	900	860
		A. niger	12	200	250	275

Table 2. Changes of acidic, neutral and alkaline protease activities according to incubation periods of koji prepared with flour, Korean wheat powder and soybean powder inoculated with *A. oryzae*, *A. sojae* and *A. niger* and incubated at 30°C (Unit/g)

Enzyme activity	Koji materials	Strains	Incubation periods (day)			
			2	3	4	5
Acidic protease	Flour	*A. oryzae*	257	330	337	339
		A. sojae	264	343	324	277
		A. niger	207	248	211	169
	Korean wheat powder	*A. oryzae*	226	369	360	301
		A. sojae	220	288	343	283
		A. niger	143	214	284	156
	Soybean powder	*A. oryzae*	120	286	126	130
		A. sojae	60	203	105	110
		A. niger	50	150	60	80
Neutral protease	Flour	*A. oryzae*	379	418	542	594
		A. sojae	396	475	458	386
		A. niger	111	220	156	126
	Korean wheat powder	*A. oryzae*	368	510	550	618
		A. sojae	396	522	502	490
		A. niger	1,296	159	170	113
	Soybean powder	*A. oryzae*	270	825	297	280
		A. sojae	200	862	301	295
		A. niger	100	250	100	105
Alkaline protease	Flour	*A. oryzae*	380	451	499	429
		A. sojae	220	222	182	110
		A. niger	32	70	75	80
	Korean wheat powder	*A. oryzae*	302	465	511	402
		A. sojae	300	561	490	480
		A. niger	60	80	91	86
	Soybean powder	*A. oryzae*	770	866	1,091	906
		A. sojae	891	1,086	1,188	1,152
		A. niger	70	160	150	155

with *A. oryzae*, *A. sojae* and *A. niger*. The acidic protease activity of flour and Korean wheat powder koji increased until 3 days of incubation and maintained nearly the same level or slightly decreased on the fifth day of incubation. Signicant difference in the activity in flour and Korean wheat powder koji was not observed. However, the activities of

these were higher than those of soybean powder koji. Neutral protease activity increased until 3 days of incubation and maintained nearly the same level on the fifth day of incubation. The neutral protease activity in soybean powder koji was higher than that of flour and Korean wheat powder koji and this trend was similar to alkaline protease activity.

Lee et al. (2) reported that alpha-amylase, beta-amylase and neutral protease activities of rice koji grown with various species of *A. oryzae* at 41.83% of DG increased until 4 days of fermentation and then slightly decreased on the fifth day of fermentation. Lee et al. (30) also reported that the activities of saccharogenic and liquefying amylase and acidic protease have increase until 96 hr, but thereafter slightly decreased. These results were similar to our results.

In conclusion, these results suggested that the preparation of koji is possible by Korean wheat powder and soybean powder. Also, since protease activity of *A. sojae* as well as *A. oryzae* was high, *A. sojae* can be utilized as a new strain for fermented foods using soybean as the main material to increase functional properties such as antioxidation (23), anticancer action (24) and prevention of bone loss (25), and produce a product having a new taste and flavor not made by *A. oryzae*.

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