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New Fermentation Technique for Complete Digestion of Soybean Protein

LEE, JEONG OK[1], MI HWA PARK[1], YUNG HYUN CHOI[2], YEONG LAE HA[1], AND CHUNG HO RYU[1*]

[1]*Division of Applied Life Science, Institute of Agriculture and Life Science, Gyeongsang National University, Jinju 660-701, Korea*
[2]*Department of Biochemistry, College of Oriental Medicine, Dongeui University, Busan 614-052, Korea*

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Abstract The aim of this study was to develop a new fermentation method in order to improve the digestion of soybean protein, and to promote normal fermentation of soybean. A proximate composition, such as moisture, pH, and reducing sugar, of fermented soybeans by the new fermentation was similar to those of controls. Neutral protease activity, the most important factor for fermented soybean products, was the highest, having about 636 U/g at 54 h fermentation. The content of total free amino acid was almost 3–18 times higher than controls. The three-step fermented soybeans can be used as a functional food ingredient for human consumption, with higher protein digestibility.

Keywords: Soy protein, digestion, three-step fermentation

Soybean from the most cultivated plant in the world is rich in proteins (40–50%), lipids (20–30%), and carbohydrates (26–30%), and therefore, has been the subject of extensive scientific research [4]. Soybean protein is one of the widely used proteins, because of its abundance and bioactivities [22]. Recently, interest in the composition of soybean and its fermented products has grown, since potential anticarcinogens and other therapeutic agents have been reported [21]. However, consumption of improperly processed or uncooked soybeans can be harmful because of antinutritional compounds in soybeans including trypsin inhibitors, lectins, and flatulence-producing compounds, *etc.* [3, 17, 20]. Some of the antinutritional compounds can be destroyed or eliminated by proper processing or cooking [7, 10, 14].

Fermented soybean foods, such as *Chungkook-jang* in Korea and *Miso* in Japan, are highly digestible and nutritious, contributing important nutrients including calcium and vitamins A and B, as well as functional properties, such as laxative effect and anticancer properties [15, 18]. Nevertheless, Westerners and younger people tend not to eat fermented soybeans prepared by Korea traditional methods because of a strong ammonia odor when it is cooked. The ammonia odor is almost similar to the spoiling odor that is a volatile gas resulting from nitrogen components of soybean metabolism by some *Bacillus* spp. [13].

Many lactic acid bacteria (LAB) and other Gram-positive bacteria produce small antimicrobial and proteinaceous compounds, named bacteriocin, that typically kill or inhibit the growth of closely related bacteria. Nisin is a bacteriocin, which belongs to a group of lantibiotics and is produced by several strains of *Lactococcus lactis*. It is of special interest because of its widespread use as a natural food preservative [5].

In this study, we inoculated and pre-incubated the nisin-producing lactic acid bacteria in steamed soybeans to inhibit the growth of the odor-producing bacteria. Subsequently, it was fermented by a mold that has enzymes to hydrolyze soybean constituents and contributes to the development of a desirable texture, flavor, and aroma of the product. It was expected to decrease or eliminate antinutritional constituents by fermentation with mold. To complete digestion of nondegraded soybean proteins after the lactic acid and mold fermentation, the two-step fermented soybeans were followed by treatment with *Bacillus subtilis* that are known to extensively hydrolyze proteins into amino acids and peptides [6, 26]. In addition, properties of soybeans during the novel fermentation process were investigated.

Lactococcus lactis subsp. *lactis* ATCC 7962, ATCC 11454, and IFO 12007, nisin-producing strains, were grown in M17 supplemented with 1% glucose (M17G) at 30°C for 18 h before use. The growth of lactic acid bacteria (LAB) on the steamed soybeans was investigated. Among the above, *Lc. lactis* IFO 12007 did not strongly decrease the pH of soybeans, despite of its rapid growth. This fact would be important, because excessive decrease of pH might inhibit *koji* fermentation and cause over-acidification of the final product, *Chungkook-jang* and *Doengjang* [27]. In the subsequent experiment, therefore, we applied *Lc. lactis* IFO 12007 as a starter culture for soybean fermentation [16].

*Corresponding author
Phone: 82-55-751-5482; Fax: 82-55-753-4630;
E-mail: ryu@gnu.ac.kr

The spores of *Aspergillus oryzae* were obtained from the Chung-moo fermentation (Ulsan, Korea) and maintained at –20°C. *Bacillus subtilis* KFCC11293 was isolated from *Doenjang* and found to have an exceedingly high digesting activity of soybean proteins. Therefore, this bacterium was grown in a tryptic soy broth at 37°C for 12 h in a shaker incubator before use.

Soybeans were cleaned and soaked overnight until they doubled in weight. The soaked soybeans were steamed at 115°C for 60 min in an autoclave, and cooled to room temperature. Cooled soybeans were inoculated with 10^6 CFU/g *Lc. lactis* IFO 12007 as a lactic starter culture, before being incubated at 30°C for 24 h. This process was designated as one-step (lactic) fermentation. The one-step fermented soybeans were then inoculated with spores of *A. oryzae* at 10^6 spores/g as a *koji*-starter culture, which had been previously mixed with roasted barley powder, and incubated at 30°C until the soybeans were covered with a cottony mycelium of the mold. This process is called two-step fermentation. The two-step fermented soybeans were then inoculated with *B. subtilis* KFCC11293 at 10^6 CFU/g and incubated at 43°C for 24 h. This process is called three-step fermentation. As a control, the steamed soybeans were inoculated with *B. subtilis* KFCC11293 (BS) or *A. orzae* (AO) and incubated as described above. The samples were collected at 6-h intervals during fermentation and stored at –70°C before use.

pH and water and crude protein contents of the samples were determined by standard AOAC methods [2]. Reducing sugars in the samples were determined using the dinitrosalicylic reagent method [24]. In addition, acidic, neutral, and alkaline protease activities of the samples were measured by the procedure reported by Yi *et al.* [26]. Determination of free amino acids was performed according to the method described by Fujimaki *et al.* [9] using a Sykam model A433 automatic amino acid analyzer (Sykam, Germany).

Table 1 shows the changes in moisture, pH, total sugar, reducing sugar, and crude protein concentration during the three-step fermentation of soybeans. The levels of moisture in the samples and controls were slightly decreased. The pH value of the samples was increased after decrease depending on the progress of the three-step fermentation.

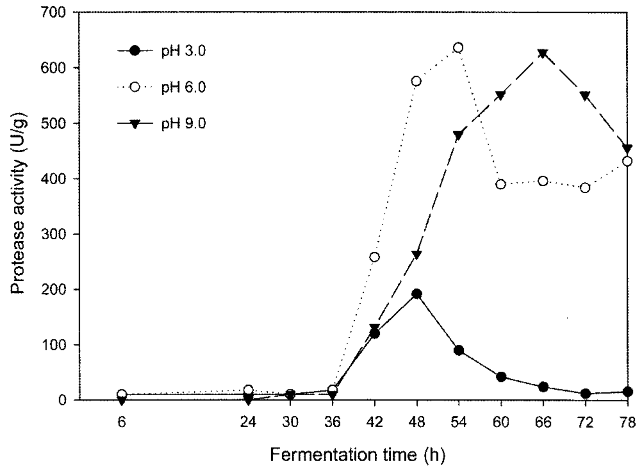

Fig. 1. The changes of acid (●), neutral (○), and alkaline (▼) protease activities during three-step fermentation.
The steamed soybeans were inoculated with *Lc. lactis* IFO 12007 before being incubated at 30°C for 24 h (one-step fermentation). The one-step fermented soybeans were then inoculated with spores of *A. oryzae* and incubated at 30°C for 30 h (two-step fermentation). The two-step fermented soybeans were then inoculated with *B. subtilis* GSK 3580 (KFCC11293) and incubated at 43°C for 24 h.

The increase of pH was presumably due to proteolysis and the release of ammonia following the utilization of amino acids by the fermenting microorganisms [8]. The difference in pH change between the samples and controls was not significant. As the fermentation progressed, the contents of reducing sugar and protein were increased. The changes of sugars content during fermentation may be due to degradation of sugar into a facile form for utilization by the microorganisms during fermentation. Zamora and Veum [28] demonstrated that the concentration of crude protein in heated soybeans increased (5%) after *A. oryzae* fermentation. Moreover, the increase of protein has been reported during soybean fermentation [25]. The increase in crude protein contents may also be due in part to the decrease of carbohydrate content by fermentation [12].

As shown in Fig. 1, the acid protease activity (pH 3.0) rapidly increased after 36 h of fermentation, showing the highest at 48 h. Neutral protease activity (pH 6.0) rapidly increased from 36 h, and the highest value of about 636 U/g

Table 1. Changes of moisture, pH, total sugar, reducing sugar, and crude protein during the three-step fermentation of soybean.

	Fermentation time (h)							
	0	12	24	36	48	60	72	78
Moisture (%)	60.4	60.3	60.1	60.7	60.3	59.1	58.6	58.2
pH	6.6	6.4	6.3	5.4	5.9	6.2	6.8	7.0
Total sugar (%)	75.5	87.5	89.5	86.5	47.5	23.5	21.5	18.0
Reducing sugar (%)	1.5	0.3	0.7	5.7	14.4	6.0	7.9	8.3
Crude protein (%)	17.8	16.4	16.3	16.3	17.2	18.3	18.2	18.8

Steamed soybeans were fermented by the serial addition of microoganisms [*Lc. lactis* IFO 12007 (10^6 CFU/g, 30°C, 24 h), *A. oryzae* (10^6 spore/g, 30°C, 30 h), and *B. subtilis* KFCC11293 (10^6 CFU/g, 43°C, 24 h)]. The samples were collected at 12-h intervals during fermentation.

Table 2. Free amino acid contents of fermented soybean by different microorganisms (mg%).

	A. oryzae fermentation	*B. subtilis* fermentation	Three-step fermentation
Threonine	199.90	20.34	1,028.36
Serine	297.47	27.90	484.27
Asparagine	209.21	54.05	324.28
Glutamic acid	1,030.66	259.13	2,231.12
Proline	385.56	18.07	1,279.71
Glycine	156.24	22.97	737.30
Alanine	440.15	48.23	1,461.38
Valine	388.88	100.59	1,475.57
Methionine	129.60	27.07	431.75
Isoleucine	282.07	31.81	1,410.29
Leucine	624.39	56.71	2,523.56
Tyrosine	334.62	121.74	1,267.37
Phenylalanine	476.68	128.83	1,690.47
Lysine	566.36	54.11	1,767.66
Histidine	147.94	67.55	802.99
Arginine	661.85	12.30	348.64
Total	6,331.59	1,033.30	19,264.72

was found at 54 h of fermentation, corresponding to 1.7 and 2.2 times of AO and BS, respectively (data not shown). It was maintained at nearly 400 U/g or slightly decreased after 60 h of fermentation. The neutral protease activity in fermented soybeans has been considered to be the most important factor for fermented soybean products such as *Chungkookjang* and *Doenjang* [11]. The activity of alkaline protease (pH 9.0) followed patterns similar to that of neutral protease. The highest protease activity of about 627.6 U/g was recorded in the sample at 66 h of fermentation and was gradually decreased thereafter. During the three-step fermentation, the protease activities of neutrality and alkalinity were higher when *A. oryzae* or *B. subtilis* was used (data not shown).

The contents of free amino acids in the samples are shown in Table 2. The amount of total free amino acids in the sample was 19,264.72 mg%, and was 3.0 and 18.6 times higher than that of AO and BS, respectively. It is highly likely that soybean proteins were completely digested more efficiently because of various proteases produced by different microorganisms. The contents of glutamic acid in the sample, which gives the savory taste components, were the highest. In *Chungkook jang meju* by fermentation with *B. subtilis*, glutamic acid, leucine, lysine, and phenylalanine were higher [19], and Aaslyng *et al.* [1] reported that soybean hydrolysates by proteolytic enzymes from *B. licheniformis* and *A. oryzae* contained large quantities of leucine, glutamic acid, and lysine.

Traditionally, the use of plant-origin proteins in young and old people's diets has been rather limited, mainly because of antinutritional factors as well as the complex structure of nutrients, including protein [3]. The fermentation processes developed presently improved the nutritional quality of soybeans, and therefore, the three-step fermented soybeans can be used as a functional food ingredient for human consumption, with higher protein digestibility and probiotic effects.

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