DOI:10.1021/jf904600a

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Some Biochemical and Physical Changes during the Preparation of the Enzyme-Ripening Sufu, a Fermented Product of Soybean Curd

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In this study, sufu, a fermented product of soybean curd, was prepared by ripening the salted tofu in the mash of *Aspergillus oryzae*-fermented rice−soybean koji possessing various hydrolytic enzymes for 16 days. It was found that protease, α-amylase, β-amylase, and lipase activities observed in the koji granules reduced most pronouncedly during the initial 4 or 8 days of ripening. Meanwhile, an increase in the activity of the various enzymes was noted in the ripening infusion and tofu cubes. During the ripening period, the content of total nitrogen, pH, and hardness of sufu decreased, while the titratable acidity, protein dissolution ratio, content of free fatty acid, and free amino acid increased with glutamic acid, showing the largest magnitude of increase at the end of the 16 day ripening period. Additionally, the color of tofu cubes changed from pale yellow to yellowish brown at the end of the ripening period.

KEYWORDS: Sufu; rice−soybean koji; biochemical and physical changes; enzyme-ripening process

INTRODUCTION

Sufu, the oriental fermented product of soybean, is a highly flavored, soft cheese-like product made from cubes of soybean curd (tofu). This product is also known as fu-su, fu-ru, tou-fu-ru, Chinese cheese, or bean cake and has been consumed for more than 1000 years in China (*1*). Sufu, with an estimated annual production of over 300 000 tons in China, is usually consumed as an appetizer or a side dish and is often eaten with breakfast rice or steamed bread. Sufu adds zest to the bland taste of a rice and flour diet (*2*). On the basis of processing technologies, sufu is divided into four types: mold-fermented sufu, naturally fermented sufu, bacteria-fermented sufu, and enzymatically ripened sufu (*2*). Mold-fermented sufu is the type of sufu produced predominantly in China. The manufacturing process consists of four steps, including the preparation of tofu, the preparation of pehtze (tofu freshly grown with the fungus, such as *Actinomucor*, *Rhizopus*, or *Mucor* on its surface), salting, and the aging of salted tofu cubes in saline solution (*2*,*3*). In contrast, most sufu in Taiwan is prepared with an enzymatically ripened process using koji as the source of enzymes. To prepare sufu in this manner, salted tofu cubes and koji mash are first prepared simultaneously (**Figure 1**). Koji mash is prepared by growing *Aspergillus oryzae* in a mixture of steamed rice and soybean and then soaking in syrup containing ca. 65% sucrose. The sufu is ripened by soaking the salted tofu cubes in the prepared koji mash containing various hydrolytic enzymes at 37 °C. In comparison to the mold-fermented sufu, the fermentation period of the koji enzyme-ripening sufu usually takes only ca. 2−3 weeks, considerably shorter than the 4−6 months required for the preparation of mold-fermented sufu. Other differences include the use of a different fungus in the manufacture process. The koji enzyme-repening sufu is sold packed in jars together with the koji mash, which contains granules of rice and soybean koji. Besides, it is generally sweeter and not as salty as the mold-fermented sufu.

Although numerous papers related to the mold-fermented sufu were documented (*4*−*11*), no scientific data concerning the koji enzyme-ripening sufu are currently available. To provide information essential to improvements in quality control and the manufacturing process of this enzyme-ripened sufu, this study was attempted to investigate some biochemical and physical changes during the fermentation (ripening) of the koji enzyme-ripening sufu.

MATERIALS AND METHODS

Preparation of the Sufu. In this study, the koji enzyme-ripening sufu was prepared in a manner as performed in the sufu manufacture plant. It included the preparation of salted tofu cubes, the preparation of koji mash, and the ripening of the tofu cubes, as shown in **Figure 1**. For the preparation of salted tofu cubes, tofu cubes approximately 2.5 × 2.5 × 2 cm in size were transferred to plastic containers. Each layer of tofu cube was sprinkled with a layer of salt at a ratio of 4:1 (w/w) tofu and salt. Water, 3 times the weight of salt, was then added. After the salted tofu cubes were storing for 24 h at room temperature, they were removed from the container and air-dried for 24 h.

To prepare the rice−soybean koji mash, soybeans and rice at a ratio of 2:1 (w/w) were soaked in hot water at ca. 70 °C for 1 h and then steam-cooked for 1 h. Koji was prepared by inoculating 22.5 g of powder of seed koji of *A. oryzae*, obtained from San-Yi Chemical Co., Taichung, Taiwan, into 50 kg of a steamed rice−soybean mix. The inoculated rice and soybean mix was placed evenly in bamboo trays (thickness of about 1 cm)

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 Published on Web 03/10/2010

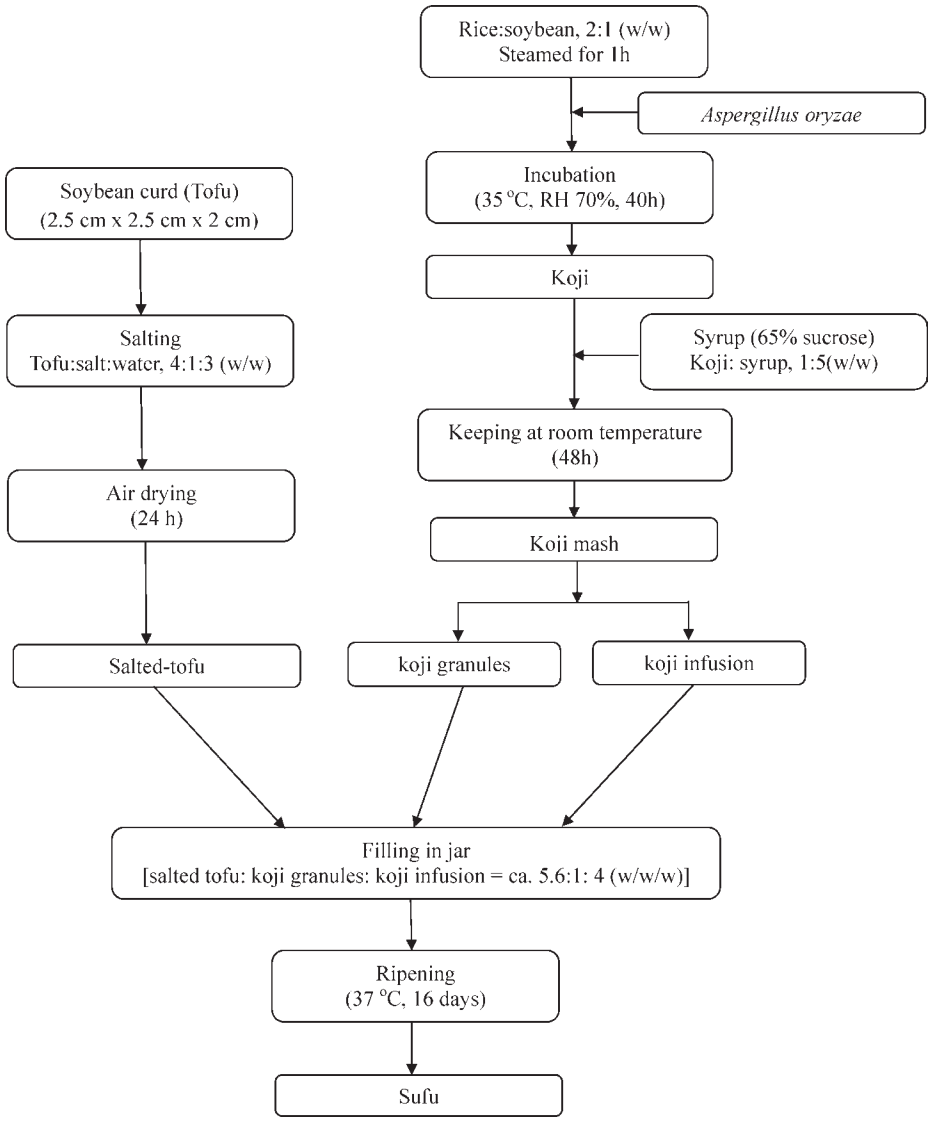

Figure 1. Schematic diagram for the production of the koji enzyme-ripening sufu.

and placed in an incubation room, where the temperature was controlled at 35 °C, with a relative humidity of 70% for 40 h. The koji mash was then prepared by mixing the prepared koji with syrup (65% sucrose) at a ratio of 1:5 (w/w) and then kept at room temperature for 48 h. The koji mash was then separated into koji granules and infusion by filtering through a screen.

To perform the ripening of sufu, salted tofu cubes (195 g, ca. 14−15 cubes) placed in a glass jar (400 mL) were mixed with koji mash (175 g), where the ratio of koji granules/syrup was 1:4 (w/w). To ripen the sufu, they were then incubated at 37 °C for 16 days. In the present study, koji mash and salted tofu cubes were prepared in a local sufu manufacture factory, while ripening of sufu was performed in our laboratories.

Sampling. During the sufu ripening period, bottled sufu samples were withdrawed at specific intervals. Contents of the bottle were separate into three parts: sufu (tofu cubes), koji granules, and infusion. Tofu cubes were rinsed and soaked in distilled water for 2 min, then the water was decanned. Koji mash was filtered through a screen to separate the koji granules and infusion. They were subjected to analysis immediately or kept at −20 °C until analyses were performed.

Measurement of the Enzyme Activity. In the present study, protease, α-amylase, β-amylase, and lipase activities of koji granules, sufu (tofu), and infusion samples collected during the ripening period were examined. To measure the enzyme activities of koji granules and sufu, enzymes were first extracted from the 10 g of sufu or koji samples with 50 mL of 0.9% NaCl solution by shaking (200 rpm) at room temperature for 60 min as described by Su et al. (*12*). They were then centrifuged (5000*g*, 20 min). The supernatant and the infusion were then used as the enzyme sources.

Protease activity was determined according to the method described by Keay and Wildi (*13*), with minor modification. Essentially, 1 mL of enzyme solution and 1.0 mL of 2% Hammersten milk casein solution (dissolved in phosphate buffer at pH 6.0) were mixed and incubated at 37 °C for 20 min. One unit of protease activity is defined as the amount of enzyme that liberates 1 μg of tyrosine per minute.

Lipase activity was assessed by modification of Bier's method (*14*). Essentially, 10 mL of enzyme solution, 10 mL of freshly prepared polyvinyl alcohol emulsion (pH 6.0), and 5.0 mL of McIlvaine buffer (pH 6.0) were mixed and shaken (100 rpm) at 37 °C for 4 h. One unit of lipase activity is defined as the amount of enzyme that liberates 1 μg of oleic acid per minute.

The activity of α-amylase was determined according to that described by Narahara et al. (*15*), with a minor modification. The method used is essentially based on monitoring the degree of reduction in the color of an iodine−starch complex after a definite reaction time. A reaction mixture was prepared consisting of 1.0 mL of enzyme solution, 5.0 mL of 3% soluble starch solution, and 3.0 mL of 0.2 M acetate buffer (pH 5.0), incubated at 40 °C for 5 min. One unit of α-amylase activity is defined as the amount of enzyme that catalyzes the hydrolysis of 10 mg of soluble starch per 30 min under the assay conditions. To determine β-amylase activity, maltose in the above-mentioned reaction mixture was measured by the dinitrosalicylic acid (DNS) method (*16*). One unit of β-amylase

activity was defined as the amount of enzyme that catalyzed the liberation of 1 mg of maltose per hour under the assay conditions.

Measurement of Total Nitrogen, Amino Nitrogen, and Free Fatty Acid. The content of total nitrogen and amino nitrogen in sufu samples was determined according to the Kjeldahl method and the formal titration method, respectively, as described by the Association of Official Analytical Chemists (AOAC) (*17*).

To determine the free fatty acid content, sufu lipid was first extracted with *n*-hexane following the method described by Wiese and Snyder (*18*). The extraction lipid was dissolved in alcohol containing phenolphthalein as an indicator and titrated with standard NaOH solution as described by AOAC (*17*). The amount of fatty acid was then calculated as oleic acid and expressed as a percentage of lipid.

Analysis of Free Amino Acids. To determine the composition of free amino acids, samples were first mixed with 0.9% NaCl solution and kept at room temperature with stirring for 60 min. They were then centrifuged at 8500*g* for 10 min. The supernatant was then collected and filtered through a membrane filter (0.45 μm).

The high-performance liquid chromatography (HPLC) method using precolumn derivatization with orthophthaldialdehyde (OPA) (*19*) was then employed to determine the amino acids in the samples. A kit containing OPA-3 column, reagents, borate buffer, and amino acid standards was obtained from GROM (Rottenburg-Hailfingen, Germany). The amino acid derivatives of amino acids in the samples and the amino acid standards formed by a reaction with OPA were detected using the HPLC system.

The HPLC system consisting of a degassing system (model DG-2410, Sanwa Tsusho Co., Tokyo, Japan), a pump (880-LC, Jasco Co., Tokyo, Japan), a OPA-3 column (GROM), a column heater (800-LC, Jasco Co.), a fluorescence detector (FP-920, Jasco Co.), and a solvent mixing module (880-PU, Jasco Co.). The chromatographic conditions are as follows: flow rate, 1.1 mL/min; volume of injection, 20 μL; and solvents A, sodium phosphate buffer (25 mM, pH 7.2)/tetrahydrofuran (1000:7.5) and B, sodium phosphate buffer (25 μm, pH 7.2)/acetonitrile/methanol (50:15:35). Fluorimetric detection is carried out using excitation and emission wavelengths of 330 and 450 nm, respectively. The gradient consists of 100% A for 2 min, 0−50% B in A for 8 min, 50−60% B in A for 5 min, 60−100% B in A for 5 min, and 0% B in A for 1 min.

Measurement of pH, Acidity, and Dry Weight. A total of 5 g of tofu sample was mixed with 50 mL of distilled water, heated to boil, cooled, and then centrifuged (8960*g*, 10 min). The pH of the supernatant was measured directly with a pH meter, while acidity was measured by titrating the supernatant with 0.1 N NaOH solution and was expressed as a percentage of the citric acid.

The dry weight of sufu and koji granules was measured by drying to a constant weight at 105 °C as described by AOAC (*17*).

Measurement of Hardness and Color. Hardness of sufu was determined according to the method described by Bourne (*20*) using a texture analyzer (TA-XT2, SMS, Inc., Surrey, U.K.). Indices of color (*L*, *a*, and *b* values) were determined using a color differential meter (model 985917, AC adapter AC-A12, BYK-Gardner, Geretsried, Germany) according to that described by Almela et al. (*21*).

Statistical Analysis. The mean value and standard deviation were calculated from the data obtained from the three separate experiments. Means were compared using Duncan's multiple range test method in SAS, version 9.1 (SAS Institute, Gary, NC).

RESULTS AND DISCUSSION

Changes of Enzyme Activity in Koji Granules, Ripening Infusion, and Tofu Cubes during Ripening. Enzymes, including protease, amylase, and lipase, are important for the production of the characteristic flavors, colors, and textures of traditional oriental fermented products of soybean, such as sufu, miso, and soy sauce (*22*). In the preparation of mold-fermented sufu, enzymes were produced by molds, such as *Actinomucor*, *Rhizopus*, or *Mucor*, acting directly on tofu cubes during phetze preparation and aging. On the other hand, rice−soybean koji was first prepared by growing *A. oryzae* on a mixture of steamed rice and soybean during the manufacture process of koji enzyme-ripening sufu (**Figure 1**). The prepared koji served as the source of the hydrolytic enzymes. Before the ripening of tofu cubes started, koji mash was first prepared by mixing the rice−soybean koji with syrup at room temperature for 2 days. During this period, enzymes leached out of the koji granules into the syrup, which was later used as the ripening solution. It was found that the rice−soybean koji contained protease, α-amylase, β-amylase, and lipase with an activity of 305.8, 1541.4, 3614.3, and 433.3 units/g of dried koji, respectively, before mixing with the syrup for the preparation of koji mash. However, the enzyme activity detected in the koji granules reduced to 62.3, 740.4, 1460.4, and 114.6 units/g of dried koji, respectively, 2 days after having been mixed with the syrup. Originally, the syrup itself showed no enzyme activity, while the prepared infusion of the koji mash showed an enzyme activity of 12.9, 282.7, 264.9, and 8.2 units/mL of infusion, respectively, after 2 days of mixing with koji granules.

Figure 2 shows the change in enzyme activity detected in the koji granules, ripening infusion, and tofu cubes during the 16 day ripening period. It was found that the protease, α-amylase, β-amylase, and lipase activities detected in koji granules reduced during the ripening period, while the extent of decline in the enzyme activity in the koji granules was generally most marked during the first initial 4 or 8 days of ripening. Thereafter, the reduction in enzyme activity was less marked. At the end of the ripening period, the koji granules showed protease, α-amylase, β-amylase, and lipase activities of 15.7, 484.4, 630.1, and 47.0 units/g of dried koji, respectively. In contrast to the reduced enzyme activity detected in koji granules during ripening, generally, an increase in the activities of the various enzymes was noted in the ripening infusion and tofu cubes. Although no enzyme activity was detectable from tofu cubes before ripening, considerable enzyme activity was detected after 4 days of ripening. However, little change in the enzyme activity was detected in the tofu cubes thereafter. The leaching out of enzymes from the prepared koji granules into the koji mass infusion and tofu cubes might lead to the decline of enzyme activity detected in koji granules and to the increase of enzyme activity in both the ripening infusion as well as tofu cubes observed during the ripening period.

In the manufacture process of mold-fermented sufu, the aging solution usually contained a relatively high concentration of ethanol and NaCl. These components retarded or inactivated the action of the hydrolytic enzymes and, thus, led to a decline in the activity of protease, amylase, and lipase, which was noted in the sufu infusion (*7*) and during the hydrolysis of the tofu substrate (*5*, *6*), while such a phenomenon was not observed in the tofu cube and infusion during the ripening of the koji enzyme-ripening sufu (**Figure 2**).

Change of Total Nitrogen and Amino Nitrogen Contents and Protein Dissolution Ratio of Sufu during Ripening. Figure 3 shows the change in the contents of total nitrogen, amino nitrogen of tofu, and the dissolution ratio of protein (ratio of amino nitrogen/total nitrogen content) during the 16 days of the sufu ripening period. Protein is the largest major component in the sufu product, except water. It is generally accepted that the fungal protease catalyzing the degradation of protein into smaller molecular elements contributes to the particular flavor and texture of the sufu product (*11*, *23*). Similar to the mold-fermented sufu (*9*, *24*), a decline in the total nitrogen content of tofu with an increase in the amino nitrogen content and dissolution ratio of protein was noted with the enzyme-ripening sufu during the ripening period (**Figure 3**). However, the koji enzyme-ripening sufu showed a higher protein dissolution of 19.0% after only 16 days of ripening (**Figure 3**) compared to 13−18% noted with the mold-fermented sufu after 4 months of aging (*25*).

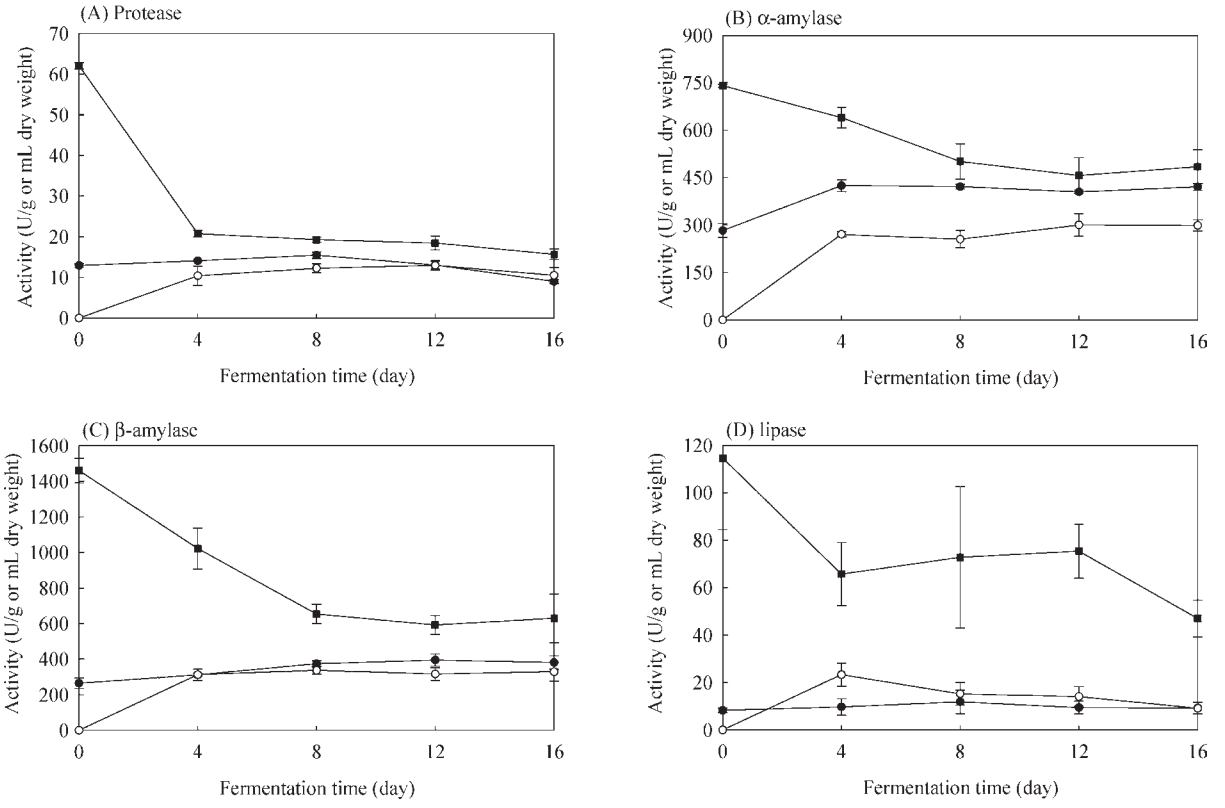

Figure 2. Changes of enzyme activity during the ripening of sufu: (■) koji granule, (●) infusion, and (○) tofu cube.

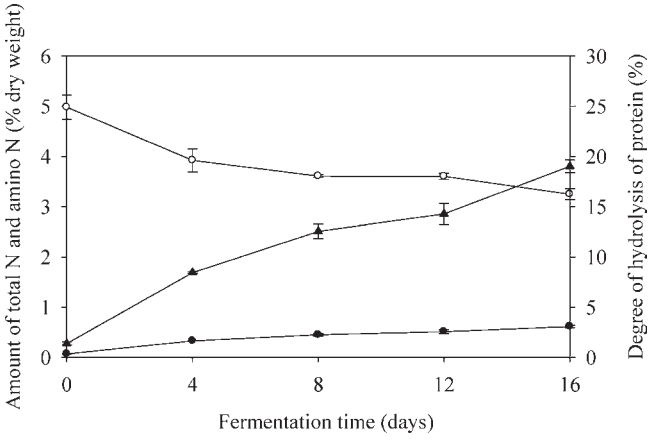

Figure 3. Hydrolysis of protein during the ripening of sufu: (●) amino N, (○) total N, and (▲) degree of hydrolysis of protein.

This result demonstrated that protein hydrolysis proceeded more rapidly in the enzyme-ripening process controlled at 37 °C than in the mold-fermented sufu manufacture process proceeded at room temperature. In addition to the effect of NaCl, ethanol, and ripening temperature, differences in the fungus involved during the manufacture process might also contribute to this discrepancy.

Table 1 shows the content of individual free amino acids of tofu cubes before and after the 16 days of ripening. Generally, the content of total free amino acids and most of the individual free amino acid increased at the end of the ripening periods. These results were consistent with that observed with sufu prepared with the mold-fermented process (*9*) and other oriental fermented products of soybean, such as miso, soy sauce, and tou-pan-chiang (*26*, *27*). Among the various free amino acids examined, a relatively large increase in the content of glutamic acid was noted in the 16 day ripened sufu. Glumatic acid has generally been considered to be one of the most important contributors toward the flavor of the oriental fermented products of soybeans (*23*, *26*). The koji mold, *A. oryzae*, is able to produce glutaminase, which converts glutamine to glutamic acid (*28*, *29*). Therefore, the action of glutaminase in addition to the hydrolytic release of glutaminic acid from the raw material by protease might contribute to the large increase in the content of glutamic acid.

Table 1. Free Amino Acid Contents of Sufu before and after 16 Days of Ripening

	content (mg/g of dry sufu)	
free amino acid	before ripening	after ripening
Asp	2.10 ± 0.21 b[a]	3.37 ± 0.89 a
Glu	0.39 ± 0.22 b	4.12 ± 1.26 a
Ser	0.04 ± 0.00 c	0.39 ± 0.07 a
His[b]	0.04 ± 0.03 d	0.59 ± 0.01 a
Gly	0.05 ± 0.02 b	0.26 ± 0.03 a
Thr[b]	0.05 ± 0.03 ab	0.11 ± 0.07 ab
Arg[b]	0.07 ± 0.09 c	1.44 ± 0.10 a
Ala	0.02 ± 0.02 a	0.02 ± 0.02 a
Tyr	0.03 ± 0.02 c	0.74 ± 0.10 a
Val[b]	0.11 ± 0.04 b	0.15 ± 0.02 b
Met[b]	0.03 ± 0.03 c	0.55 ± 0.06 a
Phe[b]	0.05 ± 0.03 c	0.64 ± 0.05 a
Ile[b]	0.02 ± 0.02 c	0.22 ± 0.01 a
Leu[b]	0.05 ± 0.05 c	1.95 ± 0.14 a
Lys[b]	0.03 ± 0.00 c	0.52 ± 0.12 a
Pro	0.03 ± 0.01 a	0.01 ± 0.01 b
total	3.10 ± 0.43 c	15.66 ± 2.26 a

[a] Values are expressed as means ± standard deviation (SD) ($n = 3$). Means in the same row with different lowercase letters (a, b, c, and d) are significantly different by Duncan's multiple range test ($p < 0.05$). [b] Essential amino acid.

Change of the Free Fatty Acid Content of Sufu during Ripening. **Figure 4** shows the change in the free fatty acid content expressed

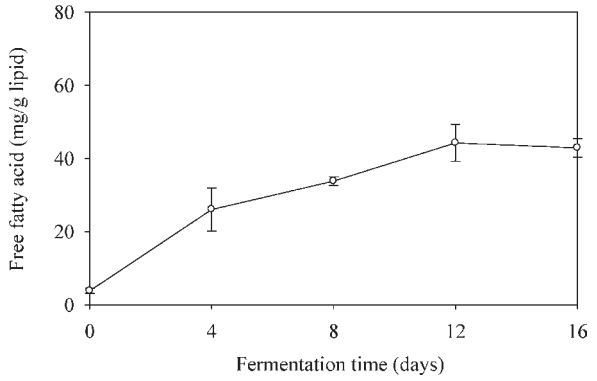

Figure 4. Change of the free fatty acid content during the ripening of sufu.

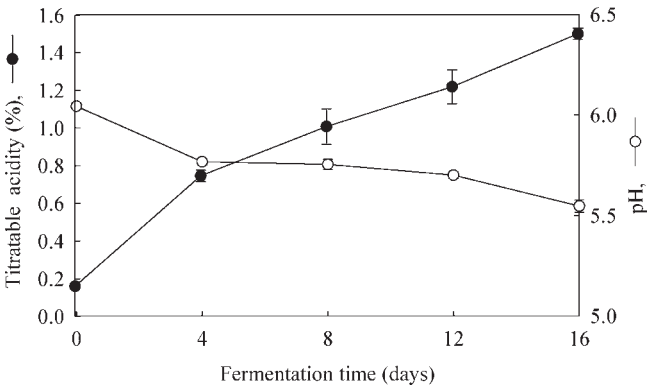

Figure 5. Change in the titratable acidity content and pH during ripening of sufu.

as a fraction of crude lipid during the ripening of tofu cubes. It was found that the free fatty acid content increased from the start of ripening until about 12 days of ripening. Further extending the ripening period did not show a marked change in the free fatty acid content. The free fatty acid content increased from 3.8 to 42.9 mg/g of lipid after the 16-day ripening period. The action of fungal lipase had apparently resulted in the increase of the fatty acid content observed during the sufu ripening period.

Changes of Acidity and pH of Sufu during Ripening. Figure 5 shows the changes of acidity and pH of tofu cubes during the ripening period. The tofu cubes showed an initial acidity and pH of 0.16% and 6.04, respectively. The acidity increased while the pH decreased as the ripening period extended. At the end of 16 days of ripening, the tofu cubes exhibited an acidity of 1.50%, while the pH dropped to 5.55. This phenomenon of increasing acidity and decreasing pH of tofu observed is in accordance with other fermented soybean products, such as miso, soy sauce, and tou-pan-chiang, that use *A. oryzae* as the starter organism during fermentation (*12*, *27*, *30*) Autolysis of microbial cells, the accumulation of free fatty acids, amino acids, and peptides containing carboxylic side chains as a result of hydrolysis of tofu constituents, and the microbial fermentation of carbohydrate (*31*) during the ripening period may all have led to the increased acidity and decline of pH observed in the present study. While probably because of the difference in the starter organism involved, this phenomenon is not consistent with the aging of the mold-fermented sufu (*11*, *25*). They all found that acidity and pH only changed slightly in sufu fermented by *Actinomucor elegans* or *Actinomucor taiwanensis* during the manufacture process. After 75 days of aging, this mold-fermented sufu showed an acidity and pH of 0.02% and 6.4−6.8, respectively (*25*).

Table 2. Change of Color during the Ripening of Sufu

	color		
fermentation time (days)	*L*	*a*	*b*
0	56.17 ± 5.83 A[a]	−0.30 ± 0.88 A	−0.30 ± 0.88 D
4	52.85 ± 3.75 A	−1.72 ± 0.49 B	−0.84 ± 1.05 D
8	53.57 ± 2.37 A	−1.23 ± 0.86 B	1.11 ± 0.82 C
12	53.04 ± 2.69 A	−1.16 ± 0.93 B	3.84 ± 0.86 B
16	52.17 ± 4.02 A	−0.19 ± 0.88 A	5.89 ± 0.88 A

[a] Values are expressed as means ± SD ($n = 3$). Means in the same column with different uppercase letters (A, B, C, D, and E) are significantly different by Duncan's multiple range test ($p < 0.05$).

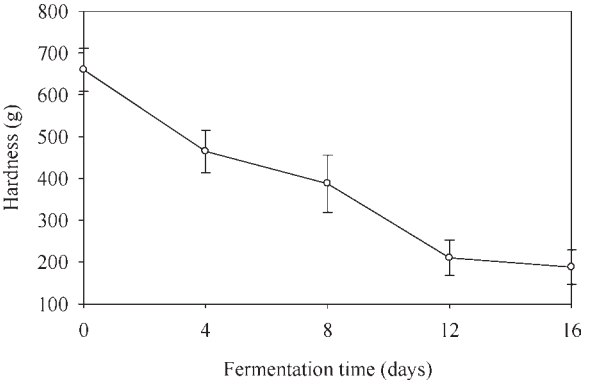

Figure 6. Change of hardness during the ripening of sufu.

Color Change of the Sufu Cube during the Ripening Period. Table 2 shows the change in the color of tofu cubes during the ripening period. Positive *L* values signify that color is approaching white, while negative *L* values indicate that the color is approaching black. A positive *a* value signifies red, whereas a negative *a* value signifies green. Positive and negative *b* values signify yellow and blue, respectively (*21*). As shown in **Table 2**, the value of *L* decreased while values of *a* and *b* increased during the ripening period. It was found that the color of tofu cubes changed from pale yellow to yellowish brown after 16 days of fermentation. The change in the color observed on the tofu cubes may be attributed to the result of the enzymatic and non-enzymatic browning reactions that occurred during the ripening period (*32*).

Change of Hardness during Ripening. Han et al. (*11*) proposed that hardness could be used to judge the extent of the ripening of sufu. They observed that, during the ripening period, the hardness of the mold-fermented sufu decreased, which varied with dressing mixture composition. A reduction in the hardness of sufu was also noted by Hwan and Chou (*25*) during the aging of the mold-fermented sufu prepared with *A. taiwanensis* or *A. elegans*. They also found that the kind of starter organism in addition to the aging period affected the hardness of the sufu products prepared. As shown in **Figure 6**, a continuous reduction in the hardness of the enzyme-ripening sufu was also observed during the ripening period. Hardness of the sufu reduced from ca. 659.6 to 187.6 g after 16 day of ripening. The decline in the hardness of sufu during the ripening period may be attributed to the degradation of tofu components caused by the action of hydrolytic enzymes.

This is the first scientific report concerning the biochemical and physical changes of sufu prepared with the enzyme-ripening method. This study demonstrated the behavior of hydrolytic enzymes that leached out from the *A. oryzae* prepared rice−soybean koji into the ripening infusion and tofu cubes during the manufacture process. It also showed that the rate of the hydrolytic action of the enzymes, especially the protease, in

this sufu manufacture process is higher than that in the mold-fermented sufu manufacture process using phetze as the source of enzymes. This finding explains why less processing time is required for the preparation of sufu with the enzyme-ripening method. Furthermore, the information provided in this paper can serve as the basis for future improvements in the manufacturing process and quality of sufu.

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Received for review December 31, 2009. Revised manuscript received February 17, 2010. Accepted February 18, 2010. This research was financially supported by The National Science Council, Republic of China (Taiwan), NSC 97-2313-B-002-015-MY3.