JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2005,** *53,* 1521−1525 **1521**

Effects of Temperature and Sodium Chloride Concentration on the Activities of Proteases and Amylases in Soy Sauce Koji

NAN-WEI SU,† MEI-LING WANG,§ KAM-FU KWOK,† AND MIN-HSIUNG LEE*,†

Department of Agricultural Chemistry, National Taiwan University, Taipei 106, Taiwan, and Department of Food Science and Technology, Chia Nan University of Pharmacy and Science, Tainan 717, Taiwan

This study investigated the effects of temperature and sodium chloride concentration on the proteolytic and amylolytic activities of soy sauce koji. The optimal temperatures for both protease and amylase were found in the range of 50−55 °C. The protease was not stable at 55 °C and retained only ∼20% residual activity after incubation at 55 °C for 4 h. The protease was labile in sodium chloride solution, whereas the amylase was quite stable. The residual protease activity in an 18% NaCl solution was only ∼3%. The harvested koji was mixed with 1.5 volumes of water (v/w) and incubated at 45 °C for 48 h; the total nitrogen and amino nitrogen contents were 1.3 and 0.56%, respectively. The results indicated that the hydrolysis of koji at the critical temperature of 45 °C could be employed as a rapid fermentation method to reduce the time for soy sauce manufacturing. According to this study, the combination of 5% sodium chloride and fermentation at 45 °C was considered as the best condition for the prohydrolysis of koji for making soy sauce. In addition, the critical temperature of 45 °C was very important when used in the preparation of protein hydrolysates for the flavoring industry and for the preparation of biologically active peptides.

KEYWORDS: Soy sauce; protease; amylase; total nitrogen; amino nitrogen

INTRODUCTION

Soy sauce is a traditional fermentation product of Asia. Its main ingredients are salt and protein hydrolysates (amino acids and peptides). In the production of soy sauce, steamed defatted soybean flakes and baked wheat grains are used as the main starting materials. The starting materials are then well mixed and inoculated with ∼0.1% of *Aspergillus oryzae* and/or *Aspergillus sojae* to make a soy sauce koji. After incubation at 25−30 °C for ∼2−3 days, the koji is mixed with 1.2−1.5 volumes of 22−23% saline to make a soy sauce mash with a final sodium chloride concentration of 16−18%, followed by aging at room temperature for about half a year. The soy sauce mash is then pressed to obtain the raw soy sauce. During the aging period, the proteins and polysaccharides of the starting materials are hydrolyzed by the fungal protease and amylase, and the unique soy sauce flavor is artfully developed. The total nitrogen (TN) and amino-type nitrogen (AN) contents are generally used as the quality indices for the soy sauce products. According to the national standard of Taiwan, the first-grade soy sauce products should contain >1.4% TN and >0.56% AN (*1*). In Japan, the Japanese Agricultural Standard (JAS) specifies three grades of soy sauce: special, upper, and standard. The most popular type of soy sauce in Japan is the koikuchi soy sauce of special grade (*2*). According to the JAS, the TN values of special grade, upper grade, and standard grade should be more than 1.5, 1.35, and 1.2% (w/v), respectively (*3*). Recently, the fermentation techniques for soy sauce production have been improved very quickly. By means of the appropriate technique for treating the soy sauce raw materials, the total nitrogen utilization ratio (TNUR) has been raised to >90%. However, the long aging time is still a disadvantage in soy sauce manufacturing. This drawback is due to the high concentration of sodium chloride in the mash. The high concentration of sodium chloride inhibits the protease activity very significantly and, subsequently, prolongs the fermentation time required for soy sauce production. To remedy the above-mentioned problems, several methods have been tried, such as using extruded raw materials for soy sauce manufacturing (*4*, *5*), screening a novel soy sauce koji mold that could produce the salt-tolerant protease (*6*, *7*), rapid fermentation at an elevated temperature for 2−3 days after the addition of sodium chloride to make the soy sauce mash (*8*−*13*), and/or using the combinations of various NaCl concentrations and ethanol in soy sauce fermentation to prevent microbial contamination (*14*). However, none of these methods produced any satisfactory results, particularly with regard to flavor and microbial contamination. Additionally, up to now, there were no data concerning the effects of sodium chloride concentration on the proteolytic and amylolytic activities of soy sauce koji. Therefore, the objective of this study was to investigate the effects of temperature and sodium chloride

* Author to whom correspondence should be addressed (telephone +886-2-23630231, ext. 2490; fax +886-2-23632714; e-mail mhlee@ntu.edu.tw).
† National Taiwan University.
§ Chia Nan University.

10.1021/jf0486390 CCC: $30.25
Published on Web 02/10/2005

concentration on the hydrolytic activities of koji protease and amylase and the effects of rapid fermentation at an elevated temperature on the protein hydrolysis of raw materials. The results of this study would be useful for improving the method of soy sauce fermentation and effectively reducing the time needed for the industrial production of soy sauce by a suitable incorporation of lactobacteria and/or yeasts. Recently, food scientists have been very concerned with the HVP produced by HCl hydrolysis, because it produced the harmful carcinogenic monochloropropanediol and dichloropropanol (*15*−*17*). Therefore, many food chemists are urgently conducting research on the enzymatically hydrolyzed vegetable proteins. Meanwhile, many scientists recently reported a lot of peptides with biological functions, such as opiate, anti-opiate, ACE inhibitor, inhibition of platelet aggregation, and immunostimulant (*18*). Therefore, the basic data in this study would be very useful in the field of protein hrydrolysates and peptides.

MATERIALS AND METHODS

Chemicals. Glucose, 3,5-dinitrosalicylic acid (DNS), and tyrosine were obtained from Sigma Chemical Co. (St. Louis, MO). Rochelle salt was obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Formaldehyde, sodium chloride, sodium hydroxide, silver nitrate, potassium sulfate, cupric sulfate, boric acid, and phosphoric acid were obtained from Merck Co. (Darmstadt, Germany). All above chemicals used in this study were purchased from their local agencies and were of reagent grade.

Koji Mold and Preparation of Spore Inoculum. An *Aspergillus oryzae* strain provided by a local soy sauce factory was employed in this study and was maintained with a potato−dextrose agar (PDA; Difco Laboratories, Detroit, MI) containing 0.9% (w/v) sodium chloride. The spore suspension was prepared by adding 10 mL of sterilized distilled water into a slant tube (diameter = 16 mm) containing a fully grown mycelial mat on saline PDA medium and mixing vigorously. The solid fermentation substrate was prepared by mixing 100 g of defatted soybean flakes with 120 mL of water and autoclaving at 1.2 kg/cm^2 for 20 min, followed by mixing with 100 g of slightly ground roasted wheat. The substrate was then inoculated with 5 mL of spore suspension and mixed thoroughly. The mixture was then transferred to a perforated stainless steel tray and cultured at a controlled temperature of 30 °C and a relative humidity of 92% for 2−3 days. When a green-yellow mass appeared as a result of mold growth and sporulation, the culture was transferred to a hot-air oven maintained at 40 °C for 6 days. Subsequently, the dried culture was blended as spore inoculum and stored in a tightly sealed plastic bottle at 4 °C until use.

Preparation of Koji by Solid-State Fermentations. One kilogram of defatted soybean flakes mixed with 1.2 L of distilled water was autoclaved and then combined with 1 kg of slightly ground roasted wheat to prepare the koji medium. The medium was cooled to room temperature, inoculated with 0.1% (w/w) of spore inoculum, and subsequently dispersed onto several perforated stainless steel trays. Each tray was loaded with fermenting koji to an ∼3-cm thickness and incubated first at 30 °C for 36 h and then at 25 °C for 30 h. During the koji making, the internal temperature of the koji was always kept below 40 °C by occasional turning. The preparation of koji was completed in ∼60−72 h when the culture began turning greenish yellow in color.

Preparation of Crude Enzyme Extracts from Koji and Determination of Proteolytic and Amylolytic Activities. The harvested koji was immediately processed to prepare the enzyme extracts according to the following method modified from that of Hirose (*19*). One hundred grams of koji was mixed with 2 L of distilled water containing 0.9% sodium chloride. The mixture was left standing at ambient temperature for 4 h with occasional stirring and then centrifuged at 5000*g* for 20 min. The supernatant was assayed for proteolytic and amylolytic activities. Protease activity was assayed according to a method modified from that of Anson (*20*), using 1.5% Hammarsten-milk casein (Merck) containing different NaCl concentrations (*6*) as the substrate (dissolved in 100 mL of 0.1 M, pH 7.0, phosphate buffer). A standard tyrosine solution (in 0.1 M, pH 7.0, phosphate buffer) was used to make a calibrated curve for quantitative analysis. One unit of protease activity was defined as the enzyme needed for the release of 1 μg of tyrosine from 1.5% casein solution per minute at 30 °C. The amylase activity assay was conducted according to the DNS method (*21*). One milliliter of 1% soluble starch (1 g of soluble starch dissolved in 50 mL of distilled water followed by the addition of 0.1 M phosphate buffer, pH 6.5, to 100 mL) was added into a test tube and incubated for 5 min in a 40 °C water bath. When NaCl was considered as a variable, different amounts of NaCl were contained in the substrate mixture. One milliliter of properly diluted enzyme solution was then added to the substrate and incubated at 40 °C for exactly 3 min. Thereafter, 2 mL of DNS reagent (1 g of DNS dissolved in 20 mL of 2 N NaOH and 50 mL of distilled water at room temperature, followed by adding 30 g of Rochelle salt and quantitated to 100 mL with distilled water) was added and incubated in boiling water for 5 min to colorize. After cooling with cold water, distilled water was added to the reaction mixture to a volume of 20 mL, and the OD_{540} was measured by using distilled water as a blank control. One unit of amylase activity was defined as the enzyme needed to release 1 μmol of glucose per minute at 40 °C. The 0−10 μmol/mL glucose solutions were used to make the standard curve.

Analytical Methods. Total nitrogen was determined according to the AOAC method (*22*) with a little modification. Five milliliters of the sample, 1 g of catalyst ($K_2SO_4/CuSO_4 \cdot H_2O$ = 10:1), and 15 mL of concentrated sulfuric acid were added into a digestion tube and then heated at 350 °C for digestion. When the digestion was completed, the sample was mixed with 10 mL of 30% NaOH solution and subjected to distillation. The distillate was absorbed with 50 mL of a 4% boric acid solution. The TN was determined by back-titrating the boric acid solution with 0.2 N sulfuric acid. Formol nitrogen (FN) and ammonium nitrogen were determined according to the Chinese National Standard methods (*23*). Sodium chloride was determined by using a silver nitrate titration method modified from the AOAC method (*24*). A 0.5 mL of a 10% potassium chromate solution was added into a 250-mL flask containing 50 mL of a properly diluted sample. The sample was titrated with a 0.1 N of silver nitrate solution (made by dissolving 4.25 g of $AgNO_3$ in 250 mL of double distilled H_2O) until the appearance of a brownish color. The standard curve of NaCl was prepared with 0.1−0.5% (w/v) of NaCl solutions. AN was determined by subtraction of ammonium nitrogen from FN according to the Chinese National Standard methods (*19*). Degree of hydrolysis (DH) was defined as the percent ratio of FN to TN. Total nitrogen utilization ratio (TNUR) was calculated by using NaCl as the quantitative internal standard according to the following equation:

$$\text{TNUR (\%)} = \frac{\text{TN of soy sauce/NaCl concn in soy sauce}}{\text{total amount of N used in starting material/ total amount of NaCl used in starting material}} \times 100\%$$

Data Analytical. All results were analyzed by analysis of variance (ANOVA) using the general linear model. Duncan's multiple-range test was used to determine differences among the samples. Significant levels were defined as probabilities of 0.05 or less. All processing treatments were carried out in triplicate.

RESULTS

Effect of Temperature on the Activities of Crude Enzyme Extracts from Koji. The relationship between reaction temperature and proteolytic and amylolytic activities of crude enzyme extracts from soy sauce koji are shown in **Table 1**. The best proteolytic activity appeared at ∼50−55 °C with a proteolytic activity of ∼1150 units/g of koji. When the temperature exceeded 60 °C, the proteolytic activity dropped significantly. The highest activity of amylase also appeared at ∼50−55 °C with an activity of ∼195 units/g of koji. When the temperature exceeded 65 °C, the activity decreased significantly. The thermal stability tests of the crude enzyme extracts of koji were conducted by incubating the extracts at 45 and 55 °C for

Table 1. Effects of Reaction Temperature on Proteolytic and Amylolytic Activities of Crude Enzyme Extract from Soy Sauce Koji[a]

temp (°C)	proteolytic activity (units/g of koji)	amylolytic activity (units/g of koji)
30	379 ± 9	112 ± 5
35	488 ± 16	140 ± 6
40	703 ± 12	146 ± 5
45	947 ± 11	172 ± 4
50	1150 ± 16	195 ± 4
55	1150 ± 14	193 ± 5
60	873 ± 18	185 ± 4
65	99 ± 4	122 ± 3
70	85 ± 3	51 ± 2

[a] Data are expressed as mean ± standard deviation.

Table 2. Effects of Incubation Time and Temperature on Protease and Amylase Activities of Crude Enzyme Extract from Soy Sauce Koji[a]

temp (°C)	time (min)	proteolytic activity (units/g of koji)	amylolytic activity (units/g of koji)
30 (control)		380 ± 8 (100)[b]	115 ± 4 (100)
45	10	359 ± 8 (95)	115 ± 4 (100)
	20	316 ± 7 (84)	114 ± 5 (100)
	40	301 ± 7 (80)	112 ± 5 (98)
	120	277 ± 6 (73)	108 ± 6 (94)
	240	260 ± 5 (69)	105 ± 5 (91)
55	10	265 ± 6 (70)	107 ± 3 (93)
	20	146 ± 4 (39)	105 ± 4 (91)
	40	115 ± 4 (30)	93 ± 3 (81)
	120	85 ± 2 (22)	68 ± 4 (59)
	240	63 ± 3 (17)	54 ± 4 (47)

[a] Data are expressed as mean ± standard deviation. [b] Numbers in parentheses are the relative percentage of residual activity.

0−240 min, and the proteolytic and amylolytic activities were determined at various intervals. The results are shown in **Table 2**. When the crude enzyme extracts were treated at 45 °C for 240 min, 70% of the proteolytic activity remained, whereas after incubation at 55 °C for 240 min, there was only 17% of proteolytic activity left. In the case of amylolytic activity, when treated at 45 °C for 240 min, there was 90% of activity left, whereas after treatment at 55 °C for 240 min, there was only 47% of activity remaining. These results showed that the protease was more sensitive to heat as compared to the amylase.

Although 55 °C was the optimal proteolytic temperature for koji protease(s), the thermal stability at 55 °C was not satisfactory. In consideration of both the effect of temperature on enzyme activity and the thermal stability of the enzyme, the temperature of 45 °C was considered to be more suitable for rapid fermentation (warm-brewing).

Effect of NaCl Concentration on the Activities of Crude Enzyme Extracts. The effects of sodium chloride concentration on proteolytic and amylolytic activities of crude enzyme extracts from soy sauce koji are shown in **Table 3**. The concentration of sodium chloride affected protease activity very significantly. When the reaction mixture contained 5% sodium chloride, the proteolytic activity was repressed to ~60%. When the concentration of sodium chloride increased to 18%, the protease activity was reduced to 3%. Under the same conditions, the effect of NaCl concentration on amylase was much smaller. When the concentration of sodium chloride was 18%, there was still ~70% of amylase activity left.

Effects of Temperature on the General Composition of Soy Sauce Koji Hydrolysate during Rapid Fermentation (Warm-Brewing) of Koji. The harvested koji was mixed with 1.5 volumes of water based on the weight of raw materials to make a mash and then incubated, respectively, at 45 or 55 °C for 24 or 48 h with occasional stirring to hydrolyze the koji. The resulting hydrolysate was centrifuged at 5000*g* for 20 min. The characteristics of the supernatant including TN, FN, AN, DH, TNUR, reducing sugar, and pH were measured. As shown in **Table 4**, the koji hydrolysate obtained from rapid fermentation at 45 °C had the higher formol nitrogen, amino nitrogen, degree of protein hydrolysis, and total nitrogen utilization rate than those obtained at 55 °C. After 48 h of rapid fermentation, the degree of protein hydrolysis, total nitrogen utilization rate, reducing sugar content, and pH of the hydrolysate were about 50%, 81%, 3.24%, and 5.4, respectively. The degree of hydrolysis and the total nitrogen utilization rate of the mash incubated at 55 °C for 48 h were about 40% and 75%, respectively. Although the total nitrogen content was a little higher in the hydrolysate incubated at 55 °C for 48 h, the contents of formol nitrogen and amino nitrogen were both lower than those incubated at 45 °C for 48 h. These results indicated that rapid fermentation at 45 °C could obtain higher amounts of amino acids and short-chain oligopeptides in koji hydrolysate and consequently could offer better flavor-enhancing contribution.

Table 3. Effects of Sodium Chloride Concentration on the Proteolytic and Amylolytic Activities of Crude Enzyme Extract from Soy Sauce Koji[a]

NaCl (%)	proteolytic activity (units/g of koji)	amylolytic activity (units/g of koji)
0	379 ± 5 (100)[b]	145 ± 3 (100)
5	145 ± 3 (38)	142 ± 3 (99)
10	65 ± 3 (17)	127 ± 4 (87)
14	39 ± 3 (10)	114 ± 5 (78)
18	13 ± 3 (3)	104 ± 3 (72)

[a] Data are expressed as mean ± standard deviation. [b] Numbers in parentheses are the relative percentage of residual activity.

Effects of NaCl Concentration on the General Composition of Soy Sauce Koji Hydrolysate during Rapid Fermentation (Warm-Brewing) of Koji. The methods for making mash were similar to those described in the above paragraph. The only difference was using various sodium chloride solutions to replace the water to make a final NaCl concentration in mash of 5, 10, 14, or 18%. Moreover, 50 °C was employed instead of 55 °C because of the unsatisfactory results shown in **Table 4**. Two groups of mash with the above various NaCl concentrations were separately incubated at 45 and 50 °C for 48 h. The results are shown in **Table 5**. When incubated at 45 °C, the TNURs of the koji hydrolysates with 5 and 10% NaCl were ~80%, and the DHs were 54.3 and 50.1%, respectively. When the salt concentrations were increased to 14 and 18%, the TNURs were significantly reduced to 75.5 and 72.4%, respectively, and DHs were decreased to 46.3 and 44.7% in that order. When considering the prevention of deterioration of koji during rapid fermentation, we proposed that the rapid fermentation of soy sauce koji at 45 °C with 5% NaCl concentration was applicable for the industry. In the case of incubation at 55 °C, the results were all unsatisfactory.

DISCUSSION

The main flavoring ingredients in soy sauce are amino acids and short-chain peptides derived from the protein of soy sauce raw materials through the hydrolysis of koji during mash fermentation periods. For this reason, the proteolytic activity of koji is very important in the process of soy sauce making.

Table 4. Effects of Warm-Brewing Temperature and Time on the General Composition of Soy Sauce Koji Hydrolysate[a]

temp (°C)	time (h)	TN[b] (%)	FN (%)	AN (%)	DH (%)	TNUR (%)	reducing sugar[c] (%)	pH
original koji	0	1.04 ± 0.03 c	0.44 ± 0.02 d	0.35 ± 0.01 e	43.0 ± 0.5 c	59.6 ± 0.6 d	6.20 ± 0.07 a	5.70 ± 0.13 b
45	24	1.26 ± 0.04 b	0.60 ± 0.02 ab	0.50 ± 0.01 b	48.0 ± 1.1 b	75.8 ± 1.0 b	3.18 ± 0.06 f	5.55 ± 0.06 b
	48	1.30 ± 0.04 ab	0.65 ± 0.03 a	0.56 ± 0.02 a	50.1 ± 0.9 a	81.3 ± 1.2 a	3.24 ± 0.08 ef	5.36 ± 0.05 c
55	24	1.34 ± 0.03 a	0.53 ± 0.02 c	0.48 ± 0.01cd	39.6 ± 0.9 d	73.6 ± 0.8 b	3.59 ± 0.08 d	6.01 ± 0.11 a
	48	1.32 ± 0.02 ab	0.54 ± 0.04 bc	0.46 ± 0.01 d	40.9 ± 1.0 c	75.4 ± 0.9 b	3.46 ± 0.06 de	5.11 ± 0.17 d

[a] Data are expressed as mean ± standard deviation. Different letters in the same column indicate significant difference ($p < 0.05$). [b] TN, FN, AN, DH, and TNUR are the abbreviations of total nitrogen, formol nitrogen, amino nitrogen, degree of hydrolysis, and total nitrogen utilization rate, respectively. [c] Reducing sugar is expressed as glucose equivalent.

Table 5. Effects of Warm-Brewing Temperature and Sodium Chloride Concentration on the General Composition of Soy Sauce Koji Hydrolysate[a,b]

temp (°C)	NaCl (%)	TN[c] (%)	FN (%)	AN (%)	DH (%)	TNUR (%)	reducing sugar[d] (%)	pH
45	5	1.27 ± 0.05 a	0.69 ± 0.03 a	0.59 ± 0.04 a	54.3 ± 0.4 a	79.9 ± 1.8 a	3.14 ± 0.05 b	5.40 ± 0.10 b
	10	1.22 ± 0.04 b	0.61 ± 0.02 b	0.54 ± 0.03 ab	50.1 ± 0.6 b	80.5 ± 1.3 a	3.24 ± 0.08 b	5.91 ± 0.08 a
	14	1.21 ± 0.04 b	0.56 ± 0.02 c	0.50 ± 0.02 b	46.3 ± 1.4 c	75.5 ± 0.9 b	3.25 ± 0.04 b	5.88 ± 0.08 a
	18	1.12 ± 0.03 c	0.50 ± 0.03 d	0.44 ± 0.03 c	44.7 ± 0.9 cd	72.4 ± 0.9 c	3.27 ± 0.06 b	5.89 ± 0.11 a
50	5	1.23 ± 0.03 ab	0.56 ± 0.02 c	0.48 ± 0.03 b	45.5 ± 1.1 c	75.2 ± 1.1 b	3.51 ± 0.03 a	5.94 ± 0.07 a
	10	1.18 ± 0.03 bc	0.55 ± 0.02 cd	0.47 ± 0.04 b	46.6 ± 0.9 c	72.7 ± 1.5 c	3.44 ± 0.04 a	5.92 ± 0.12 a
	14	1.09 ± 0.06 c	0.49 ± 0.03 d	0.43 ± 0.02 c	45.0 ± 0.6 cd	71.8 ± 0.8 c	3.25 ± 0.03 b	5.90 ± 0.18 a
	18	1.08 ± 0.04 c	0.44 ± 0.03 e	0.38 ± 0.04 d	40.7 ± 0.5 e	68.0 ± 0.9 d	3.34 ± 0.05 ab	5.88 ± 0.11 a

[a] The warm-brewing was carried out for 48 h. [b] Data are expressed as mean ± standard deviation. Different letters in the same column indicate significant difference ($p < 0.05$). [c] TN, FN, AN, DH, and TNUR are the abbreviations of total nitrogen, formol nitrogen, amino nitrogen, degree of hydrolysis, and total nitrogen utilization rate, respectively. [d] Reducing sugar is expressed as glucose equivalent.

Many researchers have studied the fractionation and purification of proteases from koji molds, classified the purified proteases as alkaline, neutral, and acid types according to their optimal pH, and demonstrated their roles in the manufacture of soy sauce (*25*−*29*). However, all of these protease activities declined significantly when the harvested koji was mixed with a high level of sodium chloride (∼18% NaCl) solution to make a mash. The high concentration of NaCl retarded the proteolytic hydrolysis of soy protein and prolonged the overall maturation time for soy sauce production. Many alternative soy sauce making methods such as autolysis of soy sauce koji at high temperature have been investigated in Japan (*9*−*13*). There have been some reports that the TN of the hydrolysate could be increased (*10*, *11*, *13*); however, most of these researches were concentrating on the glutaminase activities of koji (*8*) and prevention of microbial contamination (*8*, *10*−*12*, *14*). Some papers have mentioned that the protease activity in soy sauce koji was inhibited by the sodium chloride (*25*−*28*). However, up to now, no exact numerical data have been reported. In this study, we revealed the exact results to confirm the inhibition effects of NaCl on the proteolytic activity of koji. Many past studies had investigated the methods for elevating koji-mold protease activity, so as to promote the utilization efficiency of raw materials and to shorten the time for the maturation process. For example, Nakadai and Nasuno (*28*) and Sekine et al. (*30*) tried to obtain high proteolytic mutants from *A. sojae* and *A. oryzae* by traditional induced mutation and attempted to improve the proteolytic hydrolysis of raw material in soy sauce making. Moreover, Nakadai used the statistical estimation to illustrate the relationship between various proteases of harvested koji and the nitrogen utility of raw materials during soy sauce manufacturing (*31*). Nevertheless, to date, no satisfactory results have been applied to the practical use in soy sauce manufacture. According to our results, the proteolytic activity of harvested koji was indeed affected extensively by NaCl concentration. At 18% sodium chloride concentration (the general salt concentration of soy sauce mash), only 3% of the protease activity remained. The active proteases in koji were possibly salted out and denatured in such high NaCl concentration solutions. To dissolve this retarded hydrolysis problem of soy protein in mash, we tried an alternative method to effectively accelerate the proteolytic hydrolysis of the harvested koji by means of incubation at 45 °C for 48 h before the addition of salt needed to make a final concentration of 18% NaCl. According to our results, the quality indices, TN and AN, of koji hydrolysate were significantly increased from 1.04 to 1.30% and from 0.35 to 0.56% (**Table 4**), respectively. These data showed that warm-brewing at 45 °C enables the protease(s) of harvested koji to solubilize and hydrolyze the soy protein of koji efficiently. Meanwhile, the above TN and AN were very close to those of the first-grade soy sauce according to the national standard of Taiwan. Therefore, rapid fermentation of soy sauce koji at 45 °C for 48 h should be able to be employed as the preliminary process to reduce the time for soy sauce making. After the preliminary fermentation is properly carried out, the salt-tolerant yeast (e.g., *Zygosaccharomyces rouxii* and *Candida versatilis*) and/or the lactobacteria (e.g., *Pediococcus halophilus*) can be added to develop the unique flavor of soy sauce. Muramatsu et al. (*10*) used high-protein wheat and defatted soybeans as raw material to make koji and subjected it to autolysis at 55 °C for 48 h. Although they obtained hydrolysates with high TN of >1.6%, the FN/TN ratios were all <43.1%, which were not good for flavor quality. In another research, Muramatsu et al. (*11*) carried out an intermediate-scale fermentation test with an initial autolysis of koji at 55 °C for 48 h. The results indicated that an extra ∼8% of glucose should be added for the development of the unique soy sauce aroma. The TN and AN were not mentioned in this paper. Kitamura et al. (*12*) carried out a research concerning "short-term shoyu brewing from shoyu koji digested successively at low and high temperature and their systemization". The high-temperature fermentation was carried out at 45, 50, and 55 °C with a salt concentration of 13, 15, or 16%. The results indicated their TNs were of 1.19−1.37%. However, in all cases, the total nitrogen utilization rates were all relatively low (71.42−75.6%). Furthermore, there were no

numerical data concerning the effects of temperature and salt concentration on the enzymatic activities of the koji proteases and amylases in any of the papers.

In conclusion, it is clear from our results that the proteolytic activity of soy sauce koji was adversely affected by the addition of NaCl. A 5% NaCl solution reduced the proteolytic activity by 62%. The rapid fermentation of harvested koji at the optimal temperature of 45 °C for 48 h was a very useful method to promote the protease(s) of koji to solubilize and hydrolyze the soy protein efficiently. These data of this study would be very useful for the industrial application. We suggest that a preliminary rapid fermentation of soy sauce koji at 45 °C combined with a suitable NaCl concentration of <5% would be a satisfactory means to make soy sauce within a shorter time. The critical temperature of 45 °C would be also very useful for the preparation of protein hydrolysates and biologically active peptides.

LITERATURE CITED

(1) Chinese National Standard. *Soy Sauce*; 4235, N5006. Central Standard Bureau, Ministry of Economic Affairs, Executive Yuan: Taipei, Taiwan, 1994.

(2) Yokotsuka, T. Soy sauce biochemistry. *Adv. Food Res.* **1986**, *30*, 195−329.

(3) Japanese Agricultural Standard. *Shoyu* (revision); Ministry of Agriculture, Forestry and Fisheries: Tokyo, Japan, 1997.

(4) Chou, C. C.; Ling, M. Y. Biochemical changes in soy sauce prepared with extruded and traditional materials. *Food Res. Int.* **1998**, *31*, 487−492.

(5) Ling, M. Y.; Chou, C. C. Biochemical changes during the preparation of soy sauce koji with extruded and traditional raw materials. *Int. J. Food Sci. Technol.* **1996**, *31*, 511−517.

(6) Su, N. W.; Lee, M. H. Screening and characterization of koji molds producing saline-tolerant protease. *J. Ind. Microbiol. Biotechnol.* **2001**, *26*, 230−234.

(7) Su, N. W.; Lee, M. H. Purification and characterization of a novel salt-tolerant protease from *Aspergillus* sp. FC-10, a soy sauce koji mold. *J. Ind. Microbiol. Biotechnol.* **2001**, *26*, 253−258.

(8) Ohtsuki, H.; Hirakuri, Y.; Haara, T.; Kanematsu, Y.; Honkawa, Y. Studies on effect of brewing temperature in fermentation of shoyu mash. *J. Jpn. Soy Sauce Res. Inst.* **1981**, *7*, 260−264 (in Japanese).

(9) Yokotsuka, T.; Iwasa, T.; Fujii, S. Studies on high-temperature digestion of shoyu koji (part 1): (I) the role of glutaminase in shoyu brewing and (II) investigation for heat and salt resistant glutaminase producing microbes. *J. Jpn. Soy Sauce Res. Inst.* **1987**, *13*, 18−25 (in Japanese).

(10) Muramatsu, S.; Sano, Y.; Uzuka, Y. Selection of raw food materials for koji making to yield high nitrogen content in the hydrolyzate made by autodigestion at high temperature. *J. Brew. Soc. Jpn.* **1992**, *87*, 219−223 (in Japanese).

(11) Muramatsu, S.; Ito, N.; Sano, Y.; Uzuka, Y. Soy sauce making from soy sauce koji autolyzated at high temperature. *J. Brew. Soc. Jpn.* **1992**, *87*, 538−544 (in Japanese).

(12) Kitamura, E.; Eguchi, U. Short-term shoyu brewing from shoyu koji digested successively at low and high temperature and their systemization. *J. Brew. Soc. Jpn.* **1993**, *88*, 929−937 (in Japanese).

(13) Sano, Y.; Ito, N.; Muramatsu, S.; Uzuka, Y. Large-scale production of soy-sauce by the use of soy-sauce koji autolyzate prepared at high-temperature. *J. Jpn. Soc. Food Sci. Technol.* **1993**, *40*, 519−524 (in Japanese).

(14) Baba, H.; Baba, K.; Shimizu, T.; Kimura, E. Studies on the moromi mash of soy sauce (part 4): Fermentation of Exwb-40 as raw material under the condition of low NaCl concentration on the producing soy sauce containing high nitrogen. *J. Jpn. Soy Sauce Res. Inst.* **1983**, *9*, 24−28 (in Japanese).

(15) Jones, A. R. The antifertility actions of α-chlorohydrin in the male. *Life Sci.* **1978**, *23*, 1625−1646.

(16) Morris, I. D.; Williams, L. M. Some preliminary observations of the nephrotoxicity of the male antifertility drug (±)α-chlorohydrin. *J. Pharm. Pharmacol.* **1980**, *32*, 35−38.

(17) Weteburger, E. K.; Ultand, B. M.; Nam, J.-M.; John, J. G.; John, H. W. Carcinogenicity tests of certain environmental and industrial chemicals. *J. Natl. Cancer Inst.* **1981**, *67*, 75−88.

(18) Linden, G.; Lorient, D. Chapter 14, Amino acids and peptides. In *New Ingredients in Food Processing*; Woodhead Publishing: Cambridge, U.K., 1999.

(19) Hirose, Y. Analytical method of soy sauce. In *Experimental Method of Soy Sauce*; Japan Soy Sauce Research Institute: Tokyo, Japan, 1985; p 286 (in Japanese).

(20) Anson, M. L. The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. *J. Gen. Physiol.* **1938**, *22*, 79−89.

(21) Bernfeld, P. *Methods in Enzymology*; Colowick, S. P., Kaplan, N. O., Eds.; Academic Press: New York, 1955; Vol 1, pp 149.

(22) AOAC. *Official Methods of Analysis*, 14th ed.; Sidney, W., Ed.; Association of Official Analytical Chemists: Washington, DC, 1984; method 30.049.

(23) Chinese National Standard. *Methods of Test for Soy Sauce*; 995, N6008; Central Standard Bureau, Ministry of Economic Affairs, Executive Yuan: Taipei, Taiwan, 1976.

(24) AOAC. *Official Methods of Analysis*, 14th ed.; Sidney, W., Ed.; Association of Official Analytical Chemists: Washington, DC, 1984; method 32.034.

(25) Nakadai, T.; Nasuno, S.; Iguchi, N. Purification and properties of alkaline proteinase from *Aspergillus oryzae*. *Agric. Biol. Chem.* **1973**, *37*, 2685−2694.

(26) Nakadai, T.; Nasuno, S.; Iguchi, N. Purification and properties of neutral proteinase II from *Aspergillus oryzae*. *Agric. Biol. Chem.* **1973**, *37*, 2703−2708.

(27) Impoolsup, A.; Bhumiratana, A.; Flegel, T. W. Isolation of alkaline and neutral proteases from *Aspergillus flavus* var. *colmnaris*, a soy sauce koji mold. *Appl. Environ. Microbiol.* **1981**, *42*, 619−628.

(28) Nakadai, T.; Nasuno, S. Hyperproduction of peptidase and proteinase by mutants of *Aspergillus oryzae* [enzymes for digestion of soybean proteins to produce good quality soy sauce]. *J. Ferment. Technol.* **1977**, *55*, 273−276.

(29) Nasuno, S.; Ohara, T. Comparison of alkaline proteinase from hyperproductive mutants and parent strain of *Aspergillus sojae*. *Agric. Biol. Chem.* **1971**, *35*, 836−843.

(30) Sekine, H.; Nasuno, S.; Iguchi, N. Isolation of highly proteolytic mutants from *Aspergillus sojae*. *Agric. Biol. Chem.* **1969**, *33*, 1477−1482.

(31) Nakadai, T. Statistical estimation of key-enzymes determining the nitrogen utility of soy sauce and total nitrogen of pressed residue. *J. Jpn. Soy Sauce Res. Inst.* **1984**, *10*, 93−96 (in Japanese).

Received for review August 13, 2004. Revised manuscript received December 26, 2004. Accepted January 5, 2005. We are very thankful for the financial support provided by the National Science Council of the Republic of China (Taiwan) through Grant NSC 88-2313-B-002-111.

JF0486390