

# ACCELERATED PROTEOLYSIS OF SOY PROTEINS DURING FERMENTATION OF THUA-NAO INOCULATED WITH *BACILLUS SUBTILIS*

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## Abstract

*Thua-nao is a traditional Thai-fermented soy product. It was prepared by the conventional method and also with starter inoculation. Inoculated soybean exhibited a higher rate of fermentation than did the natural fermentation as indicated by greater rate of pH increase, higher extent of proteolysis, ammonia-nitrogen content and nitrogen solubility. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns indicated the extensive degradation of soy proteins during fermentation. Activity staining of Thua-nao extract suggested that proteolysis of soybean was mediated by various proteinases. Active proteinases with estimated molecular weight (MW) of 29,000, 27,000 and 19,000 Da were present during natural fermentation of soybeans. However, proteinases with MW of 40,000 and 29,000 Da were predominant in inoculated soybeans and like the activity bands observed with spent culture broth of Bacillus subtilis BIOTEC 7123. The results suggested that proteinases released by the dominant species in the inoculum, especially B. subtilis, play an important role in proteolysis of soy proteins during fermentation. Inoculation with B. subtilis resulted in an increased proteolysis, which is useful for subsequent reactions leading to development of Thua-nao characteristics.*

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## INTRODUCTION

Thua-nao is a traditional fermented soy product widely consumed in Northern Thailand. Because of its unique meat-like flavor and taste, Thua-nao is mainly used as a condiment in northern Thai cuisine. Thua-nao is generally produced as a home-based practice in which the fermentation techniques have been passed from one generation to another. Traditionally, the manufacturing process generally consists of soaking, boiling and fermenting. Soybean is soaked in tap water overnight and boiled in aluminum pots over wood fires for approximately 7 h to soften the seeds. After boiling, the water is drained off and the seeds are transferred to woven bamboo baskets. Then, cooked beans are covered with leaves and incubated at room temperature. The traditional fermentation is generally complete within 3 days, when the fermented soybeans are covered with a slightly sticky, brownish layer of bacteria and have a unique flavor with a noticeable odor of ammonia (Sundhagul *et al.* 1972; Leejeerajumnean *et al.* 2001).

Thua-nao has increasingly gained interest in food applications because of its possible nutritional and health benefits and the potential use as a food-flavoring agent. Because traditional Thua-nao fermentation mainly relies on adventitious microorganisms, an inconsistency of product quality is a primary limitation. *Bacillus subtilis*, a Gram-positive, endospore-forming bacteria, has usually been found as the predominant microorganism in Thua-nao fermentation (Sundhagul *et al.* 1972; Leejeerajumnean *et al.* 2001). To improve product uniformity, a pure starter culture was developed by Sundhagul *et al.* (1973) based on the ability to ferment and the sensory quality of the final product. Among the selected cultures, *B. subtilis* TISTR10 (presently BIOTEC 7123) has been shown to be one of the effective strains as Thua-nao starter culture.

Enzymatic degradation of proteins is an important process in fermentation of foods containing high protein content especially milk (Bockelman 1995), meat (Molly *et al.* 1997) and legume seeds (Odunfa 1985). Proteolysis mainly contributes to product attributes including texture, appearance, as well as characteristic aroma and taste of fermented products. In addition to Thua-nao, other soy products of *B. subtilis* fermentation include Japanese natto and kinema made in Nepal and Sikkim (Sarkar *et al.* 1993). Unlike other indigenous fermented soy foods, e.g., soy sauce, natto, Indonesian tempeh and Javanese oncom, which have been extensively studied, only a few studies on Thua-nao have been carried out (Sundhagul *et al.* 1972; Leejeerajumnean *et al.* 2001). Nevertheless, basic information related to biochemical and chemical changes, particularly proteolysis during fermentation of Thua-nao and other *B. subtilis* fermented soy products, has not been reported. The objective of this study was to investigate the changes in soy proteins during fermentation

of Thua-nao fermented with and without inoculation of *B. subtilis* BIOTEC 7123.

## MATERIALS AND METHODS

### Preparation of Starter Culture

Pure culture of *B. subtilis* BIOTEC 7123 was obtained from the BIOTEC Culture Collection, Bangkok, Thailand. Freeze-dried culture was streaked onto the nutrient agar (NA) plate and incubated at 37C for 15 h. To maintain culture throughout the study, a single colony was subcultured onto NA slants, and stored at 4C. Inoculum was prepared by transferring a loop full of microorganisms from a slant culture into 50 mL of sterile nutrient broth. The culture was then incubated for 24 h at 37C with shaking at 150 rpm in an orbital shaker. Cells were harvested by centrifugation at 4C at  $10,300 \times g$  for 5 min using an Eppendorf refrigerated centrifuge 5403 (Eppendorf, Hamburg, Germany) and washed with 5 mL of sterile deionized water. Finally, the cells were resuspended in sterile distilled water to obtain an approximate cell concentration of  $1 \times 10^7$  cfu/mL.

### Preparation of Thua-nao

Dry soybean (*Glycine max*) was purchased from a local market in Mae Hong Sorn, Thailand. Soybean was washed and soaked overnight (~12 h) with clean water at the ratio of 1:10 (w/v). The soaked soybean was boiled for 4 h to soften the seeds. For conventional fermentation (Control), cooked soybean (500 g) was then transferred to a sterile screener overlaid with two layers of sterile cheesecloth. The screener was then covered on top with two layers of sterile cheesecloth. To prevent the excessive evaporation, a polyethylene sheet was placed on the top before incubation at 37C. For pure culture fermentation, cooked beans were further sterilized by autoclaving for 20 min at 121C. The liquid that occurred was drained. Sterile cooked beans were transferred into sterile aluminum bowl and inoculated with 9 mL of the bacterial suspension to obtain approximate cell concentrations of  $10^2$  cfu/g (BS102) or  $10^4$  cfu/g (BS104). Inoculated soybean was transferred into a sterile screener and incubated as previously described. During fermentation, soybean samples were taken at 6, 12, 18, 24, 36 and 48 h for microbiological and chemical analyses.

### Microbiological Analyses

Sample (25 g) was aseptically transferred to a sterile plastic bag and pummeled for 1 min at 200 rpm in a stomacher (IUL Instrument, Barcelona, Spain), with 225 mL of 0.1% sterile peptone water. Appropriate decimal

dilutions of the suspension were prepared using the same diluent and 0.1 mL of each dilution was plated in triplicate on different growth media. The following media and incubation conditions were used: (1) plate count agar (Oxoid Ltd., Hampshire, England) incubated at 37C for 18–24 h for total viable count (TVC) and (2) yeast malt agar pH 3.5 incubated at 25C for 3–4 days for yeast and mold count (YM). Spore counts (SC) were determined using the suspensions heated at 85C for 20 min on plate count agar incubated at 37C for 18–24 h.

### Chemical Analyses

The fermented soybean was ground with a grinder (Osterizer, Sunbeam Products Inc., Niles, IL) into a paste. Direct pH measurement was taken using a standard pH meter (Mettler Teledo, Schwerzenbach, Switzerland). The extent of proteolysis was monitored by the modified method of Greene and Babbitt (1990). The TCA-soluble peptide content was measured by the method of Lowry *et al.* (1951) and expressed as mmol of tyrosine/g dry matter. Free  $\alpha$ -amino acids were determined by using 2,4,6-trinitrobenzene sulfonic acid (TNBS) according to the method of Benjakul and Morrissey (1997). Free  $\alpha$ -amino acids were calculated as  $\mu$ mol of leucine/g dry matter. Nitrogen solubility at pH 4.5 and 7.0 was determined by the method of Bera and Mukherjee (1989). Nitrogen content was determined by Kjeldahl method (AOAC 1999) and percentage of nitrogen solubility was calculated based on total nitrogen in the sample. Ammonia nitrogen was determined by distillation method as described by Parris and Foglia (1983).

### Electrophoretic Analysis of Soy Proteins

Soy proteins were extracted according to the method of Cai and Chang (1999) with a slight modification. Samples were homogenized in 1% sodium dodecyl sulfate containing 50 mM  $\beta$ -mercaptoethanol ( $\beta$ -ME) at 8000 rpm for 1 min using an Ultra Turrax homogenizer (IKA Labortechnik, Selangor, Malaysia). The homogenate was sonicated at 60C for 90 min to extract the proteins. The extract was then centrifuged at  $17,600 \times g$  for 30 min. The protein content of the supernatant was analyzed according to the method of Lowry *et al.* (1951). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out by the method of Laemmli (1970). Solubilized samples were mixed at a ratio of 1:1 (v/v) with the sample buffer (0.125 M Tris–HCl, pH 6.8 containing 20% [v/v] glycerol) with 1.5 M  $\beta$ -ME and boiled for 3 min. The samples (25  $\mu$ g) were loaded on a gel made of 4% stacking and 5–20% gradient separating gels and then subjected to electrophoresis at a constant voltage of 110 V using a mini vertical Hoefer apparatus (Hoefer Pharmacia Biotech Inc., Uppsala, Sweden). After electrophoresis, the

gels were stained with 0.125% Coomassie Brilliant Blue R-250 in 25% ethanol and 10% acetic acid and destained with 25% ethanol and 10% acetic acid.

### **Proteolytic Activity Staining of Soybean Extract**

Samples (3 g) were homogenized in 9 mL of deionized water at 8000 rpm for 1 min using an Ultra Turrax homogenizer (IKA Labor Technik). The homogenate was agitated with a magnetic stirrer for 1 h at room temperature and centrifuged at  $10,000 \times g$  for 30 min. Supernatants were subjected to SDS-PAGE (Laemmli 1970) and stained for protease activity by the modified method of Garcíá-Carreno *et al.* (1993). Supernatant was mixed with the sample buffer at 1:1 (v/v) ratio. Proteins (100  $\mu$ g) were loaded into the gel made of 4% stacking and 5–20% gradient separating gels and then subjected to electrophoresis at a constant voltage of 110 V using a mini vertical Hoefer apparatus (Hoefer Pharmacia Biotech Inc.). After electrophoresis, the gels were immersed in 100 mL of 2% (w/v) casein in 50 mM Tris buffer, pH 7.5 for 1 h at 0C to allow the substrate to penetrate into the gels. The gels were then immersed in 2% casein (w/v) in McIlvaine's buffers, pH 7.5 at 45C for 1 h. The gels were fixed and stained with 0.125% Coomassie blue R-250 in 50% ethanol and 10% acetic acid and destained in 25% ethanol. Development of clear zones on blue background indicated proteolytic activity.

### **Statistical Analysis**

Analysis of variance (ANOVA) was performed and mean comparisons were run by Duncan's multiple range test (Steel and Torrie 1980). Analysis was performed using a SPSS package (SPSS 8.0 for Windows, SPSS Inc., Chicago, IL).

## **RESULTS AND DISCUSSION**

### **Microbiological Changes during Thua-nao Fermentation**

Inoculation of pure culture of *B. subtilis* BIOTEC 7123 at the levels of  $10^2$  and  $10^4$  cfu/g resulted in a higher initial TVC of approximately 1 and 3 log cycles, respectively, compared with that of the control (Fig. 1). TVC substantially increased up to 24 h of fermentation without any noticeable changes in YM ( $< 10$  cfu/g), suggesting that only bacterial growth was essential for Thua-nao fermentation. Thereafter, no marked changes in TVC were observed. Compared with naturally fermented soybean (control), inoculation with the higher level of starter resulted in a greater TVC throughout fermentation.

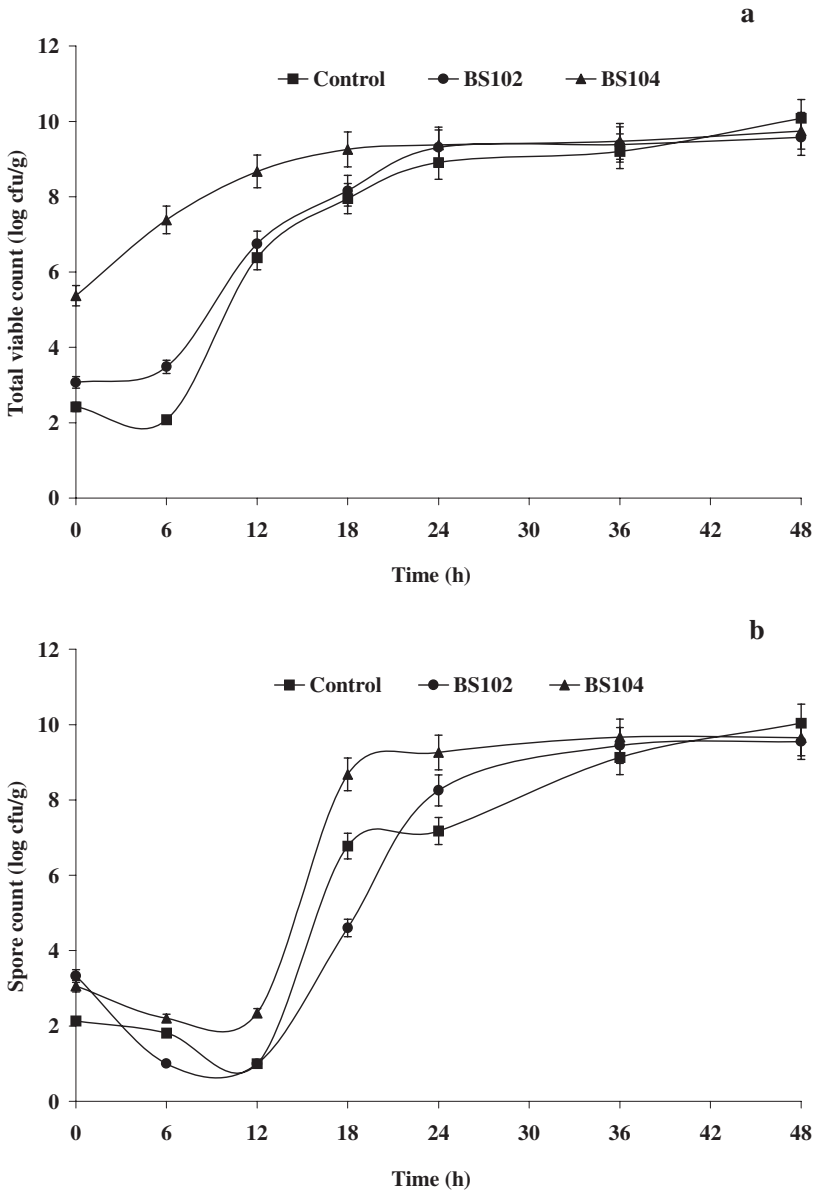


FIG. 1. CHANGES IN TOTAL VIABLE COUNT (a) AND SPORE COUNT (b) OF SOYBEAN DURING FERMENTATION

Control: soybean naturally fermented; BS102 and BS104: soybean inoculated with *B. subtilis* at the levels of  $10^2$  and  $10^4$  cfu/g, respectively. Bars represent standard deviation from triplicate determinations.

Unlike TVC, inoculation at both levels resulted in only 1 log cycle increase in the initial SC of soybeans. SC remained relatively unchanged during the first 12 h of fermentation. Thereafter, a subsequent increase in SC was observed and reached a maximum level of  $10^9$ – $10^{10}$  cfu/g within 36–48 h. Greater SC was observed with soybean inoculated with higher level of starter. The increase in SC was in accordance with that of TVC, particularly after 12 h of fermentation, making up the majority of total counts. The result suggested that spore-forming bacteria are predominant in Thua-nao fermentation. Microorganisms involved in Thua-nao fermentation have been isolated and characterized to be *B. subtilis* (Sundhagul *et al.* 1972). Hara *et al.* (1986) reported that four strains of aerobic, Gram-positive and spore-forming rods isolated from Thua-nao produced in Thailand were taxonomically similar to *B. subtilis* (natto).

In general, complete fermentation of Thua-nao is indicated by the occurrence of a slightly sticky brownish layer with a pungent, ammonia-like odor. Based on the appearance and smell, natural fermentation of cooked soybean in this study required 48 h to exhibit characteristics of Thua-nao. On the other hand, cultured fermentation was accomplished within 24 h for BS102 and 18 h for BS104, respectively. The results suggested that bacterial growth was essential for Thua-nao fermentation, which could be accelerated by using microbial inoculum.

### Changes in pH

An increase in pH of fermented soybean was observed during the course of fermentation from the initial pH of 6.2 to above 8 (Fig. 2). From the results, the marked increase in pH was observed after 24, 18 and 12 h for naturally fermented soybean and those inoculated with *B. subtilis* at the levels of  $10^2$  and  $10^4$  cfu/g, respectively. The pH of soybean inoculated with pure culture of *B. subtilis* generally increased with a higher rate than that of natural fermentation. Soybean inoculated with  $10^4$  cfu/g showed the highest pH after 48 h of fermentation. Most fermentation of protein-rich materials with *Bacillus sp.* caused a characteristic increase in pH (Steinkraus 1995). It is postulated that the deamination of amino acid induced by *B. subtilis* with resulting ammonia led to the increased pH. Ammonia formed also makes the substrate unsatisfactory for invasion by microorganisms that might spoil the products (Steinkraus 1995).

### Changes in TCA-soluble Peptides and Alpha-amino Acids

An increase in TCA-soluble peptides and free  $\alpha$ -amino acids of fermented soybean was observed during the time course of fermentation (Fig. 3a,b). The higher rate and extent of proteolytic degradation of soy pro-

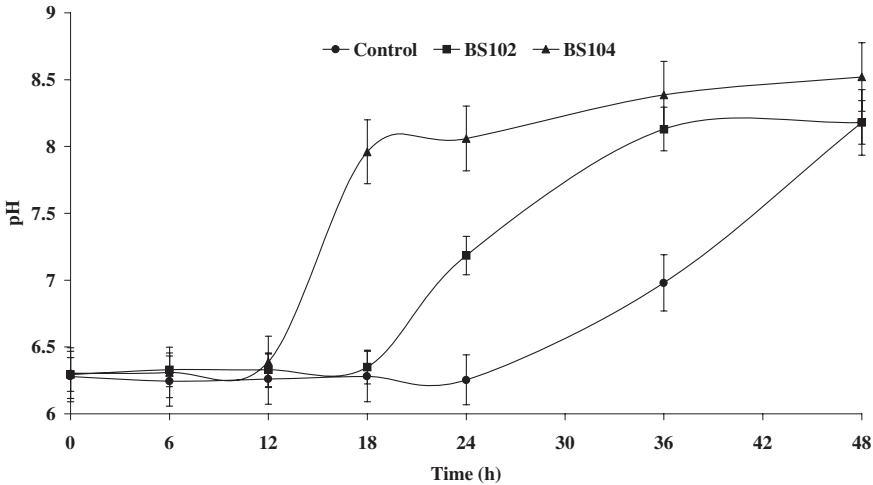


FIG. 2. CHANGES IN pH OF SOYBEAN DURING FERMENTATION  
 Control: soybean naturally fermented; BS102 and BS104: soybean inoculated with *B. subtilis* at the levels of  $10^2$  and  $10^4$  cfu/g, respectively. Bars represent standard deviation from triplicate determinations.

teins during fermentation was correlated with the higher amount of starter culture inoculated. The result indicated that *B. subtilis* probably played an essential role in protein degradation. Proteases secreted by *B. subtilis* growing on soybean surface might hydrolyze soy proteins to oligopeptides and amino acids. These products were subsequently converted to  $\gamma$ -polyglutamic acid ( $\gamma$ -PGA), a major component of a viscous material on the soybean surface (Fujii 1963; Yamaguchi *et al.* 1996). Furthermore, those compounds possibly contribute to the characteristic taste and flavor of Thua-nao, especially dimethylpyrazine and tetramethylpyrazine, which are the main pyrazines detected in soybean-based fermented foods (Leejeerajumnean *et al.* 2001). Threonine is a precursor of 2,5-dimethylpyrazine while tetramethylpyrazine can be derived from acetoin and ammonia (Larroche *et al.* 1999). The pleasant and palatable taste is considered to be related to the content of free amino acids, mainly glutamic acid (Chou *et al.* 1993).

### Changes in Nitrogen Solubility of Soybean during Fermentation

Nitrogen solubility of fermented beans at pH 4.5 and 7.0 increased with the increasing time and level of inoculum added (Fig. 4). Generally, inoculation with  $10^4$  cfu/g resulted in the highest rate of increase in nitrogen solubility at both pHs. Slightly higher nitrogen solubility was observed at pH 7.0,

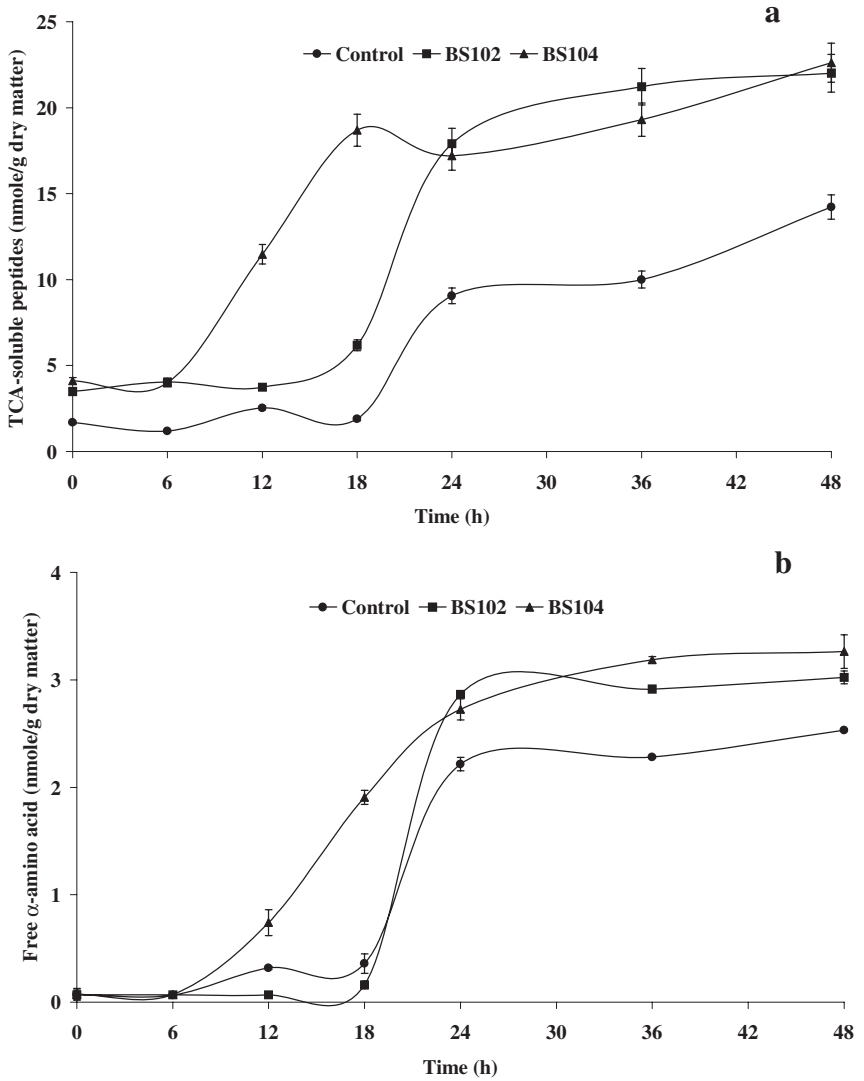


FIG. 3. CHANGES IN TCA-SOLUBLE PEPTIDE (a) AND FREE  $\alpha$ -AMINO ACIDS (b) OF SOYBEAN DURING FERMENTATION

Control: soybean naturally fermented; BS102 and BS104: soybean inoculated with *B. subtilis* at the levels of  $10^2$  and  $10^4$  cfu/g, respectively. Bars represent standard deviation from triplicate determinations.

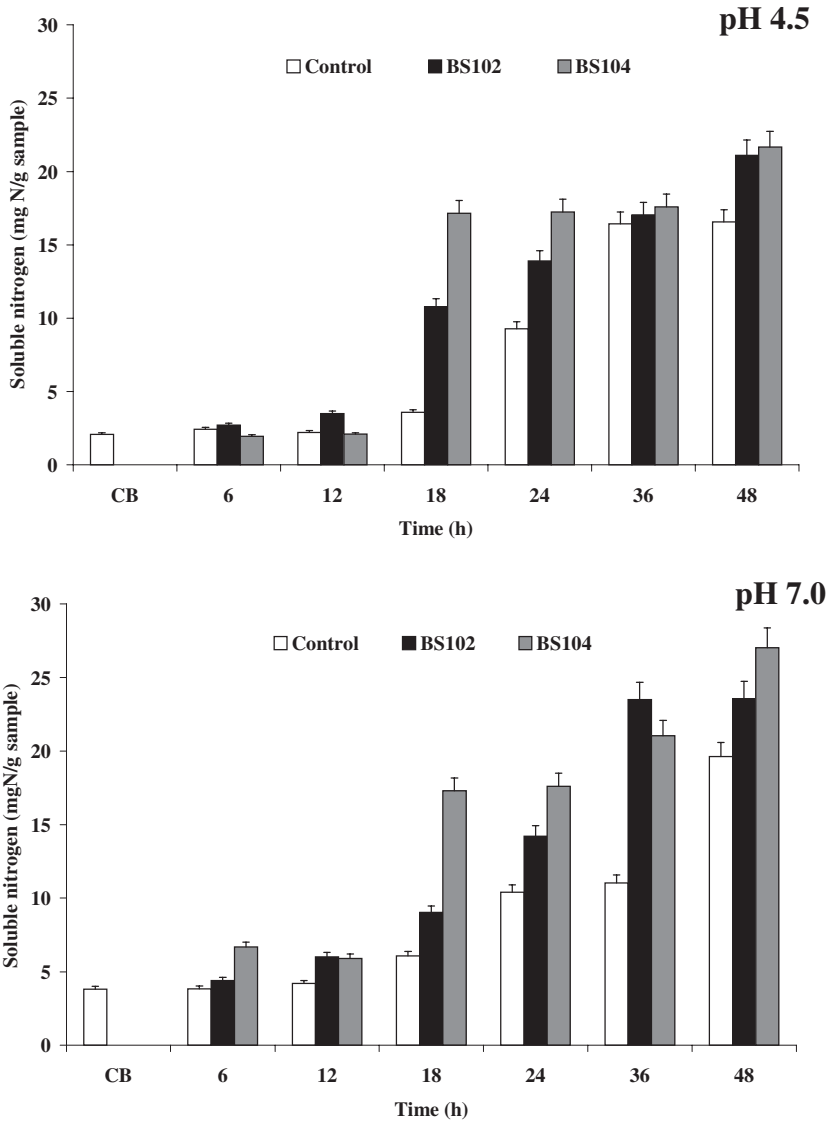


FIG. 4. CHANGES IN NITROGEN SOLUBILITY AT pH 4.5 AND 7.0 OF SOYBEAN DURING FERMENTATION

Control: soybean naturally fermented; BS102 and BS104: soybean inoculated with *B. subtilis* at the levels of  $10^2$  and  $10^4$  cfu/g, respectively. Bars represent standard deviation from triplicate determinations.

compared with that at pH 4.0. Nitrogen solubility of soybean proteins was generally high at pH below 3.5 and pH above 7, while solubility of soy nitrogen was minimal (about 10% or less) at pH 4.5–4.8, an isoelectric point of soy proteins (Bera and Mukherjee 1989). Corresponding to an increase in both TCA-soluble peptides and free  $\alpha$ -amino acids, especially with increasing fermentation time, an extensive degradation of proteins resulted in an increase in nitrogen solubility.

### Changes in Ammonia Nitrogen

The rate and magnitude of increase in ammonia nitrogen of soybean inoculated with starter was greater than that of naturally fermented soybean (Fig. 5). The marked changes in ammonia nitrogen were observed after 12, 18 and 36 h of fermentation for the control and those inoculated with *B. subtilis* at  $10^2$  and  $10^4$  cfu/g, respectively. The increase in ammonia content was coincidental with the increased pH (Fig. 2). Inoculation generally accelerated an increase in ammonia nitrogen. The result is in agreement with Van Buren *et al.* (1972) and Sparringa and Owen (1999) who reported that the increase in pH of tempeh, a *Rhizopus* spp. fermented soy product was caused by the liberation of ammonia. Ammonia is a product of the utilization of amino acids by the bacteria as sources of carbon and energy. Stillings and Hackler

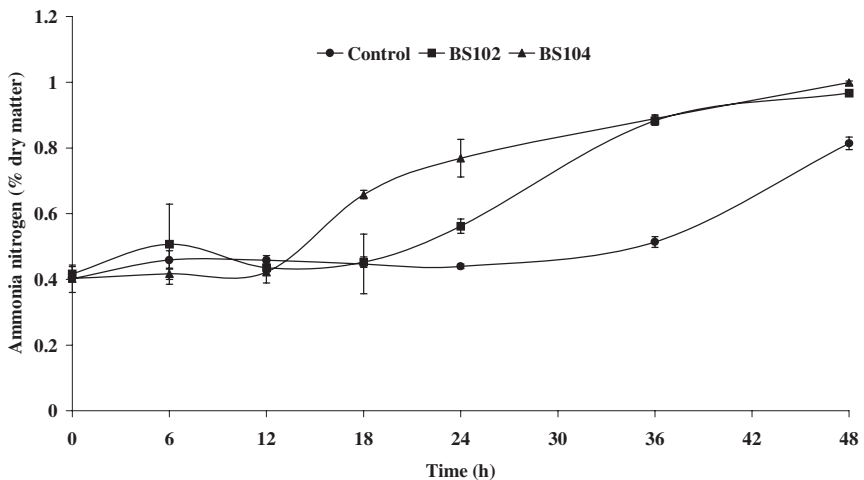


FIG. 5. CHANGES IN AMMONIA-NITROGEN CONTENT OF SOYBEAN DURING FERMENTATION

Control: soybean naturally fermented; BS102 and BS104: soybean inoculated with *B. subtilis* at the levels of  $10^2$  and  $10^4$  cfu/g, respectively. Bars represent standard deviation from triplicate determinations.

(1965) observed an increase in free amino acid and ammonia contents as fermentation time increased. Ammonia concentrations in kinema may rise as high as 200 mg/100 g (Sarkar *et al.* 1993), indicating a high level of proteolytic activity with release of free amino acids, followed by deamination. During fermentation, ammonia is formed by deamination but more than 0.2% ammonia nitrogen is unacceptable to consumers. When the pH value reaches about 8–8.3, sufficient ammonia is present as the volatile. As a result, the products have an unpleasantly strong ammoniac odor, which readily reaches objectionable levels (Campbell-Platt 1980; Sarkar *et al.* 1993). Because the volatilization of ammonia occurred, particularly at higher pH, ammonia concentrations were lower in fermentation with access to air than in those with limited oxygen (Allagheny *et al.* 1996).

### **Proteolysis of Soy Proteins during Fermentation**

SDS–PAGE patterns of soy proteins indicated marked changes in proteins caused by cooking and extensive proteolysis during the time course of fermentation (Fig. 6a–c). Proteins in soybean consist of  $\beta$ -conglycinin ( $\alpha'$ ,  $\alpha$  and  $\beta$  subunits). Glycinin (11S) and  $\beta$ -conglycinin (7S) are the major storage proteins in soybeans. Glycinin has an estimated molecular weight (MW) of 309,000–393,000 and consists of an acidic subunit (MW ~ 37,000–40,000) and a basic subunit (MW ~ 19,900–20,000) (Nielsen 1985).  $\beta$ -conglycinin has a trimeric structure having a MW of 140,000–170,000 and consists of subunit  $\alpha'$  (MW ~ 19,900–20,000),  $\alpha$  (MW ~ 19,900–20,000) and  $\beta$  (MW ~ 19,900–20,000) (Brooks and Morr 1985). However, most of these protein bands, except acidic and basic subunits of glycinin, were not found after prolonged cooking. It is postulated that  $\beta$ -conglycinin could be totally removed during prolonged cooking process, whereas glycinin remained to some extent. During fermentation, proteins underwent hydrolysis to a high extent as the incubation time increased. Disappearance of acidic and basic subunits of glycinin coincided with the appearance of low MW bands (~6500 or lower). No acidic and basic subunits were observed after 18, 12 and 6 h of the control fermentation and those inoculated with *B. subtilis* at the levels of  $10^2$  and  $10^4$  cfu/g, respectively. The results indicated that hydrolysis of soy proteins of sample inoculated with high dose of starter underwent hydrolysis more rapidly, suggesting the presence of higher proteolytic activity during fermentation. Therefore, *B. subtilis* was postulated to produce proteinases, which effectively hydrolyze acidic and basic subunits of glycinin.

### **Proteolytic Activity Staining of Fermented Soybean Extract**

The proteinases in fermented soybean extract were identified by separation on gradient gel electrophoresis followed by staining for proteolytic

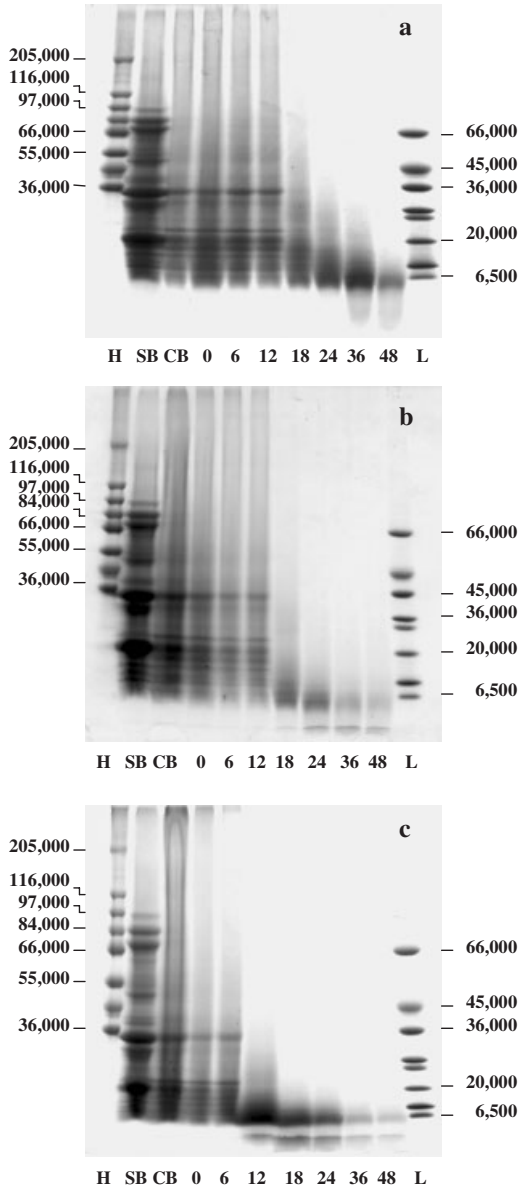


FIG. 6. SDS-PAGE PATTERNS OF SOY PROTEINS DURING FERMENTATION (a) Soybean naturally fermented; (b) and (c) soybean inoculated with *B. subtilis* at the levels of  $10^2$  and  $10^4$  cfu/g, respectively. SB and CB designate protein extract of soaked soybean and cooked soybean. H and L designate high and low molecular weight protein standards, respectively. The numbers indicated fermentation time (h) when the samples were taken.

activity (Fig. 7). Activity bands of proteinase were shown as clear zones on a dark background. Different patterns of activity bands indicated various proteinases involved in Thua-nao fermentation with and without starter inoculation (Fig. 7a–c). Activity bands of naturally fermented soybean were observed at 48 h, while those of inoculated soybean were observed at 18 and 12 h for those inoculated with *B. subtilis* at the levels of  $10^2$  and  $10^4$  cfu/g, respectively. The appearance of activity bands at different times of fermentation also showed different proteinases involved in the fermentation. Proteinases with estimated MW of 29,000, 27,000 and 19,000 Da were involved in soy protein degradation of naturally fermented soybeans. For inoculated soybeans, activity bands with MW of 40,000 Da were dominated at 12–18 h of fermentation. Thereafter, activity bands at MW of 29,000 Da were dominated with the concomitant disappearance of activity band with MW 40,000 Da. Proteinase with MW of 40,000 Da was presumed to be inactivated, caused by an increased pH or inhibitory compounds produced during fermentation. With an extended fermentation period, proteinase with a MW of 29,000 Da was found to function in hydrolysis of protein under the more alkaline condition. Coincidentally, activity bands observed were similar to those of spent culture broth of *B. subtilis* BCC7123 (Fig. 7d). The results suggested that proteolysis of soy proteins was mainly mediated by proteinases produced by *Bacillus sp.*, which is normally dominated in Thua-nao fermentation. Almost all species of bacilli are able to secrete proteinases. The maximal rate of exoenzyme synthesis is usually observed in the late exponential or early stationary phase of growth, at which the cells begin the preparation for sporulation (Debabov 1982). Two prominent proteases produced during the onset of sporulation are the alkaline protease or subtilisin (Millet 1970) and neutral metalloproteinases (Tsuru *et al.* 1966; Uehara *et al.* 1979). The combined activities of these two enzymes account for 96–98% of the total proteinase activity present in the culture supernatants of wild-type sporulating cells (Kawamura and Doi 1984). Neutral proteinases are optimally active near a pH of about 7.0, and their activities show an absolute dependence on the presence of divalent metal ions (Matsubara and Feder 1971). *Bacillus* strain grown on soybean during the production of natto was reported to produce a fibrinolytic enzyme (Miyamura *et al.* 1998).

## CONCLUSION

Thua-nao fermentation primarily involved proteolytic degradation of soy proteins, yielding peptides and amino acids, which can be deaminated to ammonia. Proteolysis of soy proteins can be accelerated by inoculation of *B. subtilis* and was generally mediated by several proteinases, especially excreted

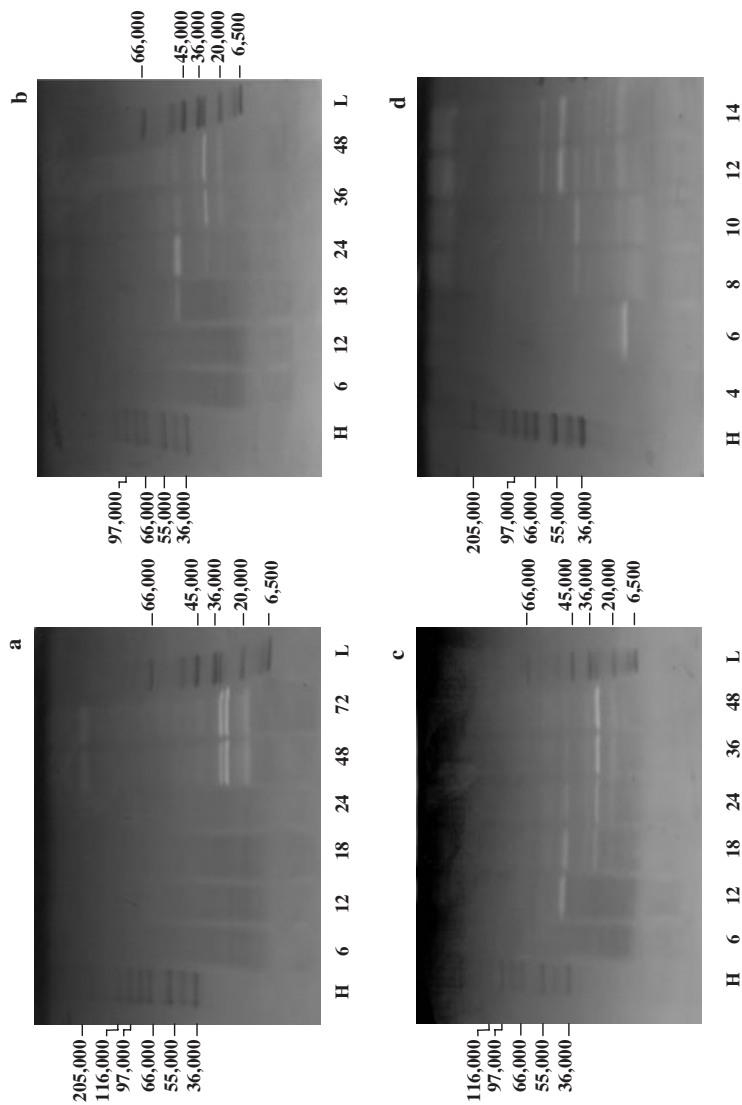


FIG. 7. PROTEOLYTIC ACTIVITY STAINING OF SOYBEAN EXTRACTS AT DIFFERENT FERMENTATION TIMES (a) Soybean naturally fermented; (b) and (c) soybean inoculated with *B. subtilis* at the levels of 10<sup>2</sup> and 10<sup>4</sup> cfu/g, respectively; (d) spent culture broth of *B. subtilis* at different times. H and L designate high and low molecular weight protein standards, respectively. The numbers indicated fermentation time (h) when the samples were taken.

by *B. subtilis*. Inoculation with *B. subtilis* resulted in an increased proteolysis, which is useful for subsequent reactions leading to development of Thua-nao characteristics.

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