ScienceAsia 28 (2002) : **241-245**

Characterization of proteases of *Bacillus subtilis* strain 38 isolated from traditionally fermented soybean in Northern Thailand

Panuwan Chantawannakul[a,*], Anchalee Oncharoen[a], Khanungkan Klanbut[a], Ekachai Chukeatirote[b] and Saisamorn Lumyong[a]

[a] Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200 Thailand.
[b] Department of Biotechnology, School of Science, Mae Fah Luang University, Chiang Rai 57100 Thailand.
* Corresponding author, E-mail: panuwan@yahoo.com

Received 12 Dec 2001
Accepted 27 Feb 2002

ABSTRACT Eighty-two bacillus strains were isolated from Thai fermented soybeans (*thua nao*), of which thirty-nine were identified as *Bacillus subtilis*. Crude proteins from these *B. subtilis* strains were investigated for their proteolytic activity. When tested on skim milk agar, the crude proteins of *B. subtilis* strain 38 exhibited the highest proteolytic activity (a clear zone with an average area of 480 mm^2). Optimal pH and temperature of the proteases from *B. subtilis* strain 38 were found to be at 6.5 and 47 °C, respectively. The proteases were unstable and rapidly decreased in activity when heated at 60 °C. Various protease inhibitors were tried, only 1, 10-phenanthroline decreased the enzyme activity indicating the presence of metalloproteases. In addition, we attempted to produce fermented soybeans using the *B. subtilis* strain 38 as a starter culture.

KEYWORDS: *Thua nao*, fermented soybean, *B. subtilis*, protease.

INTRODUCTION

In Northern Thailand, fermented soybeans, the so-called "*thua nao*", have been produced and consumed locally for several decades. Conventional production of *thua nao* is as follows: soybeans are washed, soaked overnight, cooked by boiling for about 3 – 4 h, gently smashed and wrapped inside banana leaves. The fermentation generally proceeds for 2 – 3 days at ambient temperature.[1,2] Alternatively, the soybean products are allowed to ferment outdoors exposed to sunlight, resulting in a dried form of *thua nao* that can be kept for several months. Its use is versatile, and fresh *thua nao*, for example can be consumed by steaming or roasting while the dried products are important ingredients in a variety of local dishes.

Similar fermented soybean products have been described in several countries, ie *kinema* in India,[3-5] *schuidouchi* in China,[6] and *natto* in Japan.[7] Other related products (ie *ugba* and *iru*) made with legume seeds instead of soybeans have also been reported in Western Africa.[8,9] Among these, only a few fermented soybean products have been studied systematically and manufactured industrially. The best-characterized fermented soybean product is probably *natto*—the Japanese styled fermented soybeans. *Natto* is commercially prepared using a pure starter culture of *B. subtilis* natto strain.[7] It has been estimated that the consumption of *natto* in Japan alone exceeds 50,000 tons annually.

Several *Bacillus* species have been found to be strongly associated with these fermented soybean products. For example, Ogbadu et al[10] identified a variety of *Bacillus* species (ie *B. subtilis*, *B. laterosporus*, *B. pumilus*, *B. brevis*, *B. macerans*, *B. licheniformis*, *B. polymyxa*, and *B. coagulans*) from Nigerian fermented soybean foods. It should also be noted that other bacterial species such as lactic acid bacteria or *Enterococcus* species may exist.[4] However, due to the initial boiling of beans in preparation, the existence of non-*Bacillus* species is likely to be a contamination during and/or after the fermentation process. *B. subtilis*, a Gram-positive, endosporeforming bacteria , has usually been found as the predominant microorganism in these fermented soybean foods. The ability of *B. subtilis* to grow over a wide pH range (with an active growth between pH 5.5 – 8.5) and to produce several enzymes (ie proteases) and other useful biological compounds[11] seems a likely reason for its superiority in the soybean fermentation. In addition, the beneficial effect of *B. subtilis* fermentation, particularly on *natto*, has been well- established including the presence of genistein (an anti-tumor

agent) and some proteolytic enzymes that can degrade staphylococcal enterotoxin.[12-16]

In contrast, *thua nao* production is still practiced locally with little information regarding microbial population and fermentation process. Our aim was to examine the microorganisms responsible for such fermentation. In this study, several *B. subtilis* isolates were screened from *thua nao* and investigated for their proteolytic activity. It was found that *B. subtilis* strain 38 showed the highest protease activity. Some fundamental characteristics of the proteases were also determined. In addition, we attempted to develop a laboratory scale process for making fermented soybeans using *B. subtilis* strain 38 as a pure starter culture.

Materials and Methods

Isolation and classification of spore-forming bacteria

Thai fermented soybean products (*thua nao*) were collected from six provinces in Northern Thailand: Chiang Mai, Chiang Rai, Lampang, Phrae, Payao, and Mae Hongson. About 0.1 g of sample was transferred to 3.0 ml sterile distilled water, shaken vigorously and held at 75 °C for 20 min to destroy all vegetative microbial cells. The suspension was then plated out on nutrient agar (1% peptone, 0.5% beef extract, 0.5% NaCl, and 1.5% agar) and incubated at 37 °C for 24 h. Bacterial colonies were isolated and characterized by their morphological and physiological properties.[11,17] These included cell shape, Gram staining, presence of spore, and growth conditions (aerobic or anaerobic). Further classification was performed using biochemical tests as described by Norris *et al*.[17]

Preparation of crude enzyme

Isolated bacteria were cultivated in 50 ml nutrient broth with vigorous shaking (150 rpm) at 37 °C for 24 h. The culture was then centrifuged at 10,000 rpm for 5 min at 4 °C. The supernatant was collected and used as the enzyme solution.

Determination of proteolytic activity

Initially, protease activity was tested using skim milk agar (1% skim milk, 0.02% sodium azide, and 2.0% agar). For this, 5 µl of crude proteins produced from isolated bacteria were spotted on skim milk agar plates. The plates were then incubated at 37 °C for 18 h. Proteolytic activity of the crude proteins was detected by observing the presence of clear zones. According to Cooper,[18] activity of biological substances (ie, antibiotics and enzymes) can be expressed in terms of the square of the diameter of the clear zone.

After initial screening for proteolytic activity, *B. subtilis* strain 38 exhibited highest activity of proteases and was used throughout the experiment. Determination of the enzyme activity was performed using the azocasein method.[19] 20 µl of the crude enzymes were incubated at 37 °C in a mixture (400 µl) containing 2% azocasein (230 µl), and 0.2 M N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES) buffer pH 7.0 (150 µl) for 2 h. To terminate the reaction, 1.2 ml of 10% trichloroacetic acid (TCA) was added. All samples were allowed to stand for 15 min and the supernatant (ca 1.4 ml) was collected after centrifugation (10,000 rpm; 5 min). An equivalent volume of 1 M NaOH was then added and the solution was mixed thoroughly prior to measuring the absorbance at 440 nm.

Influence of protease inhibitors

Inhibitors, known to affect different classes of proteases,[20, 21] that were used were follows: 3,4-dichloroisocoumarin (DCI), phenylmethylsulfonyl-fluoride (PMSF), Pepstatin A, E-64 (trans-epoxy-succinyl-leucylamido(4-guanidino)butane), and 1, 10-phenanthroline. To determine whether the activity of proteases could be affected, each inhibitor was added to the crude enzymes and incubated at 4 °C for 20 min. Protease activity was then measured using the azocasein method.

Fermentation of soybean (thua nao) using *B. subtilis* strain 38 as inoculant

Soybeans (approx. 150 g for each experiment) were washed thoroughly and soaked in water for 16 h at room temperature. After decanting the water, soaked soybeans were sterilized by autoclaving at 121 °C for 15 min. Three ml-of spore suspension (ca 7 x 10^{10} spores/ml) of the *B. subtilis* strain 38 was added to the sterilized soybeans. The fermentation was then carried out at 37 °C and 45 °C for 72 h in the traditional Thai-style fermentation[1,2] whereas, in the *natto*-style fermentation, the fermented soybeans were kept in a refrigerator (6 – 8 °C) for 48 h after the first 24 h fermentation. The pH of the fermented soybeans was monitored every 6 h.

Results and Discussion

In this study, several samples of *thua nao* were collected locally from different sources in six provinces in the Northern part of Thailand. To

destroy all vegetative cells and thus selectively isolate bacteria in the genus *Bacillus*, each sample was resuspended in distilled water, incubated at 75 ˚C for 20 min and spread on nutrient agar plates. Eighty-two bacterial isolates were obtained and further identified using biochemical assays as suggested by Norris et al.[17] Key identification of *Bacillus* species included catalase assay, Voges Proskaur (VP) test, growth under anaerobic condition, and starch hydrolysis. Of all isolated bacteria, thirty-nine (47.5%) isolates were classified as *B. subtilis* (yielding positive r eaction for catalase, VP, and starch hydrolysis tests, and unable to grow under anaerobic condition). The *B. subtilis* species is of particular interest due to its prevalence in these fermented foods.[2, 10] In addition, use of *B. subilis* in the food industry is recommended by the US FDA as one of the GRAS (Generally Recognized As Safe) organisms.[22, 23]

The *B. subtilis* isolates were then characterized for protease production. Using skim milk agar, the proteolytic activity could be detected by the presence of a clear zone. It was found that *B. subtilis* strain 38 yielded the highest protease activity with a clear zone of approximately 480 mm^2 (Fig 1). Relationship between protease production and growth of *B. subtilis* strain 38 was determined. For this, *B. subtilis* strain 38 was cultivated in nutrient broth. Cell density (as measured by absorbance at 660 nm) and protease activity (as measured by presence of the clear zone on skim milk agar) were monitored every 4 h. The results showed that the production of proteases was dependent on bacterial growth (Fig 2). During the bacillus growth, cell morphology was also examined: all bacterial cells appeared in vegetative forms during the first 12 h, but spore formation was observed between 16 and 24 h of the incubation period. This result suggested that production of proteases reached the highest level during the exponential phase and continued constant level when spores were formed (stationary state).

The effect of some physical factors, such as pH and temperature, on the activity of the crude enzyme were investigated using azocasein as a substrate (Fig 3 and 4). The optimum temperature and pH for the enzyme activity were 47 ˚C and 6.5, respectively. In addition, when testing the enzyme stability was tested at different temperatures (40 ˚C, 50 ˚C, and 60 ˚C), with the *B. subtilis* proteases being most affected at 60 ˚C as they exhibited a rapid decrease in enzyme activity. Incubation at 40 ˚C and 50 ˚C showed slight effect on enzyme activity as more than 80 and 50% of proteolytic activity respectively could still be detected after 60 min assay (Fig 4B).

Protease enzymes of *B. subtilis* generally can be categorized into two groups with respect to their optimal pH neutral and alkaline proteases. The former exhibit optimal pH at 7.0, whereas the latter have pH optima between 9 – 11.[20] Therefore, the crude protease enzymes produced from *B. subtilis* strain 38 belong to the neutral group since they had optimal pH at 6.5. To further characterize the

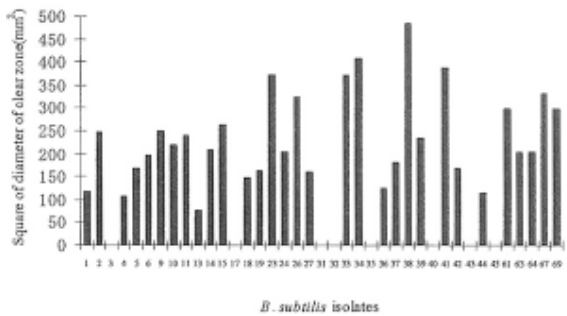

Fig 1. Comparative results of proteolytic activity of *B. subtilis* strains isolated from *thua nao*, Thai fermented soybeans. The proteolytic activity was assayed using skim milk agar and expressed as a square of the clear zone diameter.[18]

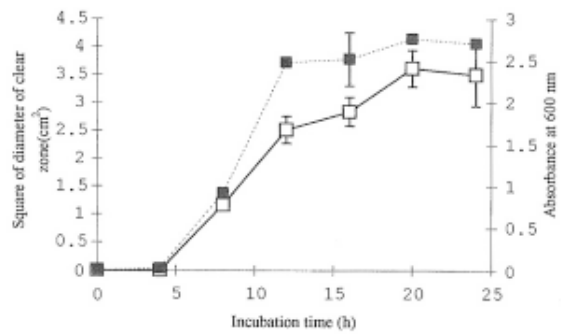

Fig 2. Relationship between cell growth and protease production of *B. subtilis* strain 38. Cell growth (■) was measured by absorbance at 660 nm and protease activity (□) was determined by presence of the clear zone on skim milk agar.

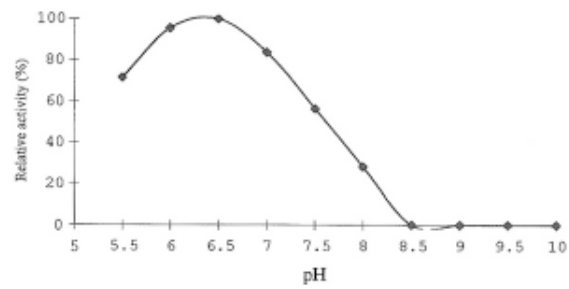

Fig 3. Effect of pH on activity of the *Bacillus subtilis* 38 proteases. Good's biological buffers (MES, PIPES, TES, TAPS, CHES (Sigma)) were used to cover a pH range 5.5-10 following the manufacture's recommendation. The crude extract used for each buffer was 20 µl.

proteases, various types of inhibitors were used. Only 1, 10-phenanthroline was clearly able to inhibit the enzyme activity (Table 1) indicating that the protease is a metalloprotease(s).

For many decades, *thua nao* has been produced in a conventional manner. The fermentation process thus mainly depends on a mixture of bacterial species

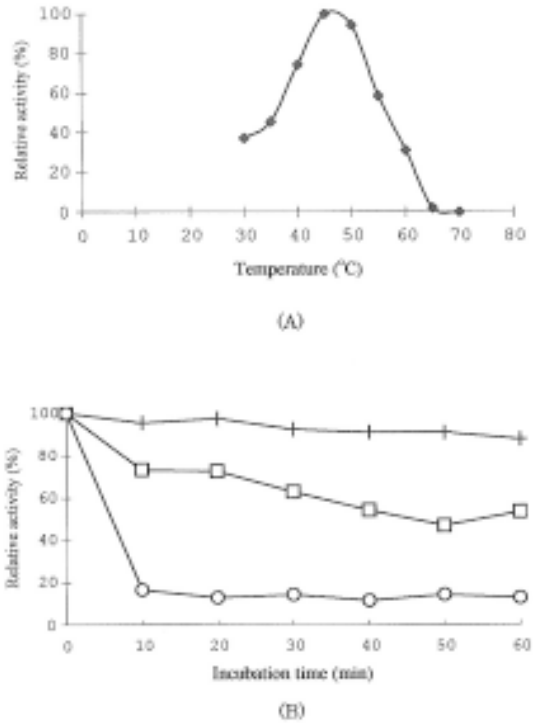

Fig 4. Effect of temperature on protease activity (A) and stability (B). For temperature stability (B), the crude enzyme solution were held at 40 °C (+), 50 °C (□), and 60 °C (O), and at intervals subsamples (20µl, 0-60 min) were removed and then assayed by the azocasein method at 37 °C. MES buffer (pH 6.5) was used for both experiments.

Table 1. Effect of inhibitors on activity of protease enzymes obtained from *B. subtilis* strain 38.

Inhibitors used[a]	Specificity	Relative activity[b]
None	—	100
1,10-phenanthroline	Metalloprotease	10.39
DCI	Serine protease	84.87
E64	Cysteine protease	93.18
PMSF	Serine protease	94.07
Pepstatin A	Aspatyl protease	100

[a] The final concentration of these inhibitors in the reaction is as follows: 1 mM for 1,10-phenanthroline; 0.1 mM for DCI and E64; 0.5 mM for PMSF, and 0.5 µg/ml for Pepstatin A.

[b] This assay was performed using azocasein method[19]. The relative activity was calculated using the A440nm value of the control reaction (no inhibitor) as 100 % enzyme activity. The crude extract used in each treatment was 15 µl.

although, not surprisingly, the *Bacillus* species have played a key role. There is also a lack of systematic procedure including no temperature control. Hence, the quality of the fermented products is not uniform. Using a suitable *B. subtilis* species as a pure starter culture is a promising approach to improve the fermentation process, which may lead to commercial-scale production. Consequently, we attempted to utilize *B. subtilis* strain 38 isolated in a laboratory-scale soybean fermentation. For this, fermentation of soybeans was carried out in both traditional Thai and *natto* styles at 37 °C and 45 °C. During the fermentation process, changes in pH were monitored every 6 h. Generally, the trends of pH change of each fermentation batch were similar with initial pH of 6.0 and gradually increased to approximately 8.0 after 72 h fermentation (Fig 5). The increased in pH of each batch was possibly due to proteolysis and ammonia release.[3] According to Fig 5, the pH changes were most dramatic at 12 h of fermentation. The difference due to temperature conditions (37 °C and 45 °C) was also clearly observed at this period, both fermented soybean samples (obtained by the *thua nao* and *natto* methods) showed higher pH (7.59 and 7.85) at 45 °C than their counterparts at 37 °C (7.24 and 7.01). The higher pH values at 45 °C were likely to be the result of higher activity of *B. subtilis* proteases. Therefore, fermentation at high temperature (45 °C) may represent a rapid means for soybean fermentation. By statistical analysis (SPSS/Friedman), soybean fermentation by *B. subtilis* 38 at 45 °C a gave more satisfactory odor and color of final products than that at 37 °C ($p<0.01$). It will be worthwhile examine the nutritional values of fermented soybeans in the future experiments. From this study, the selected strain may be regarded as a potential starting culture due to its highly active proteolytic activity and hence rapid fermentation

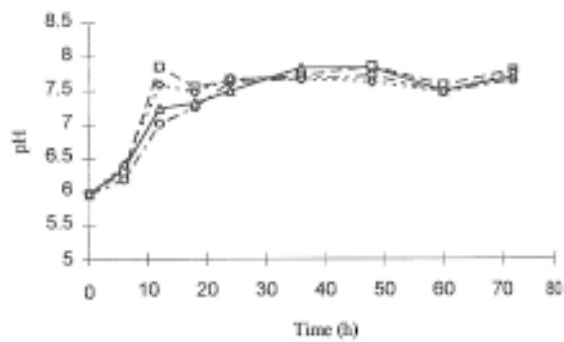

Fig 5. Change in pH during soybean fermentation. Fermentation of soybeans was carried out in the traditional Thai (*thua nao*) and natto styles; each fermentation batch was performed at 37 °C (Δ-*thua nao*, 37 °C; ◊-*thua nao*, 45 °C; O-*natto*, 37 °C; □ -*natto*, 45 °C.

would be expected. The neutral proteases produced during the outgrowth or log phase presumably play an important role in soybean fermentation. This strain holds great promise for the product, and provides attractive possibilities for further optimization as an industrial strain to improve the socio-economic benefits of traditional foods.

ACKNOWLEDGEMENTS

We would like to acknowledge the Prof Dr Natth Bhamarapravati research fund, Chiang Mai University, Chiang Mai, Thailand, for financial support. Thanks also to Associate Professor Dr Arayar Jatisatienr for her advice in statistical analysis. In addition, we would like to express our thanks to Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand for providing laboratory facilities.

REFERENCES

1. Sundhagul M, Smanmathuroj P and Bhodacharoen W (1972) Thua-nao: a fermented soybean food of Northern Thailand. *Thai J Agric Sci* 5, 43-56.
2. Chantawannakul P (1995) *Microorganisms related in Thai fermented soybeans* BSc Dissertation, Department of Biology, Faculty of Science, Chiang Mai University (in Thai).
3. Sarkar PK, Cook PE and Owens JD (1993) *Bacillus* fermentation of soybeans. *World J Microbiol Biotechnol* 9, 295-9.
4. Sarkar PK, Tamang JP, Cook PE and Owens JD (1994) Kinema—a traditional soybean fermented food: proximate composition and microflora. *Food Microbiol* 11, 47-55.
5. Nout MJR, Bakshi D and Sarkar PK (1998) Microbiological safety of kinema, a fermented soya been food. *Food Control* 9, 357-62.
6. Yokotsuka T (1991) Proteinaceous fermented foods and condiments prepared with koji moulds. In: *Handbook of Applied Mycology Vol 3, Foods and Feeds* (Edited by Arora DK, Mukerji KG and Marth EH), pp 329-73. Marcel Dekker, New York, NY.
7. Ohta T (1986) Natto. In: *Legume-based Fermented Foods* (Edited by Reddy NR, Pierson MD and Salunkhe DK), pp 85-93. CRC Press.
8. Odunfa SA (1985) African fermented foods. In: *Microbiology of Fermented Foods Vol 2* (Edited by Wood BJB), pp 155-91. Elsevier, London.
9. Njoku HO, Ogbulie JN and Nnubia C (1990) Microbiological study of the traditional processing of African oil bean (*Penackethra macrophylla* Bentham) for ugba production. *Food Microbiol* 7, 13-26.
10. Ogbadu LJ, Okagbue RN and Ahmad AA (1990) Glutamic acid production by *Bacillus* isolates from Nigerian fermented vegetable proteins. *World J Microbiol Biotechnol* 6, 377-82.
11. Sneath PHA (1986) Section 13. Endospore-forming Gram-positive rods and cocci. In: *Bergey's Manual of Systematic Bacteriology Vol 2, 9th ed* (Edited by Sneath PHA, Mair NS, Sharpe ME and Holt JG), pp 1104-39. Williams & Wilkins, Baltimore, MD.
12. Fujita M, Keiichi N, Hong K, Ito Y, Asada A and Nishimuro S (1993) Purification and characterization of a fibrinolytic enzyme (nattokinase) in the vegetable cheese natto, a popular soybean fermented food in Japan. *Biochem Biophys Res Comm* 197, 1340-7.
13. Fukutake M, Takahashi M, Ishida K, Kawamura H, Sugimura T and Wakabayashi K (1996) Quantification of genistein and genistin in soybeans and soybean products. *Food Chem Toxicol* 34, 457-61.
14. Okamato A, Hanagata H, Kawamura Y and Yanagida F (1995) Anti-hypersensitive substances in fermented soybean, natto. *Plant Foods Human Nutr* 47, 39-47.
15. Osawa R and Matsumoto K (1997) Digestion of staphylococcal enterotoxin by *Bacillus natto*. *Antonie Van Leeuwenhoek* 71, 307-11.
16. Sumi H, Yatagai C, Wada H, Yoshida E and Maruyama M (1995) Effect of *Bacillus natto* fermented product (biozyme) on blood alcohol, aldehyde concentration after whisky drinking in human volunteers, acute toxicity of acetaldehyde in mice. *Arukoru Kenkyuto Yakubutsu Izon* 30, 69-79. (in Japanese)
17. Norris JR, Berkeley RCW, Logan NA and O'Donnell AG (1981) The genera *Bacillus* and *Sporolactobacillus*. In: *The Prokaryotes Vol 2* (Edited by Starr MP, Stolp A, Truper AG, Balows A and Schlegel HG), pp 1711-42. Springer-Vertag, Berlin, Germany.
18. Cooper KE (1963) The theory of antibiotic inhibition zones. In: *Analytical Microbiology* (Edited by Kavanagh F), pp 1-86. Academic Press, New York, NY.
19. Brock FM, Forsberg CW and Buchanan-Smith JG (1982) Proteolytic activity of rumen microorganisms and effects of proteinase inhibitors. *Appl Environ Microbiol* 44, 561-9.
20. Keay L (1972) Protease of the genus *Bacillus*. In: *Fermented Technology Today* (Edited by Terui G), pp 289-98.
21. Dunn BM (1989) Determination of protease mechanism. In: *Proteolytic Proteins; A Practical Approach* (Edited by Beynon RJ and Bond JS), pp 57-81. IRL Press.
22. Kramer JM and Gilbert RJ (1989) *Bacillus cereus* and other *Bacillus* species. In: *Food-borne Bacterial Pathogens* (Edited by Doyle MP), pp 21-70. Marcel Dekker, New York, NY.
23. Denner WHB and Gillanders TGE (1996) The legislative aspects of the use of industrial enzymes in the manufacture of food and food ingredients. In: *Industrial Enzymology, 2nd ed* (Edited by Godfrey T and West S), pp 397-411. The Macmillan Press Ltd, Basingstoke, UK.