



**PROTEIN ENRICHMENT OF TRADITIONAL
FERMENTED SOY SAUCE WITH ENZYME
DIGESTED SOYBEAN MEAL**

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Soy sauce is widely used in Thailand as a flavor enhancer. The quality of soy sauce is usually judged by consumers on its flavor and aroma. However, food registration agencies usually judge soy sauce on its protein content. Also, it is known that qualities as well as quantities of soluble protein in soy sauce contribute greatly to the proper development of flavor and aroma in soy sauce. Thus, the enhancement of soluble protein content in soy sauce products should have beneficial effects for consumers as well as manufacturers. In this study, protein enhancement was carried out by the addition of soybean meal hydrolysate.

Soybean meal hydrolysate (SBH) was prepared by enzymatic hydrolysis of defatted soybean meal (SBM), a by-product from the production of soybean oil, with Kojizyme™. The optimal conditions for hydrolysis of SBM were carried out by using Kojizyme™ at 2% of protein in SBM and a total solid content at 22% and then incubated at 50°C for 12 hr. The soluble protein content and degree of solubilization of SBH product were found at 8.4% and 63% in laboratory scale hydrolysis and 5.9% and 49% in large scale hydrolysis, respectively.

The SBH was used to supplement the protein content in soy sauce in two ways. The first involved the addition of clarified soybean meal hydrolysate (cSBH) directly to the final soy sauce product. The second way involved addition of SBH during various stages of the moromi fermentation. Addition of cSBH to the final soy sauce product enhanced soluble protein content in final products but could not maintain traditional characteristics if too much cSBH was supplemented. When SBH was supplemented at primary and secondary moromi fermentation, it was found that the soluble protein content of the final product was greatly enhanced if the addition was done in the secondary moromi fermentation. In this way, the protein content increased over three fold when compared with the typical fermentation. The modified soy sauce obtained from secondary moromi fermentation gave acceptable characteristics in general appearance, odor, flavor, overall acceptability and taste at the probability level of $P > 0.05$.

The estimated cost for production of one kg each of SBH and cSBH was calculated to be at 9.50 and 13.52 Baht, respectively. Calculating the cost for production of modified soy sauce, it was shown that the addition of cSBH into finished soy sauce could save about 12.87 Baht per liter (Baht/L) for producing first grade soy sauce (3% protein) and save about 10.66 Baht/L for increasing the soluble protein content from 3% to 4% in the soy sauce product. In modified secondary cycle moromi fermentation, the addition of SBH provided a saving of 2 Baht/L for producing 3% protein soy sauce.

In spite of the small reduction in savings, SBH should be used with traditionally produced soy sauce to enhance soluble protein content, especially by addition at the secondary moromi fermentation. The soy sauce product from this modification provided significantly increased protein content and also provided a product with acceptable characteristics.

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วนิดา แซ่จิ่ง : การเพิ่มโปรตีนในซีอิ๊วขาวที่หมักด้วยวิธีดั้งเดิม ด้วยกากถั่วเหลืองที่ย่อยสลายด้วยเอนไซม์ (PROTEIN ENRICHMENT OF TRADITIONAL FERMENTED SOY SAUCE WITH ENZYME DIGESTED SOYBEAN MEAL). คณะกรรมการควบคุมวิทยานิพนธ์ : อมเรศ ภูมิรัตน, Ph.D., ทิมโมที เฟลเกล, Ph.D., นภา โล่ห์ทอง, M.Sc., อภิญญา อัครวนิก, Ph.D. 156 หน้า. ISBN 974-663-839-4

ในประเทศไทย มีการใช้ซีอิ๊วขาวกันอย่างแพร่หลายเพื่อเพิ่มรสชาติในอาหาร ผู้บริโภคมักจะตัดสินใจคุณภาพของซีอิ๊วขาวที่รสชาติและความหอม แต่กระนั้น หน่วยงานควบคุมคุณภาพอาหารมักจะตัดสินใจซีอิ๊วขาวที่ปริมาณโปรตีน เพราะทราบดีว่าคุณภาพและปริมาณของโปรตีนที่ละลายน้ำได้ในซีอิ๊วขาวนั้น จะนำไปสู่การปรับปรุงที่เหมาะสมของรสชาติและความหอมของซีอิ๊วขาว ดังนั้น การเพิ่มปริมาณโปรตีนที่ละลายน้ำได้ในซีอิ๊วขาว น่าจะส่งผลดีต่อทั้งผู้ผลิตและผู้บริโภค ในการศึกษาครั้งนี้ จึงศึกษาถึงการเพิ่มโปรตีนในซีอิ๊วขาวด้วยการเติม ซอยบินมีลไฮโดรไลเซท

ซอยบินมีลไฮโดรไลเซทเตรียมโดยการย่อยกากถั่วเหลือง (ผลพลอยได้จากการผลิตน้ำมันถั่วเหลือง) ด้วยเอนไซม์ Kojizyme™ สภาวะที่เหมาะสมในการย่อยกากถั่วเหลืองคือใช้ Kojizyme™ จำนวน 2% ของปริมาณโปรตีนในกากถั่วเหลือง, ปริมาณของแข็งรวม 22% หลังจากนั้นบ่มที่ 50 องศาเซลเซียส เป็นเวลา 12 ชั่วโมง ปริมาณโปรตีนที่ละลายน้ำได้และระดับการละลายน้ำได้ของซอยบินมีลไฮโดรไลเซท คือ 8.4% และ 63% ในการย่อยในระดับห้องทดลอง และ 5.9% และ 49% ในการย่อย ในระดับใหญ่, ตามลำดับ

ซอยบินมีลไฮโดรไลเซทได้นำไปเพิ่มปริมาณโปรตีนในซีอิ๊วขาวในสองรูปแบบ แบบแรกคือเติม ส่วนใสของซอยบินมีลไฮโดรไลเซท ลงไปในผลผลิตซีอิ๊วโดยตรง แบบที่สอง คือการเติมซอยบินมีลไฮโดรไลเซทลงไปในระยะต่าง ๆ ของขั้นตอนการหมักโมโรมิ การเติมส่วนใสของ ซอยบินมีลไฮโดรไลเซทลงไปในซีอิ๊วสำเร็จ สามารถเพิ่มปริมาณโปรตีนที่ละลายน้ำได้ ในผลผลิตสุดท้าย แต่ไม่สามารถคงไว้ซึ่งคุณสมบัติของซีอิ๊วขาวที่หมักแบบธรรมชาติ ถ้า มีการเติมส่วนใสของ ซอยบินมีลไฮโดรไลเซทมากเกินไป เมื่อเติมซอยบินมีลไฮโดรไลเซทลงในการหมักโมโรมิของซีอิ๊วขาวรอบที่หนึ่ง และรอบที่สองพบว่า ปริมาณโปรตีนที่ละลายน้ำได้ในผลผลิตสุดท้ายจะเพิ่มขึ้นอย่างมาก ถ้าการเติมนั้นเติมลงไปใน การหมักโมโรมิรอบที่สอง โดยจะเพิ่มขึ้นกว่าสามเท่าเมื่อเทียบกับปริมาณที่ได้ในการหมักแบบปกติ โดยซีอิ๊วขาวที่ได้จากการคิดแปลงโดยการเติมซอยบินมีลไฮโดรไลเซทลงในการหมักโมโรมิรอบที่สองนั้นให้ลักษณะที่ยอมรับได้ในด้านลักษณะปรากฏโดยรวม, กลิ่น, กลิ่นรส, รสชาติและ การยอมรับ โดยรวมที่ระดับความเชื่อมั่นที่ 95%

การประมาณค่าใช้จ่ายสำหรับการผลิตซอยบินมีลไฮโดรไลเซท และส่วนใสของซอยบินมีลไฮโดรไลเซทต่อหนึ่งกิโลกรัมมีค่าเท่ากับ 13.52 และ 9.50 บาท ตามลำดับ เมื่อคำนวณค่าใช้จ่ายในการผลิตซีอิ๊วขาวคิดแปลง พบว่าการเติมส่วนใสของซอยบินมีลไฮโดรไลเซทลงไปในซีอิ๊วสำเร็จสามารถประหยัดได้ 12.87 บาทต่อลิตรสำหรับการผลิตซีอิ๊วขาวเกรดหนึ่ง (3% โปรตีน) และประหยัดได้ 10.66 บาทต่อลิตรสำหรับการเพิ่มปริมาณโปรตีนที่ละลายน้ำได้ในซีอิ๊วขาวจาก 3% เป็น 4% การเติมซอยบินมีลไฮโดรไลเซทในขั้นการหมักโมโรมิรอบที่สองสามารถประหยัดค่าใช้จ่ายได้ 2 บาทต่อลิตรในการผลิตซีอิ๊วขาวที่มีปริมาณโปรตีน 3%

ทั้ง ๆ ที่สามารถประหยัดค่าใช้จ่ายได้เพียงเล็กน้อย แต่ซอยบินมีลไฮโดรไลเซทก็ยังสามารถใช้เติมลงในซีอิ๊วขาวที่ผลิตโดยวิธีดั้งเดิมเพื่อเพิ่มปริมาณโปรตีนที่ละลายน้ำได้ โดยเฉพาะอย่างยิ่งในการเติมลงในการหมักโมโรมิรอบที่สอง ซึ่งซีอิ๊วขาวที่ได้จากการคิดแปลงนี้มีการเพิ่มปริมาณโปรตีนอย่างเห็นได้ชัด รวมทั้งให้ผลผลิตที่มีคุณสมบัติที่ยอมรับได้อีกด้วย

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LIST OF ABBREVIATIONS

°C	Degree of Celsius
CFU	Colony forming unit
cm	Centimeter
<i>et al.</i>	Et alii (Latin), and others
g	Gram
hr	Hour
i.e.	Id est (Latin), that is
inch ²	Square inches
kg	Kilogram
L	Liter
µg	Microgram
mg	Milligram
ml	Milliliter
m ³	Cubic meter
/	Per
%	Percent
TM	Trade mark
w/v	Weight per volume
w/w	Weight per weight

LIST OF ABBREVIATIONS

(continued)

MMR	Moromi mash residue
SBH	Soybean meal hydrolysate
SBM	Soybean meal
SS	Soy sauce
cSBH	Clarified soybean meal hydrolysate
CSS ₁	Control soy sauce of primary moromi fermentation
ESS ₁ H ₀	Experimental soy sauce of primary moromi fermentation with SBH added at day 0
ESS ₁ H ₃₄	Experimental soy sauce of primary moromi fermentation with SBH added at day 34
ESS ₁ H ₄₉	Experimental soy sauce of primary moromi fermentation with SBH added at day 49
ESS ₁ K ₀	Experimental soy sauce of primary moromi fermentation with Kojizyme TM added at day 0
ESS ₁ K ₃₄	Experimental soy sauce of primary moromi fermentation with Kojizyme TM added at day 34
ESS ₁ K ₄₉	Experimental soy sauce of primary moromi fermentation with Kojizyme TM added at day 49

LIST OF ABBREVIATIONS

(continued)

CSS ₂	Control soy sauce of secondary moromi fermentation
ESS ₂ H ²	Experimental soy sauce of secondary moromi fermentation which 20 kg of MMR was mixed with 20 kg SBH (1:1)
ESS ₂ H ¹	Experimental soy sauce of secondary moromi fermentation which 20 kg of MMR was mixed with 10 kg SBH (2:1)
ESS ₂ M ²	Experimental soy sauce of secondary moromi fermentation which 20 kg of MMR was mixed with 8.8 kg autoclaved-SBM
ESS ₂ M ¹	Experimental soy sauce of secondary moromi fermentation which 20 kg of MMR was mixed with 4.4 kg autoclaved-SBM

CHAPTER I

INTRODUCTION

In Thailand, several kinds of condiments are used as flavor enhancers. These include fish sauce (nam-pla), soy sauce (se-iew), soybean paste (tao-chiew), fermented soybean curd (tao-hu-yee) and others. Soy sauce is one of the most popular condiments. For soy sauce, the main criteria used for classification is the protein content. The Thai Industrial Standards Institute, Ministry of Industry classifies white soy sauce into two grades, special grade and first grade which contain 5.5 and 4.5% protein by weight, respectively. Regulations set by The Food and Drug Administration indicate that soy sauce should contain at least 1.5% protein by weight. In order to obtain a high protein content in soy sauce, a lengthy period for fermentation is required. The lengthy fermentation period combined with high protein content usually leads to soy sauce products with a dark color which is not generally desired by consumers.

In the traditional process for producing soy sauce, the protein content comes from the degradation of protein in soybean during the koji and moromi fermentation processes by protease enzymes produced from the koji mold, *Aspergillus oryzae*. In the aging process, appropriate flavor and aroma are produced. It is important that good quality soy sauce should contain a high level of protein as well as acceptable flavor and aroma. Such good quality soy sauce requires a lengthy incubation time during the moromi fermentation. It was hypothesized that if protein hydrolysate could be

employed to supplement the protein content in soy sauce without interfering with the development of flavor and aroma, it could lead to a substantial improvement of the product.

Protein hydrolysate is a modified form of protein, which is prepared by hydrolysis of protein originating from plants, animals or microorganisms with either acid, alkali or enzyme. Protein hydrolysate is usually used as a supplementary food ingredient to give good flavor. Hydrolyzed vegetable protein produced from wheat gluten or soybean meal by acid hydrolysis gives meaty flavor to food because it contains a high concentration of glutamate. It is used as a flavor enhancer in the savory based foods. Soybean meal, a protein-rich defatted cake, which contains as much as 50% protein can provide hydrolysates of high protein content. Protein hydrolysates prepared from enzymatic digestion of soybean meal may give a product with a high protein content and meaty flavor, and may be suitable for supplementing soy sauce. This study was initiated to search for the most appropriate means to enhance the protein content of in traditional fermented soy sauce by adding soybean meal hydrolysate at various stages during soy sauce production.

It was hoped that a suitable method of soy sauce supplementation with Soybean meal hydrolysate either by mixing with the finished product or by adding during the fermentation process would enhance the protein content of soy sauce. The increased soluble protein content might lead to improvement in the quality without any reduction in consumer's preference for the product. If such intervention could subsequently be transferred to soy sauce manufacturers, it might lead to improved manufacturing techniques and increase the competitiveness of the industry.

CHAPTER II

BACKGROUND

In the orient, there are several kinds of indigenous fermented foods that use soybeans as a major raw material. Examples include soy sauce, miso (fermented soy paste), natto (fermented whole soybean), sufu (fermented soybean protein curd) and tempeh (fermented whole soybeans) (1). Among these products soy sauce is the most common and must widely used all over orient (2, 3) and in some western countries(2).

The production of soy sauce was first reported in China around 3,000 years ago (2, 4). This discovery was said to be one of the milestones in the history of food science because it was the first kind of meat-like flavor condiment which was produced from plant protein and lipid (2). From there, soy sauce was introduced into Japan and other countries in South East Asia by the spread of Buddhism, its vegetarian diet and trade (3, 5). The introduction of soy sauce into Japan took place about 1,000 years ago (5). The methods employed for soy sauce production differ slightly among the various oriental countries, largely due to the influence of local food cultures (2, 3).

1. Various types of soy sauce in Asian countries

Soy sauce is a light brown to dark brown liquid with a meat-like and salty flavor (5). There have been reports indicating that the amino acid profile as well as relative concentrations play a significant role in the development of flavor and aroma in soy sauce (6). The majority of the amino acids in soy sauce are generated from the

action of proteolytic enzymes produced by *Aspergillus oryzae* which is the koji mold used in the solid substrate fermentation of soybean and wheat flour (5).

Soy sauce production is usually undertaken in three major steps. The first step is an aerobic solid-substrate fermentation in which cooked soybean is mixed with roasted wheat grain or wheat flour and fermented with *A. oryzae* or *A. soyae* to obtain koji. The resulting koji is, then, mixed with brine solution in various ratios depending on the manufacturer and fermented under static conditions. This second step is called the moromi fermentation. After the moromi mash has been fermented for a certain period of time, the mash is collected and processed to harvest the soy sauce product. The third major step is the refining process which consists of pressing or filtering. Subsequently, the refined soy sauce is pasteurized prior to bottling. Pasteurization is the final step of soy sauce production (7).

The types and amount of raw materials used in the koji fermentation and the amount and ratio of koji to brine solution in the moromi fermentation stage vary depending on region and country. This results in different kinds of soy sauce products, and the name of the soy sauce in each country also differs. An outline of the soy sauce products, types and names for each country are described below.

1.1 Japanese soy sauce

In Japan, soy sauce is called shoyu. There are five kinds of shoyu recognized by the Japanese Agricultural Standard (JAS) (6). Koikuchi is a dark reddish brown color with a strong savory flavor (1, 7, 8) and it accounts for 85 % of the total shoyu production in Japan (6, 8). Usukuchi is a lighter reddish brown color and has a milder flavor and aroma (1, 7). It accounts for 13 % of total shoyu production in Japan (6).

Both of these types are made from almost equal amounts of soybean and wheat and are characterized by fermentation with lactobacilli and yeasts (6). Tamari is characterized by a slightly higher amino acid content. It is made mostly from soybeans with only a small amount of wheat. Shiro is very light in color and is made from wheat with a small amount of soybean (6) and it contains a very low content of amino acids (1). Saishikomi shoyu is made by digesting koji in raw shoyu instead of salt solution (6). Saishikomi's koji is made from equal amounts of soybeans and wheat (1).

The JAS classifies each variety of shoyu into three grades; special, upper and standard (1, 6) and also classifies production methods into three types; genuine fermentative, semi-fermentative and hydrolyzed vegetable protein (HVP) mixed. In 1986, about 65% of shoyu in Japan was of special grade and about 74% was made by the genuine fermentative method (6). The special grade of Koikuchi shoyu contains more than 1.5% total nitrogen and 1.0% alcohol and must be made by the genuine fermentative method (6, 8). The special grade is recognized as high-quality soy sauce and therefore must be made only by the genuine fermentation method (1).

The preparation of koji for shoyu is initiated with the mixing of cooked soybean or defatted soybean grit with roasted wheat kernels (6). The mixture is then inoculated with a small amount of koji mold, *A. oryzae* or *A. sojae* and fermented for 2 days. After that the koji is mixed with 110-120 % of salt water (17 to 19 % w/v of sodium chloride) (4). This results in a very vigorous moromi mash that is fermented for about 6 to 12 months at ambient temperature (15 to 20°C) or for 3 to 4 months if warmed to about 35 to 40°C (4). Finally, the mash is refined, bottled and shipped. Recently, most shoyu production has been undertaken on an industrial scale using many complex and sophisticated instruments. Much research and development to

improve the production process has been carried out. This includes work on reducing cost of raw materials by using defatted soybean meal instead of whole soybean (5) and developing cooking processes for soybean meal using high temperature for a short time to obtain a better yield of digestible protein (6).

1.2 Chinese soy sauce

Soy sauce in China is commonly known as Chiang-you. Major characteristics of Chiang-you are the same as Tamari-shoyu of Japan, primarily made from soybean and a small proportion of wheat (5). Presently, the soy sauce manufacturing method in China has been adapted for utilization of low cost raw materials and shorter fermentation periods (9). Defatted soybean meal (a by-product from soybean oil factories) and wheat bran are used for preparing koji. The koji fermentation time has been reduced from 48 hr to 24 hr by improvement in mold strains. The moromi fermentation time has also been shortened to about 2 to 4 weeks (1, 9) by using special fermentation methods (1). However, this kind of soy sauce has encountered problems due to lack of flavor because of the quick fermentation which does not allow for proper maturation of the moromi mash (9).

The consumption of soy sauce in China has been rising significantly. Therefore only the quick fermentation method can satisfy the consumer demand for soy sauce. Thus, major research efforts in China has been aimed to develop the soy sauce industry in 3 main areas; (i) development of cooking raw material method at high temperature for short times, (ii) enabling closed rectangular or circular koji culture to be used more widely, (iii) adding pure cultures of lactobacilli and yeasts into the moromi fermentation to improve the flavor of the soy sauce.

1.3 Taiwanese soy sauce

There are various soy sauce products in Taiwan. These include fermented soy sauce, blends of fermented and chemically hydrolyzed soy sauce and purely chemically hydrolyzed soybeans. The blended types are most popular because the flavor and price are reasonably acceptable to most consumers. There are some factories which produce soy sauce with modern facilities, but most are small-scale and primitively equipped (5).

Defatted soybean is cooked in a rotating cooker and mixed with roasted wheat flour. A pure *A. oryzae* culture is inoculated into the mixture which is then incubated at 28 to 30°C for 42 to 45 hr. The koji is mixed with brine solution in cement tanks and fermented for 3 to 4 months. Yeast and lactic acid cultures are also inoculated during the moromi fermentation period (5).

1.4 Soy sauce in South East Asia

Tamari-type soy sauce is widely produced in the countries of South East Asia such as Indonesia, Malaysia, Philippines, Singapore and Thailand. The soy sauce production in these countries usually carried out by very old industry participants that originated from China and handed down methods from generation to generation. The production processes are similar to those for tamari-shoyu production using rather primitive equipment such as bamboo trays and earthenware jars. Koji and moromi fermentations were carried out naturally without controlling any parameter. However, recently, there have been attempts to improve manufacturing techniques through concerted research and development activities. Some of these improvements include using pure *A. oryzae* culture in the koji fermentation stage in Malaysia (1) and

Thailand (6) and using defatted soybean meal instead of whole soybean in Malaysia (1) and Indonesia (7).

1.4.1 Indonesian soy sauce

Traditional Indonesian soy sauce is called “*kecap*” (3, 11, 12). There are two kinds. One is called *kecap asin*. It has a salty taste and is mainly consumed on Sumatra. The other is called *kecap manis*. It has a sweet taste and is consumed largely in Java (3). The *manis* variety is the most popular type of soy sauce in Indonesia (5). The koji and moromi fermentation stages in Indonesian soy sauce production are called “bungkil” and “baceman”, respectively (3, 12).

In traditional Indonesian soy sauce production, black soybeans are used as the only raw material in koji fermentation or bungkil. Boiled soybean is subjected to natural solid-state fermentation before being subjected to brine fermentation which is also carried out naturally. After the brine is filtered, the filtrate is boiled together with caramel or spices, yielding the final product, *kecap* (11).

In the last two decades, several industrial soy sauce manufactures have been established in Indonesia. These manufactures simply apply modern technology imported from Japan. They use defatted soybean flakes and wheat instead of black soybeans. The Bungkil stage is well controlled and inoculated but the bacemen stage still employs a natural process and is undertaken at ambient temperature for four months (11).

There are several research progresses projects being carried out to investigate the baceman stage of traditional and industrial soy sauce production in Indonesia (3, 11). It has been found that fermentation by yeast does not play an important role in traditional Indonesian *kecap* production. This is due to the fact that traditional *kecap*

is made only from whole soybeans which lowed in sugar content. After fermentation by *Pediococcus halophilus*, no substrate is left for growth and ethanol production by yeast (3). When compared with industrial soy sauce production which uses defatted soybean flakes and wheat with a higher sugar content, the fermentation by yeast occurs in the bacemen process and provides ethanol with a suitable aroma for the soy sauce product.

1.4.2 Malaysian soy sauce

Malaysian soy sauce is called “kicap” and can be classified into two kinds as; (i) a “thin” variety called *san-chau* or *pak-yau* (cantonese), and (ii) a “thick” variety called *sai-yau* or *lak-yau* (cantonese) (5).

The soy sauce production in Malaysia use soybeans and wheat flour as its main raw materials. The cooked soybeans are mixed with wheat flour at the ratio of 4 to 1, and the mixture is then spread on bamboo trays. The trays are placed on open shelves and naturally inoculated by air-born fungal spores for 6 days. Then the koji is transferred to earthenware vessels, covered with 20 % common salt brine in a ratio of 2 parts of brine to 1 part of koji, and fermented under direct sunlight for about 3 months. The liquor or sauce is then extracted by siphoning. The sauce is processed by adding monosodium glutamate or sugar to improve flavor and benzoic acid is added as a preservative. This sauce is now known as the “thin” variety or *san-chau*. This sauce can be blended further with caramel to form the “thick” variety or *sai-yau* (5).

1.4.3 Thai soy sauce

Thai soy sauce is called “se-iew” (10). There are four types of soy sauce classified by the Thai Industrial Standards Institute, Ministry of Industry. These are (i) ordinary soy sauce, (ii) salty black soy sauce, (iii) black soy sauce and (iv) sweet soy

sauce. Ordinary and salty black soy sauce are available in two grades, special grade and first grade. In black or sweet soy sauce, there is only one grade (10). The standards for all soy sauce varieties are outlined in Table 1.

The traditional soy sauce production in Thailand has been described by Bhumitatana et al., 1988 and Flegel et al., 1981 (10). Cooked soybeans are mixed with either wheat or rice flour in the ratio of 7 to 1. Then the mixture is left for natural inoculation by air-borne koji molds. After a few days, the finished koji is transferred to earthenware containers and immersed in brine solution (22 to 25 %) at the ratio of koji to brine of 1 to 3 (w/v). This mixture is called the moromi mash and has a salt concentration around 18 to 21 %. The moromi mash is then fermented under direct sunlight for a few months. The mature mash is then harvested to obtain the liquid part (soy sauce) by separating and filtering. The sauce is commonly pasteurized prior to bottling.

There are few large factories in Thailand that have the major soy sauce market share. They are well equipped and use sophisticated machines and facilities imported from abroad to produce soy sauce. However, larger number of Thai soy sauce factories in small and medium size with low capacity to develop or improve their production process for better quality soy sauce or to reduce the cost of production (6). Researches and developments in soy sauce production have been carried out long times ago. And about six years ago, a small research and development unit, the Quality Control and Training Center for Soybean Fermentation (QCTC-Soybean Fermentation) was set up with support from the Thailand Research Fund and a consortium of small and medium size soy sauce manufactures. The objectives of this program were to improve the quality of soybean fermentation products and to improve

Table 1 Standards for various Thai soy sauce products (13).

Properties	Type of soy sauce					
	White soy sauce	Salty dark soy sauce		Dark	Sweet	
	special grade	first grade	special grade	first grade	soy sauce	
Protein (N x 6.25) not less than, % by weight	5.5	4.5	8.5	7.5	2	1.5
Solid content not less than, % by weight	32	30	35	32	50	50
Salt (NaCl), % by weight	17 to 23	17 to 23	17 to 23	17 to 23	8 to 16	not over 1
Total sugar (as incerted sugar) not over, % by weight	7	6	12	10	25	80
pH	4.5 to 5.3	4.5 to 5.3	4.5 to 5.3	4.5 to 5.3	4.5 to 5.5	4.5 to 5.5
Specific gravity at 27 ± 3 °C not less than	1.20	1.20	1.23	1.23	1.33	not specify

manufacturing techniques. Examples of achievements of this program are the introduction of pure *A. oryzae* inoculum in the koji fermentation, the use of large fiberglass tanks instead of small earthenware jars for moromi fermentation, the introduction of new techniques to prepare koji and the introduction of cost effective waste treatment systems (10).

2. Raw materials in soy sauce production

2.1 Soybeans

Soybeans are known botanically as *Glycine max* (L) Merr (4). They constitute a major vegetable protein consumed commonly in Asia. There are slight differences in the chemical composition of soybeans from different countries. Generally, soybeans contain 34.3% protein, 26.7% carbohydrate, 17.5% fat, 4.5% fiber, 5.0% ash and 12% water (14). Also, they contain some minerals and vitamins at high levels, e.g. calcium (20.8 mg/100 g), iron (6.5 mg/100 g) and thiamin (1.03 mg/100 g) (4).

Soy sauce production can use either whole soybeans or defatted soybean meal as the substrate. In Japan, about 75 % of shoyu production uses defatted soybean instead of whole soybean because of lower cost, shorter time of moromi fermentation and higher utilization of nitrogen when compared to whole soybeans (4). However, the soy sauce product produced from whole soybeans still possesses better characteristics than that produced from defatted soybean meal and the product is more stable. Also, soy sauce prepared from whole soybean is known to better resist microbiological spoilage and to have a greater resistance to yeast invasion than that produced from defatted soybean meal (4). Soy sauce produced from whole soybeans has a lighter color, better color stability and better organoleptic properties than that produced from

defatted soybean meal (15). In Thailand, soy sauce manufacturers usually use whole soybeans as their raw material, mainly because the residue mash from the moromi fermentation is used as a low grade soybean paste.

2.2 Wheat

Either whole wheat or wheat flour may be used in soy sauce production.

Yokotsuka (4) listed the roles of wheat in soy sauce manufacture as follows:

- a) to make the moisture content of material to be cultured with mold just adequate for mold growth,
- b) to assist in obtaining the highest proteolytic activity from the koji,
- c) to serve as the major source of carbohydrate as the precursors of sugars, alcohol and organic acids,
- d) to serve as the source of lignin and glycosides, the precursors of vanillic flavor of soy sauce, and
- e) to serve as a rich source of glutamic acid (4).

The use of wheat decreases the nitrogen content of soy sauce, but it contributes aroma, flavor and glutamic acid (4). The ratio of soybean to wheat varies according to the types of soy sauce and manufacturers. The best soy sauce is generally believed to be made from a soybean to wheat ratio of 50:50 by weight (4, 16) or 52:48 by volume (16). Therefore, the Koikuchi-type shoyu of Japan which used this ratio of soybean to wheat provides the strong savory flavor and aroma of the final soy sauce product.

In Thailand, wheat flour is quite expensive because it has to be imported from overseas. Therefore, soy sauce manufacturers use rice flour instead of wheat flour

either partially or totally (17) for preparation of koji mixture. The use of rice flour in place of wheat flour results in a lower quality of soy sauce.

2.3 Salt

Commercial grade sea salt is commonly used in soy sauce production. The salt plays important roles in the moromi fermentation as follows:

- a) to act as a preservative to prevent the growth of spoilage microorganisms and to exert a selective condition for the microorganism which grow in the fermentation,
- b) to permit the exclusive development of flavor and aroma-forming yeasts and lactic acid bacteria, and
- c) to promote growth of halophilic and halotolerant bacteria (4).

The salt concentration and amount of salt solution used in moromi fermentation varies in each region, depending on the manufacturer. In Japan, brine solution of 17 to 19 % w/v (sodium chloride) is added to koji at 1 to 1.1 to 1 to 1.2 (4). In Thailand, brine solution of 22 to 25% w/v is added to the koji in the ratio of 3:1 (w/v) (10).

3. Production of fermented soy sauce

Soy sauce is made by fermentation of a combination of soybean, wheat grain, water and salt (7, 18). The processes for production of fermented soy sauce consist of three major steps, namely, koji production, brine (moromi) fermentation and refining (1, 7). The flow sheet for manufacture is shown in Figure 1.

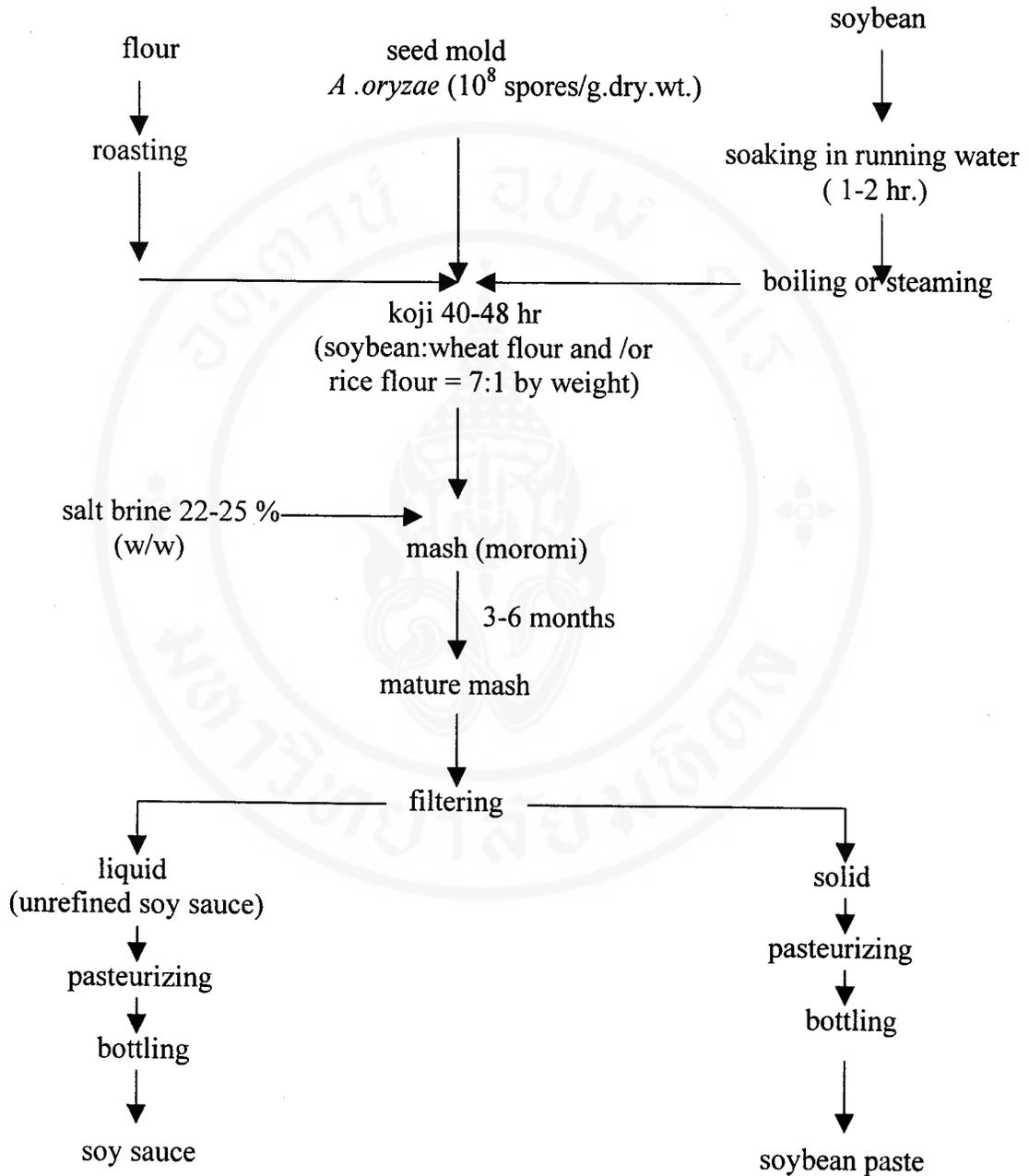


Figure 1 Process for manufacturing soy sauce and soybean paste in Thailand (10).

3.1 Koji production

Koji is a source of proteolytic enzymes for converting soybean protein into amino acids and amylase enzymes for converting carbohydrates into simple sugars. The substances converted by the enzymes in koji become the nutrients of lactic acid bacteria and yeast in the subsequent brine fermentation (1, 7).

In koji production, soybean or defatted soybean grit are soaked in water to increase the moisture content and cooked under pressure. The current soybean cooking method in Japan employs continuous cookers which allow for high pressure and short time cooking. Moistened soybeans are cooked at 6 to 7 kg/cm² pressure for 20 to 30 second and then cooled down quickly (1, 7). In Thailand, soaked soybeans are cooked with boiling water for approximately two hours. Recently, many factories have changed to cooking the soybean with steam at 10 to 13 pounds/ inch² pressure for one hour. After that, the cooked beans are drained and quickly cooled (19).

In koji making for Japanese soy sauce, wheat is cooked in a continuous roaster with hot air at 150°C for 30 to 45 s at atmospheric pressure and then cracked into 4 to 5 pieces per kernel (1, 7). For making regular Japanese soy sauce, cooked soybeans or defatted soy flakes are mixed with an equal amount of the roasted wheat and then inoculated with pure cultures of *A. oryzae* or *A. sojae* at 0.1 to 0.2%. The koji mixtures are spread at 3 to 5 cm thickness on wooden trays and incubated for 2 to 3 days in a koji making room. The temperature in this room is kept between 25 to 35°C (16). When the mold grows, it produces metabolic heat. Therefore, if the temperature of the mixture become too high (about 40°C), it is necessary to cool it down by stirring. In usual practice, the koji is stirred to reduce the temperature to about 20 to 25°C between 25 to 40 hours after the initial inoculation with mold (4). It is important

to control the temperature and moisture content of the koji mixture at an appropriate level in order to produce good quality koji. After incubation at 25°C for 48 to 72 hours, the koji becomes a greenish yellow mass that results from mold growth and sporulation (7). Nowadays, koji fermentation in Japan uses the automatic koji rooms which can control temperature and moisture content automatically. The koji making process of Thai soy sauce involves mixing of cooked soybeans with wheat or rice flour in the ratio of 7 to 1 (10). However, the ratio varies slightly depending on manufacturer. Some factories use a rotating drum or cement mixer for mixing koji. Nowadays, many factories add 0.1 % of a pure spore inoculum of *A. oryzae* into the soybean and flour mixture for making good quality koji.

3.2 Moromi fermentation

The second step in making fermented soy sauce is brine fermentation or moromi fermentation in Japanese terminology. This fermentation is unique in that it utilizes halophilic lactic acid bacteria and salt tolerant yeasts (1). The presence of brine (16 to 19%) effectively prevents growth of undesirable microorganisms, but promotes growth of halophilic lactic acid bacteria and salt tolerant yeasts (7).

In Japanese soy sauce production, the finished koji is mixed with 110 to 120% or more of brine solution. The resultant mixture is called the moromi mash and contains 17 to 19 % w/v sodium chloride (4). For Thai soy sauce, the ratio of brine solution to koji is about 3 to 1 (w/v). After mixing, the moromi mash usually contains a salt concentration of around 18 to 21 % (w/v) (10). It is dangerous to allow the

moromi mash to contain salt concentrations less than 18 %, because the low salt concentration will cause spoilage of the mash (4).

In Japanese soy sauce, the moromi mash is fermented in a wooden, concrete or steel tank (5) and it takes about one year at ambient temperature. The incubation period can be shortened to 3 to 4 months if warmed artificially to about 35 to 40°C. During the fermentation period, the mash is stirred with brief aeration to stimulate microbial growth (1). In Thailand, many factories have now adopted the use of large fiberglass tanks instead of earthenware jars for moromi fermentation. However, others still use the older process. The moromi mash is fermented in fiber glass tanks for a few months in direct sunlight (10).

During the fermentation period, enzymes from the koji hydrolyze most of the protein in the raw materials to amino acids and low molecular weight peptides (1). About 20% of the starch is used by the koji mold and the remainder is converted to simple sugars which are fermented primarily to lactic acid and ethanol by the lactobacilli bacteria and yeasts, respectively (20). The pH drops from an initial value of 6.5 to 7.0 down to 4.7 to 4.8 (1).

In the high salt moromi fermentation, only salt-tolerant microbes can grow. Halotolerant lactic acid bacteria such as *Pediococcus halophilus* is the first group of microorganism which grow in the moromi mash. The growth of this bacterium results in the formation of organic acids such as lactic acid and it leads to acidic conditions in the mash (4). The initial pH of the mash is about 6.0 to 7.0. After *P. halophilus* produces lactic acid, it decreases to about 5.0. This intense allows *P. halophilus* to reach a maximum level after which it cannot grow further due to its low tolerance to acid (13). Next, halophilic yeast species such as *Zygosaccharomyces rouxii* grow

vigorously and produce ethanol and other flavoring compounds (7, 21). After the lactic acid and ethanol fermentations are completed, the mash is aged until the moldy odor has disappeared (10, 21).

Nowadays, the Japanese soy sauce industry has developed the moromi fermentation by adding pure cultures of *P. halophilus* and *Zygosaccharomyces rouxii* to the mash at the beginning and at 1 month, respectively (7). During the aging period, another yeast, *Candida versatilis*, grows in the mash and produces phenolic compounds (1) such as 4-ethylguaiacol (4EG) and 4-ethylphenol that add typical aroma to soy sauce. Therefore, the addition of *Candida* yeasts along with or following *S. rouxii* is usually recommended (6).

3.3 Refining

The final process for production of fermented soy sauce is refining. This includes pressing or filtration and pasteurization. Then the product is bottled and packaged (7, 1).

3.3.1 Pressing

The liquid fraction on the top of the aged moromi mash is separated by drawing off or siphoning. The remaining moromi mash which contains an undigested solid soybean-flour mixture (soy cake) and some liquid fraction is processed further to remove raw soy sauce from the soy cake by filtering with a membrane filter, a filter press, or a hydraulic press machine in modern factories (4, 7). The residue moromi mash from the first drawing can be used further to produce a second grade soy sauce by adding more brine solution to it, followed by further incubation for some period of

time. This process may be repeated several times. The soy sauce from the first drawing is the best grade. In Thailand, the moromi mash residue can be used in two ways, (i) for addition of more brine solution to produce a lower grade soy sauce or (ii) for bottling as low grade soybean paste (19).

3.3.2 Pasteurization

Raw soy sauce that results from siphoning and pressing is heated to pasteurization temperature (70 to 80°C) either in kettle or in a heat exchanger (5) for a few minutes to inactivate enzymes and kill microbes (7). The pasteurization is very important for soy sauce production for many reasons as follows,

- a) to develop large numbers of compounds such as aldehydes, acetals, mercaptans, mercaptals, organic acids, pyrazines, furfural and others which contribute to the sharp flavor and aroma characteristic of soy sauce,
- b) to develop a reddish brown color,
- c) to improve clarity by precipitating heat-coagulable substances (4, 5),
- d) to increase resistance to growth of film yeasts by production of organic acids and phenolic compounds,
- e) to inactivate most of the enzymes (1, 4, 7), and
- f) to inactivate any undesirable microorganisms (1, 7).

After pasteurization, it may be necessary to clarify the soy sauce additionally by centrifugation or sedimentation (7). Then, the clear supernatant is bottled and packed. Usually a preservative such as sodium benzoate is added to the resultant filtrate before bottling (1). In Japan, Butyl-*p*-hydroxybenzoate at a concentration of 0.005 % or sodium benzoate at 0.02 % are widely used (4). In Thai soy sauce, sodium benzoate is used at a concentration of not more than 0.01 % (19).

4. Protein hydrolysate

Protein hydrolysate can be used for many purposes such as enhancing the flavor of food products (22), supplementing food for food allergy or food hypersensitive patients (23) and feeding patients (24) who can not digest intact proteins (25).

The protein raw material used in hydrolysis may originate either from plants (e.g., soybean), animals (e.g., casein) or microorganisms (e.g., yeast).

Protein hydrolysate can be produced using acid, alkali or enzymatic methods, but enzymatic method is strongly preferred over chemical methods because it destroys fewer essential amino acids (26). Hydrolyzed vegetable protein (HVP) is one example of an acid protein hydrolysate (8). Production of gelatin is an example of alkaline treatment (27). Hydrolysis of soy protein with microbial acid proteases is used to prepare a lemonade-like flavored beverage (28) and casein hydrolysis is used for hypoallergenic infant formulae (25) by using the enzymatic method. The enzymatically hydrolyzed products are used for food or nutritional purposes because they are safer than chemically hydrolyzed products.

4.1 Source of protein used as a raw material for production of protein hydrolysates

There are several kinds of proteins that can be used as raw materials for production of protein hydrolysates. These proteins originate from animals, plants or microorganisms. The proteins commonly used in nutritional protein hydrolysate

include casein, whey protein and soy protein. Other sources of protein, include gelatin, rice, potato, fish and egg albumin are less commonly used for economic reasons (26).

4.1.1 Proteins from plant origin

Wheat can be subjected to alkaline hydrolysis to produce wheat protein concentrate (WPC) which contain 60 to 80% protein of very good nutritional quality. Gluten, one of the most common proteins from wheat, was first isolated by an Italian scientist named Beccari about 200 years ago. Wheat gluten contains good functional properties after processing. These include viscoelasticity and good emulsifier activity so it can be modified chemically to enhance water binding and solubility properties. Therefore, it is used primarily as an extra source of protein in foods such as bakery and breakfast foods in the USA and Australia (29). WPC has more lysine than wheat flour. Therefore, it is suggested as a protein of good flavor (wheaty) and color (brown) to fortify wheat based food. Wheat gluten is processed by acid hydrolysis to produce HVP. It is considered as an excellent material for the production of HVP because of its high content of glutamic acid (8).

Mungbean protein, a by-product separated from the production of vitreous mungbean vermicelli, contains as high as 72% (dry basis) of protein (30) which is composed of all the essential amino acid (31). Mungbean protein accounts for about 27% of the mungbean raw material (30, 31) that is used in vermicelli industry and it is sold as an ingredient for animal feeds. It would be better if this large amount of mungbean protein were hydrolyzed to yield a favoring product that could be used as a flavoring ingredient in food products such as soups, sauces, meat, fishery product and ready-to-eat foods. Jantawat et al. (30) reported on the optimal conditions for acid hydrolysis of mungbean protein and studied the influence of hydrolysis conditions on

chemical and sensory characteristics of the product (30). Thomson (31) also studied the use of the alkaline hydrolysis to isolate mungbean protein from the whole mungbean. Mungbean is suitable for supplementing cereal-based products because its amino acid pattern is better than that in cereals. However, mungbean protein has a beany flavor and a dark color that is imparted to the final product. To overcome this problem, the beans must be sufficiently dehulled before milling into flour or the protein must be isolated from the mungbean to provide a better appearance and taste when compared with the original meal. Mungbean protein can be used as a nutritional and functional ingredient in many food products (31).

Coconut, a major industrial crop in Philippines, is a source of oil and protein. However, oil is the only component used for food in major quantities. The protein in the residue is used primarily as a feed supplement similarly to soybean meal. Pham and Rosario (32) studied the acid hydrolysis of coconut using 10 N HCl and 18 N H₂SO₄ in two separate tests and found that it took about 48 hr to complete hydrolysis at 95°C. Presently, protein hydrolysate from coconut has very limited application in the food industry.

The amount of soy protein production in the world is about equal to that of animal protein (33). However, vegetable protein is much less used as a food component since it lacks some of the desirable functional properties required for food products. Improvement in the properties of soy proteins may increase its utilization as a source of nutritive protein. Soy proteins are high in lysine and low in methionine. Thus they are appropriate to supplement cereals which tend to have a limited lysine content (33). There are several reports about the improvement of soy protein properties. Most of them involve preparation of protein hydrolysate which is one

strategy to modify soy protein and increase its utilization. Soy milk contains many kinds of unpleasant flavor components that are quite difficult to eliminate completely from it or its products. Sugimoto et al. (28) indicated that treating commercially isolated soy protein with an acid-protease derived from the higher fungus, *Trametes sanguinea*, at pH 4.6 can lead to a bland-tasting enzymatically modified soy protein soluble.

Soy protein hydrolysate can also be used in fruit juices except for the fact that the pH of fruit juice often coincides with the isoelectric pH region of soy protein. However, insoluble soy protein can be eliminated by enzymatic hydrolysis. Roozen and Pilnik (33) used ultrafiltration to select medium range molecular weight peptides of the hydrolysate mixture to introduce into fruit juice products. The molecular weight had to be low enough to ensure solubility in the acid fruit juice but not so low as to give a bitter taste. As mentioned above, that molecular size of hydrolysate products is a major factor governing functional properties (34). Therefore, it would be better if one could determine the molecular size of hydrolysate products. Deeslie and Cheryan (35) suggested an approach to hydrolyze protein by entrapping protease in a membrane reactor so that the molecular size of the hydrolysate products could be controlled by proper selection of the membrane. Zhang et al., 1996 (34) investigated the hydrolysis of soybean proteins using a vortex flow filtration membrane (VFM) reactor with hydrolyzed with *Aspergillus oryzae* protease. They found that the hydrolysate from this VFM reactor was very promising for giving value-added products from soybean protein. Soybean protein concentrates or a blend of these vegetable proteins have been used as the major raw material for producing HVP (8).

4.1.2 Proteins from animal origin

The proteins of animal origin used as substrates to produce protein hydrolysates can be classified into 2 groups. The first one is proteins from dairy products (e.g., casein, whey and cheese). The second group is proteins from meat by-product (e.g., offal of poultry, pork, fish, etc) or egg albumin.

Protein hydrolysates derived from enzymatically digested casein and whey are commonly used in hypoallergenic infant food formulae. Mahmoud et al. (25) studied the effect of the degree of hydrolysis on antigenicity and physical properties of casein-derived enzymatic hydrolysates. They found that extensive hydrolysis of casein could provide virtually nonantigenic food and also resulted in considerable reduction in emulsifying property. Haque and Mozaffar (36) studied the continuous production of casein hydrolysate by using an immobilized enzyme bioreactor. The results showed that papain, trypsin, chymotrypsin and Rhozym-41 immobilized onto amino propyl-(AP)-glass beads were stable in batch or continuous stirred tank reactors and yielded satisfactory casein hydrolysates. Whey protein hydrolysate can be prepared using either Alcalase® 0.6L (37) or a commercial pancreatic protease such as Corolase® PP (procine), Pancreatin® (porcine) and PEM 800S (bovine) (38). The α -lactalbumin, insoluble heat denatured whey protein (38) and β -lactoglobulin can also be used as substrates (37) for preparing whey protein hydrolysates.

In acidified cheese production, proteolytic enzymes alone or combined with lipolytic enzymes are used to direct and accelerate ripening and flavor development in cheese (39). Lazaridis et al. (40) has reported the successful use of protease derived from *A. oryzae* to control the meltability in directly acidified cheese products. The

optimum temperature and pH for the enzymatic reaction were reported to be 50°C and 7.0, respectively.

Eggs are commonly use in food products, and the demand for them may increase if their functional properties can be improved. Therefore, several researchers have attempted to modify egg albumin in order to enhance its functional performance. One of the methods have been enzyme hydrolysis. Kewpie (41) found that proteolytic enzymes from *A. oryzae* and *S. griceus* could enhance the foaming of frozen whole egg products.

In the meat industry, it has been estimated that the amount of protein currently discarded as process waste or merely downgraded to animal feed or fertilizer may represent the equivalent of one eighth of the total lean meat content of slaughtered animals (42). The majority of this protein is of high nutritional quality and obviously represents substantial economic loss to the meat industry. For example in 1996, Fonkwe and Singh (43) studied protein recovery from mechanically deboned turkey residue (MDTR) by enzymic hydrolysis. The hydrolysis process could recover 46% of MDTR proteins. The hydrolysate contained 78% protein, 4.6% ash and only 5.7% fat. In fish, there are several reports about recovery of protein from offal since there is a large amount of waste from finfish and shellfish fisheries (44). Fish protein has a good pattern of essential amino acids and it potentially has good nutritive value (45). The hydrolysis of whole male capelin with Alcalase® 2.4L resulted in the successful production of fish protein hydrolysate (FDH) in powder form. It was high in protein, low in fat, relatively odorless and light yellow in color (45). Fish stomachs and intestines can be utilized for food, feed and biotechnological purposes. For example, they contain a high concentration of pepsin (9 g/l) that can be obtained by

ultrafiltration of the aqueous phase. For example, a Trypsin-like enzyme concentrate could be obtained by ultrafiltration of fish sauce produced by salt fermentation of cod intestines. The hydrolysis of headed-gutted and frame mince of red hake (*Urophycis chuss*) with the commercial enzymes, Flavourzyme® and Savorase®, resulted in a seafood flavor (46).

In shellfish, protein recovery from the cephalothorax of Brazilian Lobster (*Panulirus spp*) processing wastes was performed by enzyme hydrolysis with papain, pepsin and fungal protease (47). The fungal protease gave the highest degree of hydrolysis. The protein hydrolysate product was highly soluble, with excellent wettability, some capacity for oil emulsification and no bitter off-flavor (48). Webster (48) also reported on protein hydrolysates from meat industry by-products. Bovine lungs, bovine rumen and partially defatted tissue were hydrolyzed at 50°C with either pepsin, papain, neutrase or alcalase. Papain was found to be the most effective hydrolysing agent while neutrase was the least effective.

4.1.3 Proteins from microbial origin

The most common source of microbial protein for hydrolysis is yeast. Yeast extract is a commercially available hydrolysate product produced from yeast hydrolytic enzymes (autolysis) or by other methods (plasmolysis or hydrolysis) in order to release the cell contents in a highly degrade form. Yeast extract is popular for use as a natural flavoring agent and a growth promoter of microorganisms in industrial fermentation. Yeast extract can be produced from brewers and bakers yeast, *Saccharomyces cerevisiae* or other yeast species such as *Candida utilis* or *Kluveromyces marxianus* (8).

Otero et al. (49) reported that acid hydrolysis of dried fodder's yeast, *C. utilis*, can be used to prepare an imitation soy sauce product. An aqueous yeast suspension (15%) was poured into the stainless steel reactor, mixed with 15% of HCl, and the reaction was allowed to run at 150°C for 40 min. Then the reactor was cooled rapidly, neutralized and centrifuged. This raw imitation soy sauce was supplemented with 12% NaCl, 0.1% monosodium glutamate, 2% vinegar and 0.1% sodium benzoate and evaluated by sensory analysis. It was found that the imitation sauce from yeast was at least as good as commercial hydrolyzed soybean sauce.

5. Process for production of protein hydrolysate

Protein hydrolysate can be produced by using either acid, alkaline or enzymatic methods (26). In comparison with chemical hydrolysis, enzymatic hydrolysis has several advantages. For instance, enzymes have unique specificity of action and function under mild conditions and deactivation of the enzymes after use by heating makes the downstream processing much simpler (50). Furthermore, enzymatic hydrolysis can produce hydrolysates with a well-defined peptide profile (26) and there is no destruction or racemization of amino acids so that the nutritional quality of the original protein is retained (51). While acid and alkaline hydrolysis can destroy L-form amino acids, produce D-form amino acids and can lead to the formation of toxic substances like lysino-alanine (26). Therefore, the enzyme hydrolysis is preferable method for use in food applications to render better functional properties without deterioration in nutritional value (52).

5.1 Production of protein hydrolysates by acid hydrolysis

The acid hydrolysis of mungbean protein powder (i.e., the by-product powder obtained from the production of vitreous mungbean vermicelli) was carried out by mixing 10 g of mungbean protein powder with 6 M hydrochloric acid (HCl) at the ratio of 1 to 3 in Erlenmeyer flask. It was then hydrolyzed by autoclaving at 120°C for 6 hr. The hydrolysate was cooled to ambient temperature and pH was adjusted to 5.5 to 6.0 before filtration to remove precipitates. The hydrolysed product gave high amino nitrogen and the highest amino nitrogen to total nitrogen ratio with a good odor score (30).

Hydrochloric acid is also used for preparing protein hydrolysates from defatted coconut and soybean meals (32), for acid hydrolysis of waste shrimp-shell to obtain glucosamine (53) and for preparing imitation soy sauce from hydrolyzed dried yeast *C. utilis*. In some cases, sulfuric acid (H₂SO₄) can be used instead of HCl, such as in the preparation of protein hydrolysate from defatted coconut and soybean meals. However, the conditions of hydrolysis may be different. For example, hydrolysis of soybean meal with 6 N HCl is complete after 36 hr at 95°C while it takes only 24 hr to complete the process when 18 N H₂SO₄ is used instead at the same temperature (32).

5.2 Production of protein hydrolysates by alkaline hydrolysis

Among the three methods of protein hydrolysis, alkaline hydrolysis is used least in food applications. Alkaline hydrolysis can be used for preparing gelatin type B. There are two types of gelatin; type A and type B. Type A is produced by acid processing of collagenous raw materials and has an isoelectric point between pH 7 and 9. Type B is alkaline or lime processed and has an isoelectric point between pH 4.8

and 5.2. Gelatin is widely used in food applications such as in meat products, dairy products and confectionery products, but the largest use is for making gelatin desserts (27).

In 1991, Dave et al. (54) studied alkaline hydrolysis of casein for preparing canned tuna fish. About 4.4 kg of casein was hydrolyzed with 60% w/v of sodium hydroxide solution. The casein hydrolysate was used to supplement canned tuna products (55).

5.3 Production of protein hydrolysates by enzymatic methods

Enzymatic hydrolysis of protein using selected proteolytic enzymes to split specific peptide bonds is widely used in the food industry (56). The degradation of protein into peptides increases solubility and improves functional properties such as taste, viscosity, whippability, emulsifying and foaming capability (52).

Proteolytic enzymes such as trypsin and chymotrypsin (animal proteases), papain, bromelain and ficin (plant proteases) and alcalase and neutrase (microbial proteases) can be used in hydrolytic processes (50). The protease from microorganisms seems to be more widely used than either plant or animal proteases since their production is cheap and easy to scale up and since they usually have broad specificity toward substrates. The process for production of protein hydrolysates by enzymatic methods is illustrated in Figure 2.

There have been several reports on the use of plant proteases to produce protein hydrolysates. Fonkwe and Singh (43) reported that papain could be used for recovering potentially edible protein from mechanically deboned turkey residue (MDTR). The hydrolysis was undertaken by mixing 500 g of MDTR with 1 litre of

water on a 60°C water bath. Then papain was added at the ratio of MDTR to enzyme of 250 g MDTR to 1 g of papain. The mixture was stirred for 30 to 45 min, then filtered and centrifuged. The enzyme was inactivated by heating at 95°C for 15 min and cooled down. In general, such hydrolysate products have potential for use in a wide range of applications due to excellent solubility in water over a wide range of pH's. Webster (48) also reported on the use of papain to hydrolyze protein from meat industry by-products such as bovine lungs, rumens and partially defatted tissue.

A animal proteases can be used for hydrolyzing of protein from dairy products. For example, casein can be enzymatically hydrolyzed by porcine pancreatin for potential use in hypoallergenic infant formulae. The commercial acid casein is dissolved in deionized preheated water (45 to 50°C) and brought to pH 7. Then porcine pancreatin, 4X USP is added into the mixture and hydrolyzed to obtain the final casein hydrolysate with an amino nitrogen (AN) to total nitrogen (TN) ratio of 0.55 to 0.65. The mixture is maintained at pH 7 throughout hydrolysis. The finished product is heated in a boiling water bath for 10 min, then, rapidly cooled to 20-25°C and frozen at -20°C for 1 week before analysis.

Microbial proteases can be produced from *Bacillus subtilis*, *A. oryzae*, *A. niger*, *S. cerevisiae* or *K. fragilis*. These proteases are widely used in protein hydrolysis. The fungal proteases derived from *A. oryzae* have been developed to control the meltability of direct acidified cheese products (40). The acidic-protease preparation from *T. sanguinea* is used in the hydrolysis of bland soybean protein for producing a lemonade-like flavored protein-containing beverage which contains no unpleasant flavor (28). The process for production of hydrolysates is as follows. A cooked suspension of 5% (w/w) Promine D (commercial neutral isolated soybean



protein in sodium form) is mixed with a small amount of an acid-protease preparation from *T. sanguinea*, adjusted to pH 3.3 to 3.5 with concentrated lemon juice and incubated at 50°C for 8 to 10 hr. After enzyme inactivation by heat treatment, the hydrolysate product is centrifuged to separate the insoluble part. The clear supernatant hydrolysate product possesses excellent functional properties and is almost entirely free from unpleasant flavors, including bitterness and meat-like taste and it has high buffer capacity. Alcalase®, which is a commercial microorganism protease, can be used for hydrolyzing many kinds of protein such as whole male frozen capelin fish (45), whey or whey protein concentrates (37) and blood protein (55).

In the hydrolytic process, the type of enzyme, concentration of enzyme, conditions of reaction and degree of hydrolysis can effect hydrolysis efficiency and physicochemical properties of the resulting hydrolysates (56). These conditions have to be selected for the appropriate type of enzyme, suitable concentration of enzyme and optimal conditions for the reaction. For example, in the hydrolysis of Brazilian lobster processing wastes, the enzyme papain, pepsin and fungal protease were used and each was tested at concentrations of 2, 4 and 5 mg/g (enzyme/substrate, wet weight basis). It was found that hydrolysis at 37°C by fungal protease at 5 mg/g produced the highest degree of hydrolysis (47). Webster (48) also reported that among the three enzymes papain, neutrase and alcalase used in hydrolysis of meat industry by-products, papain was the most effective while neutrase was the least effective. The degree of hydrolysis depends on the intended use of the hydrolysate. With milk protein for example, a low degree of hydrolysis is desirable for maintaining functional properties. Extensive hydrolysis may be necessary when the hydrolysate is

to be used for specific purposes such as chemically defined or hydroallergenic infant formulae (56).

6. Application of protein hydrolysates

The major application of protein hydrolysates is in the food industry. They have been used for protein enrichment of soft drinks and juice-base beverages (57) and as flavor enhancers. Protein hydrolysates are also used in medical diets such as hypoallergenic infant formulae for food allergy patients and as foods for specific diseases (24). Furthermore, protein hydrolysates can be use as growth promoters in culture media for many kinds of cell cultures.

6.1 Protein hydrolysates for food applications

Protein hydrolysates are used as food ingredients to provide meat-like savory flavors in the form of amino acids and peptides (58). They have good physicochemical properties such as high solubility, low viscosity and high protein content. Proteins increase emulsification and stabilization, increase viscosity, improve appearance, taste or texture and bind with fat or water. Nutritional benefits include lowering the caloric content of foods, increasing the protein level and balancing the amino-acid profile. The application of proteins in food products is shown in Table 2 (27).

Protein hydrolysates in food products can improve functional properties. For example, enzyme hydrolyzed soy protein curd contributes whipping and foaming properties to products and oil-free soybean flakes provide functional ingredients.

These hydrolyzed soy proteins may be used in dairy products, confectionery products,

Table 2 Functions of food proteins and their applications (27).

Function	Application	Protein type
Nutrition	Infant formula, protein-enriched foods	Soy, milk proteins
Solubility	Beverages, liquid and moist foods	Whey, soy proteins
Viscosity	Soups, sauces, salad dressings, yogurt	Various proteins
Water holding	Meat and seafood products, baked goods, yogurt	Muscle proteins, egg, milk and soy proteins
Gelation	Meat products, baked goods, dairy products, gelatin-type desserts	Muscle proteins, egg, soy and milk proteins
Adhesion/cohesion	Meats, sausages, pasta, baked foods	Muscle, egg, and whey Proteins
Emulsification	Sausages, salad dressings, Sauces, baked goods	Muscle, milk, egg, and soy proteins
Foaming/shipping/ film formation	Confectionery, baked goods, whipped toppings, frozen desserts	Egg proteins, milk and soy proteins

baked goods and beverages (27). Roozen (33) reported that the hydrolysis of soy protein can eliminate the insolubility of soy protein at isoelectric pH of them which coincides with the pH as would occur in fruit juice. Protein hydrolysates with high solubility, low viscosity and high palatability give a unique potential for protein enrichment of soft drinks and juice-based beverages (57). The emulsifying property of hydrolyzed proteins is improved by carefully controlling the extent of hydrolysis. If proteins are too extensively hydrolyzed, their emulsifying capacity will decrease substantially. Therefore, in some nutritional formulae which incorporate such hydrolysates stabilizers of the emulsion may be required (59).

6.2 Protein hydrolysates for nutritional and therapeutic purposes

Protein hydrolysates possess a number of function properties which make them attractive as a protein source in human nutrition, both in products for special medical use and in consumer products for more general use (58). Medical foods are designed to provide complete or supplemental nutritional support to individuals who are unable to ingest adequate amounts of food in a conventional form, or to provide special nutritional support to patients who have special physiological and nutritional needs (24). The use of protein hydrolysates as a dietary nitrogen source is already well established within the medical area e.g., in patients with impaired gastrointestinal function either caused by a reduced absorption surface (e.g., short bowel syndrome and Crohn's disease) or by a reduced digestive capacity (e.g., cystic fibrosis and pancreatitis). Highly hydrolyzed proteins are also used in hypoallergenic formulae, especially for infants with allergies to some kinds of foods such as milk protein (57).

Certain hospital patients such as elderly people or post-operative people may have difficulty in obtaining sufficient protein. Therefore, protein-enriched juice or soft drinks may be formulated with casein hydrolysate (27). Casein hydrolysate is used because of its quick absorption and high solubility at various pH ranges (27). Casein phosphopeptides isolated from casein hydrolysates have a potential as an enhancer for gastrointestinal absorption of calcium.

6.3 Protein hydrolysate as a growth promoter in culture medium

The protein hydrolysates from casein and tryptic meat can be used as growth promoters in culture media. Casein hydrolysates hydrolyzed by alcalase, chymotrypsin and trypsin can promote the growth of *Bifidobacterium* (60). The protein hydrolysate, Primatone RL, produced from tryptic meat digests serves as a source of amino acid, oligopeptides, iron salts, some lipids and other trace compounds of low molecular weight. It is a cost-effective multiple growth promoter of mammalian-cell culture in serum-containing and serum-free media and also displays anti-apoptosis properties (61). Singh (62) reported the enhancement of *Spirulina* biomass productivity by a protein hydrolysate. At the optimum dosage, the hydrolysate enhanced *Spirulina* productivity by almost 40% when compared to untreated cultures.

7. Proteolytic enzymes

Proteolytic enzymes have the capability to cleave peptide bonds of proteins to produce small peptides and amino acids (50). Protease is degradative enzyme which catalyzes the total hydrolysis of proteins (63). In the hydrolysis reaction, one mole of

water is added for every peptide bond cleaved and this can effect the mass balance of the amount of protein hydrolyzed and the final amount of hydrolysate product (64).

During the hydrolysis process, the protein can also be broken down by chemical and physical force such as strong chemicals, extreme pH and/or extreme temperatures. However, hydrolysis by protease enzymes is much gentler than that by chemical methods. There are several advantages of using proteases instead of physical or chemical treatments:

- 1) They have unique specificity of action, preventing potentially undesirable side reactions.
- 2) They function under mild conditions, avoiding extremes of temperature and pH and minimizing side reaction which usually occur during chemical and physical treatments.
- 3) They have an extremely high catalytic activity, requiring less processing energy than any chemical or physical treatment.
- 4) They are normally inactivated after use by subsequent processing, making additional removal steps unnecessary.

However, proteases also exhibit some serious drawbacks. For example, they are not sufficiently stable or active under normal physical properties of food, they cannot be reused because of the difficulty of separating them from the substrate (50).

Proteases have been classified according to purpose. They are grossly subdivided into two groups according to their sites of action; exopeptidases and endopeptidases. Exopeptidases cleave the peptide bond proximal to the amino (aminopeptidases) or carboxy (carboxypeptidases) termini of the substrate, whereas endopeptidases cleave peptide bonds distant from the termini or inner regions of the

substrate. Based on the functional group of the active site, catalytic residues and three dimensional structure, four major groups are recognized. These are serine proteases, aspartic proteases, cysteine proteases and metalloproteases (63). Serine proteases can be classified further into two subgroups, serine alkaline proteases that are active at highly alkaline pH and represent the largest subgroup of serine proteases, and subtilisins. Furthermore, proteases can be classified based on source, namely, plants, animals and microorganisms, and based on optimal pH profile which can be divided into three types, acid, neutral and alkaline proteases.

7.1 Source of protease enzymes

Since proteases are physiologically necessary for living organisms, they are ubiquitous and found in a wide variety of organisms such as plants, animals and microorganisms (63). Enzymes obtained from plants and animals are quite limited. Moreover, plant and animal availability also depends on season and environmental conditions and can result in erratic quality of enzyme extracts. Microbial enzymes are more economical because their production is cheap and easy to scale up. In addition, microorganisms produce a wider variety of specific proteases than either plants or animals (50).

7.1.1 Plant proteases

There are three major types of plant proteases that are commercially used. These are papain, bromelain and ficin (50). Papain is a traditional plant protease. It is extracted from the latex of *Carica papaya* fruits. Papain has a broader specificity than bromelain and ficin due to the presence of several proteinase and peptide isozymes. Bromelain is prepared from the stem and juice of pineapples. Ficin is

prepared from fig. Plant proteases act on the β -chain of insulin and are used in many industries. Applications include chill-proofing of beer, meat tenderization, manufacture of protein hydrolysates, baking, aiding digestion and medical treatment (65).

7.1.2 Animal proteases

The most familiar proteases of animal origin are pancreatic trypsin, chymotrypsin, pepsin and rennins (66). Trypsin is the main intestinal digestive enzyme. It has limited application in the food industry because it produces a strong bitter taste. Therefore, it is commonly used in the preparation of bacterial media and in some specialized medical applications. Chymotrypsin is found in animal pancreatic extract and is stored in the pancreas as the precursor, chymotrypsinogen, and is activated by trypsin in a multi step process. This enzyme is quite expensive and is used only for diagnostic and analytical purposes. Pepsin is the most acidic protease and is found in the stomachs of almost all vertebrates. The optimal pH of its function is 1 to 2, while the optimal pH of stomach is 2 to 4. Pepsin is inactivated above pH 6.0. Rennin is a pepsin-like protease found in stomach of all nursing mammals. It is produced as an inactive precursor, prorennin, and is converted to active rennin by the action of pepsin or by autolysis. Rennin is used extensively in the dairy industry to produce a stable curd with good flavor (63).

7.1.3 Microbial proteases

Proteases from microbial sources have broad biochemical diversity and are susceptible to easy genetic manipulation. Microbial proteases account for approximately 40% of the total worldwide enzyme sales (67). Most of bacterial proteases are produced by organisms belonging to the genus *Bacillus*. Bacterial neutral

proteases are active over a narrow pH range (pH 5 to 8). They provide less bitterness in hydrolyzed food protein than animal proteinases do. Therefore, they are valuable for use in the food industry. For examples, neutrase is used in the brewing industry. Bacterial alkaline proteases are active under highly alkaline conditions (e.g. pH 10) and their optimal temperatures are about 60°C. They also have broad substrate specificity and so are widely used in the detergent industry (63).

Fungi produce a wide variety of enzymes. For example, *A. oryzae* produces acid neutral and alkaline proteases. Fungal proteases are active over a wide range of pH (pH 4 to 11) and exhibit broad substrate specificity. They are conveniently produced in solid-state fermentation processes. Fungal proteases have lower reaction rates and lower thermotolerance than most bacterial proteases do. Fungal acid proteases have optimal pH at pH 4 to 4.5 and are widely used in the cheese making industry. Fungal, neutral proteases are active at pH 7.0 and are inhibited by chelating agents. They are used for reducing bitterness of food protein hydrolysates. Fungal alkaline proteases can be used in food protein modification (63).

8. Application of proteases

Proteases have a wide variety of applications, mainly in the detergent and food industries. They are also used to treat some diseases. Proteases used in the detergent and food industries are prepared in bulk quantities and used as crude preparations. By contrast, those for pharmaceutical use are prepared in small amounts and require extensive purification (63).

8.1 The detergent industry

The use of proteases in laundry detergents accounts for approximately 25% of the total worldwide sale of enzymes. The first enzymatic detergent was introduced in 1913. It consisted of sodium carbonate and a crude pancreatic extract (pepsin) (50). The first detergent containing bacterial enzymes was introduced in 1956 under the trade name BIO-40 (63). The bacterial enzymes used comprise a mixture of serine and metal microbial proteases that are less degradable by soap, alkali and high temperatures (50).

The ideal detergent proteases should provide broad substrate specificity to facilitate the removal of a large variety of stains due to food, blood and other body secretions. Also, activity and stability at high pH and temperature and compatibility with other chelating and oxidizing agents added to detergents are among the major prerequisites for proteases in detergents. Currently, all detergent proteases used in the market are serine proteases produced by *Bacillus* strains (63).

8.2 Food industry

Proteolytic enzymes are very useful substances in the dairy industry where their major application is in manufacture of cheese. Three proteases are used in cheese making. There are animal rennet, microbial milk coagulants and genetically engineered chymosin (63). Rennet refers to the standardized water extract obtained from the fourth stomach of milk-fed calves. Rennin can remove a glycopeptide from soluble calcium caseinate to yield relatively insoluble calcium paracaseinate which precipitates to form the desired curd for the production of cheese (50). Microbial

rennets derived from *Mucor miehei*, *M. pusillus* and *Endothia parasitica* are currently used in at least 60% of the cheese in the united states (68).

Whey is a by-product of cheese manufacture that contains lactose, proteins, minerals and lactic acid. The insoluble heat-denatured whey protein can be solubilized by treatment with immobilized trypsin (63).

For tenderization of meat products, thiol proteases from *A. oryzae* and *B. subtilis* are used. On individual pieces of meat, they act on muscle fiber rather than connective tissue. For tenderizing of connective tissue papain, bromelain and ficin are used. Papain, bromelain and ficin have also been used for the chill-proofing of beer. Nowadays, however, these enzymes are often substituted by microbial proteases derived from *A. oryzae* and *B. subtilis* (69).

The natural level of proteases in wheat flour are too low to have a significant effect in bread making. Wheat gluten is an insoluble protein in wheat flour which determines the properties of bakery doughs. Proteases from *A. oryzae* are added to hydrolyze and shorten the chains of gluten to reduce the mixing time and increase loaf volumes. Bacterial proteases are used to improve the extensibility and strength of the dough (50, 63).

Proteases are extensively used in the hydrolysis of food protein to yield better nutritional and functional food products. For example, in soy sauce production, alkaline and neutral proteases of *A. oryzae* hydrolyze most of the protein in soybean and wheat to form amino acids and low molecular weight peptides which provide meaty and savory flavor in the soy sauce product.

Thiol proteases are used to reduce viscosity in condensed fish hydrolysates (70) and acidic proteases are used to hydrolyze soybean for reducing the unpleasant

flavor in protein-containing lemonade-like flavored beverages (28). In some cases, protein hydrolysate is used for making the protein soluble at isoelectric pH value, for example in the hydrolysis of soybean protein or casein to make protein products suitable for use in soft drinks and liquid foods.

8.3 Health related industry

A wide diversity of specific proteases are used to develop effective therapeutic agents. Oral administration of proteases from *A. oryzae* has been used as a digestive aid to correct certain lytic enzyme deficiency syndromes. Clostridial collagenase or subtilisin is used in combination with broad-spectrum antibiotics in the treatment of burns and wounds. An asparaginase isolated from *Escherichia coli* is used to eliminate asparagine from the bloodstream in various forms of lymphocytic leukemia (71).

8.4 Other applications

Proteases also play an important role in basic research and biochemical research. Their selective peptide cleavage is used in the elucidation of the structure-function relationship, in the synthesis of peptides and in sequencing of proteins.

In the leather industry, the use of proteases instead of hazardous chemicals such as sodium sulfide can improve leather quality and reduce environmental pollution significantly. Recently, Novo Nordisk has manufactured three different proteases, Aquaderm, NUE and Pyrase for use in soaking, dehairing and bating processes of the leather industry, respectively (63).

CHAPTER III

MATERIALS AND METHODS

1. Chemicals and reagents

All chemicals and reagents used in all experiments except where indicated otherwise, were analytical grade. Plate count agar, copper sulfate (CuSO_4), potassium sulfate (K_2SO_4), sulfuric acid (H_2SO_4), boric acid (H_3BO_3), bromocresol green, hydrochloric acid (HCl), mercuric nitrate ($\text{Hg}(\text{NO}_3)_2$), absolute ethanol ($\text{CH}_3\text{CH}_2\text{OH}$), 3,5-dinitrosalicylic acid ($\text{C}_7\text{H}_4\text{N}_2\text{O}_7$) and D-glucose were purchased from E. Merck (Germany). Nitric acid (HNO_3) and diphenylcarbazone were purchased from Fluka (Switzerland). Methyl red was purchased from Difco (U.S.A.).

The commercial grade 50 % sodium hydroxide (NaOH) was obtained from Asrom company (Thailand). KojizymeTM was obtained from Novo Nordisk (Denmark). The commercial grade sodium chloride (NaCl) was obtained from Vichit Rong Raung soy sauce factory. Soybean meal was obtained from Aung Suang Ha factory.

KojizymeTM was an exo-peptidase and endo-protease complex produced by fermentation of selected strains of *Aspergillus* and *Bacillus*. It was brown, free-flowing, non-dusting granulate, which was granulated on wheat grits and NaCl . The enzyme was readily soluble in water. It had a declared activity of 800 LAPU/g. One LAPU (Leucine Aminopeptidase Unit) was the amount of enzyme which hydrolyzed 1 μmole of L-leucine-p nitroanilide per minute. The typical dosage which recommended

by manufacturer was about 2% based on protein in the substrate. This product complied with FAO/WHO JECFA and FCC recommended specifications for food grade enzymes, containing maximum limits of 5×10^4 /g for total viable count and 10^2 /g for molds.

According to the manufacturer, Kojizyme™ was developed for boosting soy sauce fermentation. When added into the fermentation tank in the beginning of liquid state fermentation, Kojizyme™ would improve the enzymatic degradation of the raw materials. Kojizyme™ had a high activity in the pH range of soy sauce fermentation which started at about 6.5 and decreased to about 4.8 at the end of fermentation period in which Kojizyme™ had maximum activity (100%) at pH 5.8 to 6.3. Kojizyme™ had a much higher activity at a high salt level than protease activity from *A. oryzae* in traditional process. Kojizyme™ had 100%, 80% and 70% relative activity in the condition that contained no NaCl, 5 to 10% NaCl and 15 to 20% NaCl, respectively (72).

2. Equipment

The major equipment used in this study included a Homoginizer (Janke Kunkel, JK RW 20 DZMP4, IKA Laboratechnik, Germany), Thermostat water bath (KW Pack Medical Laboratory Instrument, Thailand), Kettle (SKK Gearmoter type ME G22, SKK Gear works, Toshiba Corporation, Japan), Centrifuges (Sorvell RC-5C plus and RC-3B plus, Sorvall Instrument, DuPont, U.S.A), Micro Kjeldahl (Kjeltec® system, Turbosog, Kjeldtherm and Vapodest unit, Gerhardt, Germany), Spectrophotometer (Novaspec® II-Pharmacia LKB, Novaspec II, Sweden), pH meter (Orion EA940, U.S.A) and Moisture analysis (Sortorius, MA30, Germany).

3. Determination of microorganisms

Each sample was serially diluted ten fold with sterile water. After appropriate dilutions were made, 1 ml of each sample was used to prepare pour plates for agar count plates. After incubation at 37°C for 2 to 3 days, the plates containing 30-300 colonies were used to determine the number of viable bacteria in each sample. The total bacterial count in each sample was determined by direct counting with a dark background.

4. Chemical analysis

4.1 Determination of protein

Protein content was determined in terms of total nitrogen content by the Kjeldahl method (73) using a Micro Kjeldahl apparatus. One ml or one gram of sample was measured accurately and filled into a digestion tube. The catalyst (3.5 g potassium sulfate and 0.5 g copper sulfate) was added into the tube. Then, 15 ml of concentrated sulfuric acid was added into the tube. The digestion tube that was filled with sample, catalyst and concentrated sulfuric acid was digested in the digestion unit at 420°C for 45 min or until the clear green solution occurred. Then the digestion tube was cooled to room temperature. Thirty ml of distilled water was added into the cooled digestion tube. Then these digestion tube was fitted with the steam Teflon pipe of the distillation unit. The glass pipe of the condenser of distillation unit was dipped into a Erlenmeyer flask containing 25 ml of 4 % Boric acid (w/v) in distilled water that was added with a few drops of mixed indicators, bromocresol green and methyl red (see Appendix A). The distillation process was performed by adding water and 40% NaOH into the digestion tube. Then, distillation was carried out for 4 min. The

ammonium salt in the sample was evaporated into ammonia and fixed with 4 % boric acid by condensation. The solution in the Erlenmeyer flask was titrated with standard 0.1 N HCl until reaching the end point when the color of the solution changed from green to violet-red. The ammonia absorbed in the boric acid was determined throughout the reaction of titration with HCl. The protein content expressed as percent total protein in terms of total nitrogen was calculated as indicated in the following equations.

$$\text{Percent total nitrogen} = \frac{1.401 \times N_A \times V_A}{\text{weight or volume of sample}}$$

$$\text{Percent protein} = \text{percent total nitrogen} \times 6.25^*$$

N_A : normality of hydrochloric acid for titration

V_A : volume of hydrochloric acid for titration

* : index for calculation percent protein by Kjeldahl method for meat protein, fish protein, corn protein and soybean protein

4.2 Determination of sodium chloride concentration by titration with mercuric nitrate

Sodium chloride concentration was measured by titration of 25 ml of appropriately diluted sample with 0.2 M $\text{Hg}(\text{NO}_3)_2$ (see Appendix A) by using 0.1 ml of 0.1 % diphenylcarbazone (see Appendix A) as the color indicator. The end point was determined by the appearance of the permanent dark purple color. The volume of

mercuric nitrate solution used to reach the end point was used to calculate the concentration of sodium chloride in the sample by the following equation.

$$\% \text{ NaCl (w/v)} = \frac{2M_1V_2 \times W \times \text{Dilution factor} \times 100}{V_1 \times 1000}$$

V_1 : volume of sample

V_2 : volume of $\text{Hg}(\text{NO}_3)_2$

M_1 : concentration of $\text{Hg}(\text{NO}_3)_2$

W : molecular weight of NaCl

4.3 Determination of reducing sugar

The amount of reducing sugar was determined by using Dinitro Salicylic acid (DNS) method (74). A clear solution sample (0.5 ml) with appropriate dilution was mixed with 0.5 ml of DNS reagent (see Appendix A). Then, the mixture was boiled for 5 min. The reaction was terminated by cooling immediately by placing the mixture on the ice bath. Five ml of water was added into the mixture, mixed well and then measured the optical density at the absorbance of 520 nm. The reducing sugar content was calculated as follows.

$$\text{Reducing sugars (ug/ml)} = \frac{\text{OD}_{520} \times \text{Dilution factor}}{\text{slope of standard curve}^*}$$

* Standard curve of reducing sugar was prepared by using D-glucose at concentration of 16.67-66.67 ug/ml as the standard reagent.

4.4 Determination of brown color

The clear solution sample with appropriate dilution was measured at the absorbance of 420 nm against distilled water to determine the brown color (49).

5 Production of soybean hydrolysate from soybean meal

5.1 Laboratory scale production of protein hydrolysate from soybean meal

5.1.1 Preparation of soybean meal as the substrate for production of soybean meal hydrolysate

Defatted soybean meal (SBM) which was the by product from a soybean oil factory was used as the raw material for production of soybean meal hydrolysate (SBH). The SBM was obtained from Aung Suang Ha factory in a relatively large flake form and had to be grounded into fine particles in order to increase the efficiency of digestion. Prior to grinding, SBM was dried in the oven at 100 °C for 5 to 6 hr in order to decrease the moisture content and reduce difficulty in the grinding process with high moisture content it stuck in the grinding machine and interfered with the grinding process. After drying, SBM was ground using a 26-mesh sieve. The fine SBM granules were collected and kept in plastic bags until used. Samples were taken to measure moisture content and protein content by the Kjeldahl method. The protein content in each sample was calculated in terms of percent protein per gram dry weight.

In preparing the SBM for hydrolysis, finely ground SBM was mixed with water in the ratio of 1:1 (by weight). This gave moistened SBM which provided good heat transfer. It was put into the plastic bags, spread out as thin as possible and

sterilized at 121°C for 15 min. It was then kept briefly at ambient room temperature to cool before being use in the hydrolysis process.

5.1.2 Determination of optimal conditions for hydrolysis of SBM

Three parameters were studied for determining the optimal conditions for hydrolysis of SBM. These parameters were water content of the mixture, temperature and amount of Kojizyme™ used in the hydrolysis reaction.

Laboratory scale hydrolysis of SBM was performed in a stainless steel container. Two kg of ground and sterilized SBM was mixed with either 2.5 kg or 4 kg of water to bring the total solid content of the mixture to 22 or 17 %, respectively. The mixture was then put in a stainless steel container and placed in a thermostatic water bath at either 50°C or 55°C to control the temperature of digestion. When the temperature reach to the desirable value, an appropriate amount (2 % or 4 % of the amount of protein in SBM) of Kojizyme™ was added. This was the commercial mixture of exo-peptidase and endo-protease. The contents were mixed with a homogenizer at 100 rpm. At various intervals, samples were taken to determine soluble protein content and pH.

Each sample was centrifuged at 12,000 rpm (11,000 xg) for 25 min in a Sorvall RC-5C plus centrifuge. The pellet was discarded and the supernatant was collected and stored in 4°C until use for analysis. The supernatant of each SBH sample was used for determining of protein content and pH. The protein content in the supernatant was determined by the Kjeldahl method and expressed in terms of percent soluble protein (w/v).

The degree of solubilization was used to determine the efficiency of hydrolysis. Degree of solubilization was the ratio of total soluble protein which obtained from the supernatant fraction of hydrolysis product to the total protein in the substrate in dry weight basis. The ratio was multiplied by 100 to obtain value as percent solubilization.

5.1.3 Determination of bacterial contamination during the hydrolysis of SBM

The extend of bacterial contamination was determined by measuring viable bacterial counts by the Plate Count Agar (PCA) method. Soybean hydrolysate samples were collected at various intervals after starting the digestion. Each sample was appropriately diluted and plated on Plate Count Agar plate. The plates were incubated at 37°C for 72 hr. The number of the colonies were then determined on plates which contained between 30-300 colonies.

5.2 Large scale production of protein hydrolysate from SBM

Large scale production of SBH was performed in a double jacket kettle (Figure 3). The SBM (12.5 kg) was mixed with 12.5 kg water and sterilized at 121°C for 15 min, then cooled at room temperature. Prior to hydrolysis, the jacket of the kettle was circulated with 50°C water from a water bath. Then, the sterilized SBM was placed into the kettle and 31 kg of water was added. After the temperature of the mixture in the kettle reached 50°C, Kojizyme™ (at 2 % level based on the amount of protein in the SBM) was added into the mixture. The addition of enzyme marked the start of the reaction. The mixture was hydrolyzed for 12 hr and hydrolysate samples were taken at appropriate time intervals. Each hydrolysate sample was centrifuged at



Figure 3 Double jacket kettle (SKK Gearmotor Type ME G22, SKK Gear work, Toshiba Corporation, Japan) that was used in the production of large scale SBH.

12,000 rpm (11,000 xg) for 25 min, then the supernatant fraction was collected and stored at 4°C until used for further analysis. The supernatant of the hydrolysate samples was tested for protein content and pH. After completion of the reaction at 12th hr, the whole protein hydrolysate preparation was collected from the kettle and kept at 4°C until used further for use in the moromi fermentation experiments.

6. Protein enrichment of soy sauce by supplementation with SBH at various stages of fermentation

6.1 Supplementation of SBH in the final soy sauce product

Soy sauce samples from three months old primary moromi fermentation from Vichit Rung Ruong soy sauce factory were used in these experiments. The clarified SBH (cSBH) preparations from laboratory scale soybean hydrolysis were used for mixing with finished soy sauce in various ratios. Three different preparations were made to contain increasing levels of cSBH. The ratio's of finished soy sauce to cSBH in the three preparations was 1:0.5, 1:1 and 1:1.5. Finished soy sauce with a NaCl concentration of 30% (w/v) were diluted to 22% prior to mixing with cSBH and the final product after mixing was adjusted by adding NaCl to obtain a final concentration of 22%. To completely dissolve the NaCl in the final product, the sample had to be warmed during mixing until the NaCl was completely dissolved. These mixed soy sauce products were analyzed for soluble protein, NaCl, reducing sugar, pH, color and also used for sensory evaluation tests in order to determine for the consumer acceptability.

6.2 Supplementation of SBH in primary moromi fermentation

The SBH from large scale production was added into the primary cycle moromi fermentation. The moromi fermentation was carried out in 120 L earthenware jars at Vichit Rung Ruong soy sauce factory. Two day old koji was used as the starting material in these moromi fermentations. Moromi fermentation was carried out using a ratio of koji and SBH mixture to brine solution at 1:2 (by weight) and incubated for 2 months at ambient temperature. The concentration of sodium chloride was controlled in the range of 24 to 27 %. There were 3 sets of experiments as follows, (i) control soy sauce (CSS₁) in which only brine solution was added, (ii) experimental soy sauce in which SBH was added at the beginning of moromi fermentation (ESS₁H₀), on the 34th day of moromi fermentation (ESS₁H₃₄) and on the 49th day of moromi fermentation (ESS₁H₄₉), (iii) experimental soy sauce in which Kojizyme™ was added at the beginning of moromi fermentation (ESS₁K₀), on the 34th day of moromi fermentation (ESS₁K₃₄) and on the 49th day of moromi fermentation (ESS₁K₄₉). The schematic representation of these experiments is outline in Figure 4.

When SBH was added to the moromi mash, the amount added was determined based on the protein content in the koji. Half of the protein content in the moromi mash came from koji and another half came from SBH. When SBH was added at the beginning of moromi fermentation, the amount of koji used was 8.5 kg which represented a protein content of 2.0 kg (8.5 kg × 23.6 %). The SBH used in these experiments had a protein content of 8.9 % (w/v). Therefore, the added amount was 22.6 kg (2.0 kg protein at a concentration of 8.9 %) (Table 3). When SBH was added at the 34th day of fermentation, the moromi mash from koji starting materials

was divided into 2 equal parts and to each part was added 23.0 kg of SBH with a protein content of 8.7 % (2.0 kg protein at a concentration of 8.7 %). Sodium chloride was added to the mixture to obtain a final concentration between 24 to 27 % (w/v). When SBH was added on the 49th day of fermentation, the SBH was added by the same procedure as on the 34th day. The mixture was added with 22.0 kg of SBH which contained protein content of 9.1 % (2.0 kg protein at a concentration of 9.1 %).

When KojizymeTM was to be added to moromi fermentation, it was always added at a concentration of 2% of the protein content of the koji used in the moromi fermentation. For example, if 17 kg of koji containing 23.6 % (w/w) protein was used, then the amount of KojizymeTM added was equivalent to 80.3 g ($2\% \times 23.6\% \times 17\text{ kg}$). A detailed summary of the raw materials used for preparation of the protein enriched soy sauce products obtained from the primary cycle moromi fermentation appears in Table 3.

After the addition of SBH or KojizymeTM into the moromi mash, the moromi mash was incubated in earthenware containers at ambient temperature for 68 days. During the incubation period, the moromi samples were taken at the beginning, first week and then every 2 weeks during the two months period of fermentation. Before taking samples, the final volume and salinity were controlled by adding fresh water into the jars if the volume decreased from evaporation. Each moromi sample was filtered through cotton gauze and then centrifuged at 12,000 rpm (11,000 xg) for 25 min. The supernatant was collected and subsequently used to analyze for protein content, sodium chloride concentration, reducing sugar, pH and color (OD₄₂₀). At the end of incubation, the moromi mash was harvested by filtering out the insoluble part and centrifuged to collect the supernatant. The clarified soy sauce samples were used

Table 3 Summary of raw materials used for preparation of protein enriched soy sauce products obtained from the primary moromi fermentation

Sample*	Soybean containing raw material			Brine solution		Water content			Total volume (kg)	Solid content (kg)
	Koji ¹ (kg)	SBH ² (kg)	Total protein content ³ (kg)	NaCl ⁴ added (kg)	NaCl concentration ⁵ (%)	in SBH ⁶ (kg)	in brine solution (kg)	Total water content (kg)		
1. CSS ₁	17	-	4.0	34.0 ^d	26.9	-	34	34	51	33
2. ESS ₁ H ₀	8.5	22.6 ^a	4.0	9.5 ^e	22.1	17.6	10	28	51	33
3. ESS ₁ H ₃₄	8.5	23.0 ^b	4.0	10.6 ^e	30.8	17.9	15	33	46	29
4. ESS ₁ H ₄₉	8.5	21.6 ^c	4.0	4.4 ^e	26.3	16.7	16	33	46	29
5. ESS ₁ K ₀	17	-	4.0	34.0 ^d	26.3	-	34	34	51	33
6. ESS ₁ K ₃₄	17	-	4.0	34.0 ^d	26.5	-	34	34	51	33
7. ESS ₁ K ₄₉	17	-	4.0	34.0 ^d	26.2	-	34	34	51	33

¹ 2 days old koji had protein content 23.6 % (w/w)

² SBH from large scale production - ^a 1st production had protein content 8.9 % (w/w)

- ^b 2nd production had protein content 8.7 % (w/w)

- ^c 3rd production had protein content 9.4 % (w/w)

³ total protein content = protein content from koji + protein content from SBH

⁴ NaCl add - ^d saturated brine solution (31% NaCl)

- ^e solid NaCl

⁵ NaCl concentration of the mixture after mixed with brine solution or solid NaCl at the desirable date of incubation

⁶ SBH had 1 part of SBM and 3.5 parts of water

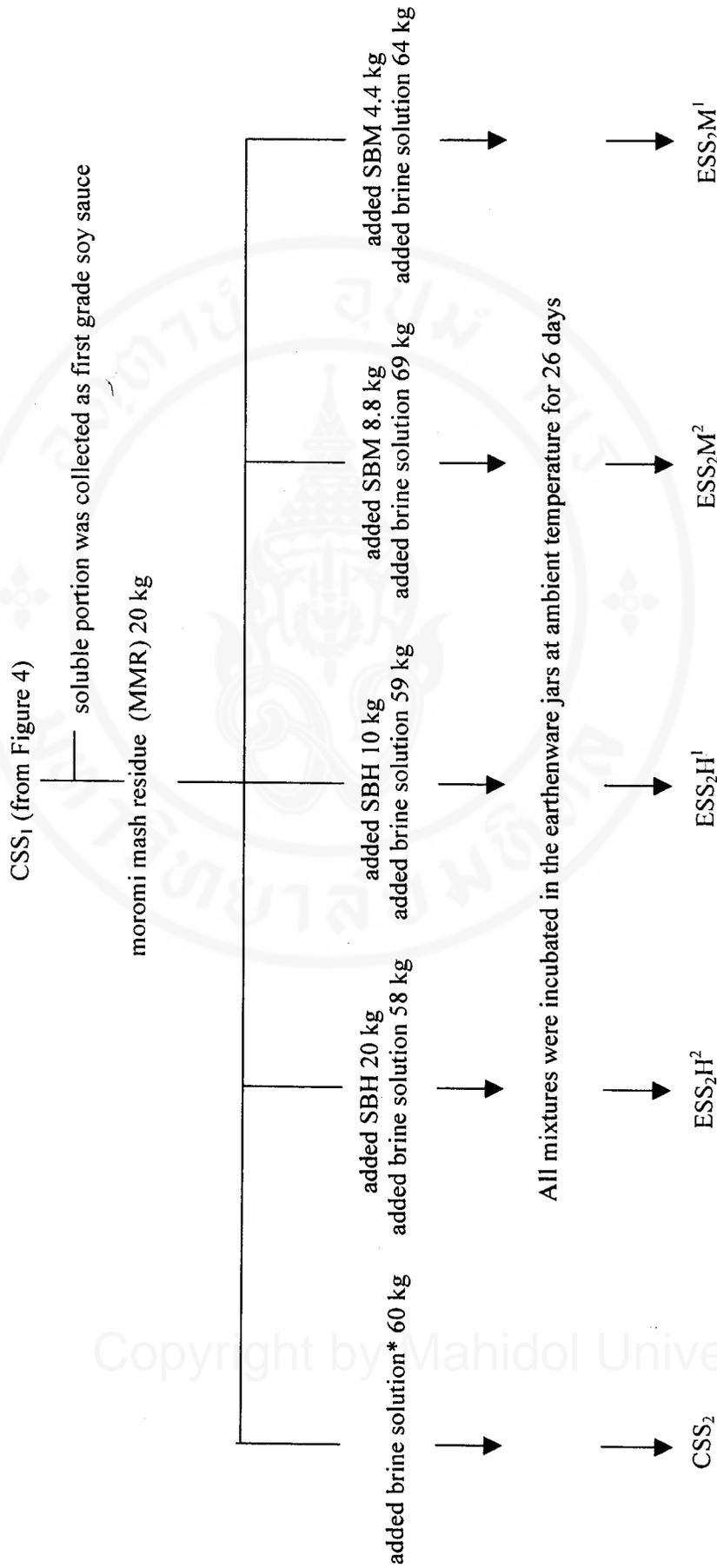
* see Figure 4 for description of abbreviation

for sensory evaluations in order to test for the acceptance and preference of consumers to the products.

6.3 Supplementation of SBH in the secondary moromi fermentation

After filtering the soluble portion from the primary cycle moromi fermentation to obtain first grade soy sauce, the residue mash (called moromi mash residue) (MMR) was used as the starting material for a secondary cycle moromi fermentation. The secondary moromi fermentation was carried out by using the ratio of substrates (MMR or mixture of MMR and SBH, or MMR and SBM) to brine solution of 1:3 (w/w). The mixtures were further fermented in earthenware jars for one month. There were three sets of experiments, namely, (i) control soy sauce (CSS₂) to which only brine solution was added, (ii) 20 kg of MMR to which 20 kg of SBH (ESS₂H²) or 10 kg of SBH (ESS₂H¹) was added and (iii) 20 kg of MMR to which 8.8 kg of autoclaved-SBM (ESS₂M²) or 4.4 kg of autoclaved-SBM (ESS₂M¹) was added. A schematic representation of these experiments is outlined in Figure 5. A detailed summary of the raw materials used for preparation of protein enriched soy sauce products obtained from the secondary cycle moromi fermentation appears in Table 4. The amount of SBH that was added into the mixture was based on the content (weight) of MMR as shown in Table 4. The amount of SBM added into the mixture was based on the amount of SBM that was used in the preparation of SBH (Table 4). In ESS₂H² which 20 kg of SBH was added, 4.4 kg of SBM was used by mixing with 4.4 kg of water and then autoclaved. When autoclaved-SBM was used in the hydrolysis process, another 2.5 times (11.2 kg) of water was added to the mixture. Therefore, in ESS₂M².

Figure 5 Outline of steps leading to preparation of protein enriched soy sauce obtained from secondary moromi fermentation.



* brine solution used in this experiment contained 31% NaCl

Table 4 Summary of raw material used for preparation of protein enriched soy sauce products obtained from the secondary moromi fermentation

Sample*	Soybean containing raw material (kg)			Brine solution		Water content (kg)			Total volume (kg)	Solid content (%)	
	MMR	SBH ²	SBM ³	Soybean meal content in SBH or SBM ⁴	Total Soybean content ⁵	NaCl ⁶ added (kg)	NaCl ⁷ concentration (%)	In SBH/ In brine solution			Total water content ⁸
1. CSS ₂	20	-	-	-	20.0	60.0	23.5	-	60.0	80.0	25
2. ESS ₂ R ₁ H ₁	20	20	-	4.4	24.4	57.6	21.4	15.6	57.6	97.6	25
3. ESS ₂ R ₂ H ₁	20	10	-	2.2	22.2	58.8	22.4	7.8	58.8	88.8	25
4. ESS ₂ R ₁ M ₁	20	-	8.8	4.4	24.4	68.8	23.0	4.4	68.8	97.6	25
5. ESS ₂ R ₂ M ₁	20	-	4.4	2.2	22.2	64.4	22.0	2.2	64.4	88.8	25

¹ MMR = moromi mash residue had protein content 5.28 % (w/w)

² SBH = soybean meal hydrolysate from the 4th and 5th large scale production, had protein content 10.1 % (w/w)

³ SBM = soybean meal mixed with 1 time of H₂O and autoclaved

⁴ soybean meal content in SBH or SBM ; SBH had SBM 1 part and 3.5 parts of water

⁵ total soybean content = soybean content of MMR or soybean content of MMR + soybean meal content in SBH or autoclaved-SBM ; autoclaved-SBM had SBM 1 part and 1 part of water

⁶ NaCl solution = saturated brine solution (31% NaCl)

⁷ NaCl concentration of the mixture after mixed with brine solution at the beginning of the incubation

⁸ total water content = water from SBH or SBM + water from brine solution

the same amount of SBM was used as in ESS₂H² which used 4.4 kg SBM mixed with 4.4 kg of water before autoclaving. In ESS₂H¹ and ESS₂M¹, the amount of SBM used was the same at about 2.2 kg.

After the addition of SBH or SBM into the moromi mash residue, the moromi mash was incubated in earthenware containers for 26 days. During the incubation period, samples were taken at the beginning, second, third and fourth week during the one month period of fermentation. Each moromi sample was filtered through cotton gauze and then centrifuged at 12,000 rpm (11,000 xg) for 25 min. The supernatant was collected and subsequently used to analyze for protein content, sodium chloride concentration, reducing sugar, pH and color (OD₄₂₀). At the end of incubation, the moromi mash was harvested by filtering out the insoluble part and centrifuged to collect the supernatant. The clarified soy sauce samples were used for sensory evaluation in order to test for consumer acceptance and preference.

7. Preparation of the soy sauce sample for sensory evaluation

After completion of moromi fermentations, the mash was filtered through a rough sieve to separate the insoluble content from the soluble part. Subsequently, the soluble part was filtered through cotton gauze again for further clarification. Then the supernatant was centrifuged at 4,500 rpm (5,900 xg) for 30 min in Sorvall RC-3B plus. The clarified supernatant after centrifugation represented raw soy sauce samples were collected and stored at 4°C until further analysis. The raw soy sauce samples were used to assay for soluble protein, NaCl concentration, reducing sugar, pH and color. Prior to sensory evaluation, the raw soy sauce samples of either primary or secondary moromi fermentations were diluted with distilled water, so that the final



sodium chloride contents were between 20 to 22 %. The primary and secondary cycle moromi fermentation raw soy sauce samples were heated to 100°C and maintained at 95°C for 15 and 20 min, respectively. The samples were then tested for soluble protein content, NaCl concentration, reducing sugars, pH and color and stored at 4°C until used for sensory evaluation tests.

8. Survey of consumer attitudes toward soy sauce products

A total of one hundred subjects, including general consumers as well as soy sauce factory owners were asked to answer the survey questionnaires. The questionnaires were divided into three sections; personal information, attitude toward traditional soy sauce products and attitude toward new soy sauce products as illustrated in Appendix B and the result illustrated in Appendix D. Subjects who familiar with soy sauce products were chosen to undertake subsequent sensory evaluation tests.

9. Sensory evaluation of soy sauce samples

In sensory evaluation tests, soy sauce samples were tested as a dipping sauce. In each test of sensory evaluation, fifty subjects were asked to conduct the test. Each subject received one tray containing four soy sauce samples labeled with 3 digit numbers and 1 cup of small pieces of cooked chicken for dipping with these samples. One piece of napkin, 1 glass of water and a sheet of questionnaire were placed in the sensory booth beside the sample tray. (Figure 6). The serving orders consisted of 15 combinations. The serving size of soy sauce was 10 ml each. Sensory evaluation of



Figure 6 Sensory evaluation booth that used to undertake the sensory evaluation test. One set of sample contained four soy sauce samples labeling with 3 digit numbers and 1 cup of small pieces of cooked chicken. One piece of napkin, 1 glass of water and a sheet of questionnaire were placed in the sensory booth beside the sample tray.

each soy sauce product was evaluated for general appearance, color and odor prior to tasting of the soy sauce products. Subsequently, the subjects were requested to evaluate for overall acceptability, flavor, taste and saltiness. Finally, they were asked to evaluate for off-flavor of products. The general appearance, odor, overall acceptability, flavor and taste were evaluated using 9 point hedonic scale (1:dislike extremely, 5:neither like nor dislike, 9:like extremely). The color intensity and saltiness were evaluated by using 5 point hedonic scale (1:much too light and less salty, 3:just about right, 5:much too dark and much too salty). Off-flavor was evaluated using a 15 cm line scale (1 cm: did not have off-flavor, 7.5 cm: had about 50 % of off-flavor, 15 cm: had extremely off-flavor) A sample questionnaire appears in Appendix C.

All sensory evaluation data were calculated as mean and standard deviation (SD). Treatment means were compared by one way analysis of variance (ANOVA) with a significance level of 95%. Also, the mean values were computed and compared by using Duncan New Multiple Range test. The computations were as follows:

$$\text{Critical difference} = dr \sqrt{\text{MSE}/n}$$

Where d represented values derived from Statistical Tables that showed the critical values of the Duncan New Multiple Range Test and r represented number of mean being compared. MSE represented Mean Square Error and n represented number of sample being compared.

CHAPTER IV

RESULTS

1. Determination of optimal conditions for hydrolysis of SBM

In order to determine optimal conditions for hydrolysis of SBM, the effects of concentration of KojizymeTM, temperature and the total solid content of the reaction mixture were studied. For study on effects of KojizymeTM, experiments were carried out by fixing the temperature at 55°C and total solid content at 22 %. The amount of KojizymeTM added was at the levels of 2 % and 4 % based on the dry weight of protein in the substrate. For study on effects of temperature, experiments were performed by fixing the amount of KojizymeTM at 2 % of protein in the substrate and total solid content at 22 %. Temperatures were 50 and 55°C. For study on effects of total solid content of the reaction mixture upon the degree of hydrolysis, experiments were undertaken by fixing the amount of KojizymeTM at 2 % based on protein content in substrate and temperature at 50°C. The total amount of solid content was tested at 17 and 22 %. The reaction mixtures under various conditions were initiated by the addition of enzyme preparation. At predetermined time intervals, samples were taken and centrifuged to obtain the supernatant fraction. The supernatant fractions were used for determining soluble protein and pH. The degree of solubilization was calculated from the ratio of total soluble protein to the total protein in the substrate multiplied by

100. The increase in degree of solubilization from the beginning of hydrolysis also used for comparing the efficiency of hydrolysis in each experiment.

1.1 Effects of various concentrations of Kojizyme™ on the hydrolysis of SBM

Based on recommendation from the manufacturer (74), Kojizyme™ should be used at the level of 2 % of the substrate (based on protein content of the substrate). In the following experiments, Kojizyme™ was added to the reaction mixtures at two concentrations (i.e. 2 % and 4%). The increase in soluble protein and the decrease in pH during 14 hr of incubation as shown in Figure 7a and 7b, respectively. The percent soluble protein contents from experiments containing of 2% and 4% Kojizyme™ experiments were about 4.5 and 4.2 % at the beginning of hydrolysis, then increased to 7.1 and 7.3 % at the peak of solubilization between 6 and 8 hr of hydrolysis, respectively. After eight hr incubation, the concentration of soluble protein was found to be maintained at maximum value during the 14 hr duration of the experiment. There was no difference in the pattern of pH decrease in the solubilization reaction whether 2% or 4% Kojizyme™ was used. The initial pH values were between 6.3 to 6.4 and decreased continuously to the level of 5.1 to 5.3 after about 12 hr of incubation and maintained at this level until the end of the experiments at 14 hr.

The time course for production of SBH as expressed in terms of degree of solubilization as shown in Figure 7c. It was found that 2% and 4% Kojizyme™ experiments had degree of hydrolysis about 31.1 and 33.8% at the beginning of hydrolysis and increase to about 52.8 and 54.8% at the 6th and 8th hr of hydrolysis, respectively. The increase in degree of solubilization (Figure 7d) showed that the

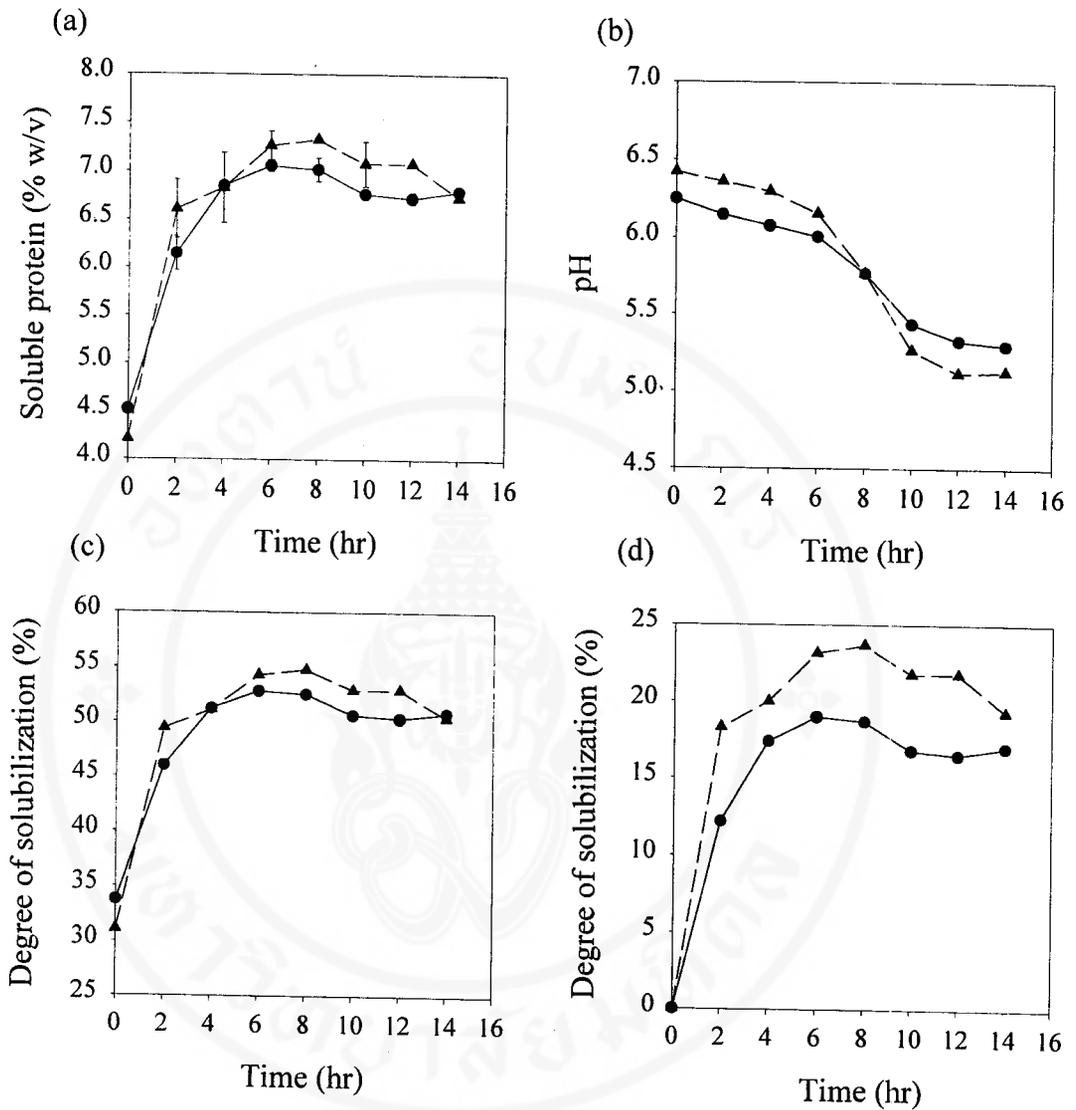


Figure 7 Effects of two different concentrations of Kojizyme™ on hydrolysis of SBM. One kg of SBM was mixed with 3.5 kg water to obtain total solid content of 22%. Then, Kojizyme™ was added at 2% (●) and 4% (▲) level based on protein content of SBM. The reaction mixtures were incubated at 55 °C, and at indicated time intervals, samples were taken and centrifuged at 12,000 rpm for 25 min. Each supernatant fraction was assayed for soluble protein (a) and pH (b). The efficiency of hydrolysis was calculated and expressed in term of degree of solubilization (c) and the increase in degree of solubilization from the beginning of hydrolysis (d).

maximum increase in degree of solubilization of 2 % and 4 % Kojizyme™ were 19.0 and 23.7%, respectively. The data indicated that when the concentration of Kojizyme™ was doubled, the increase in degree of solubilization increased only slightly about 4.7%. Thus, in order to save cost, Kojizyme™ at 2% level was used in the subsequent experiments. As stated earlier, this was the same value recommended by the manufacturer of Kojizyme™.

1.2 Effects of temperatures on the hydrolysis of SBM

From a previous study (75) it was found that the optimal temperature for hydrolysis of SBM was 55°C while other study (76) reported that optimal hydrolysis could be obtained at 50°C. Therefore, experiments were set up to investigate the effects of the temperatures at 50°C and 55°C on the hydrolysis of SBM. The increase in soluble protein and the decrease in pH during 14 hr of incubation period were shown in Figures 8a and 8b, respectively. The production of soluble protein at 50°C and 55°C was approximately 5.2% and 4.5% at the beginning of hydrolysis. This increased to maximum values of 8.4% and 7.1% for the 6th to 12th hr at 50°C and 55°C, respectively. There was no difference in the pattern of pH decrease in the solubilization mixture at 50°C or 55°C. The initial pH values were 6.2 to 6.3 at the beginning and these decreased to 5.0 to 5.3 at the 14th hr of incubation.

The time courses for production of SBH as expressed in term of degree of solubilization as shown in Figure 8c. It was found that 50°C and 55°C experiments had degree of hydrolysis about 39.5 and 33.8% at the beginning of hydrolysis and increase to about 62.8 and 52.8% at the 12th and 6th hr of hydrolysis, respectively.

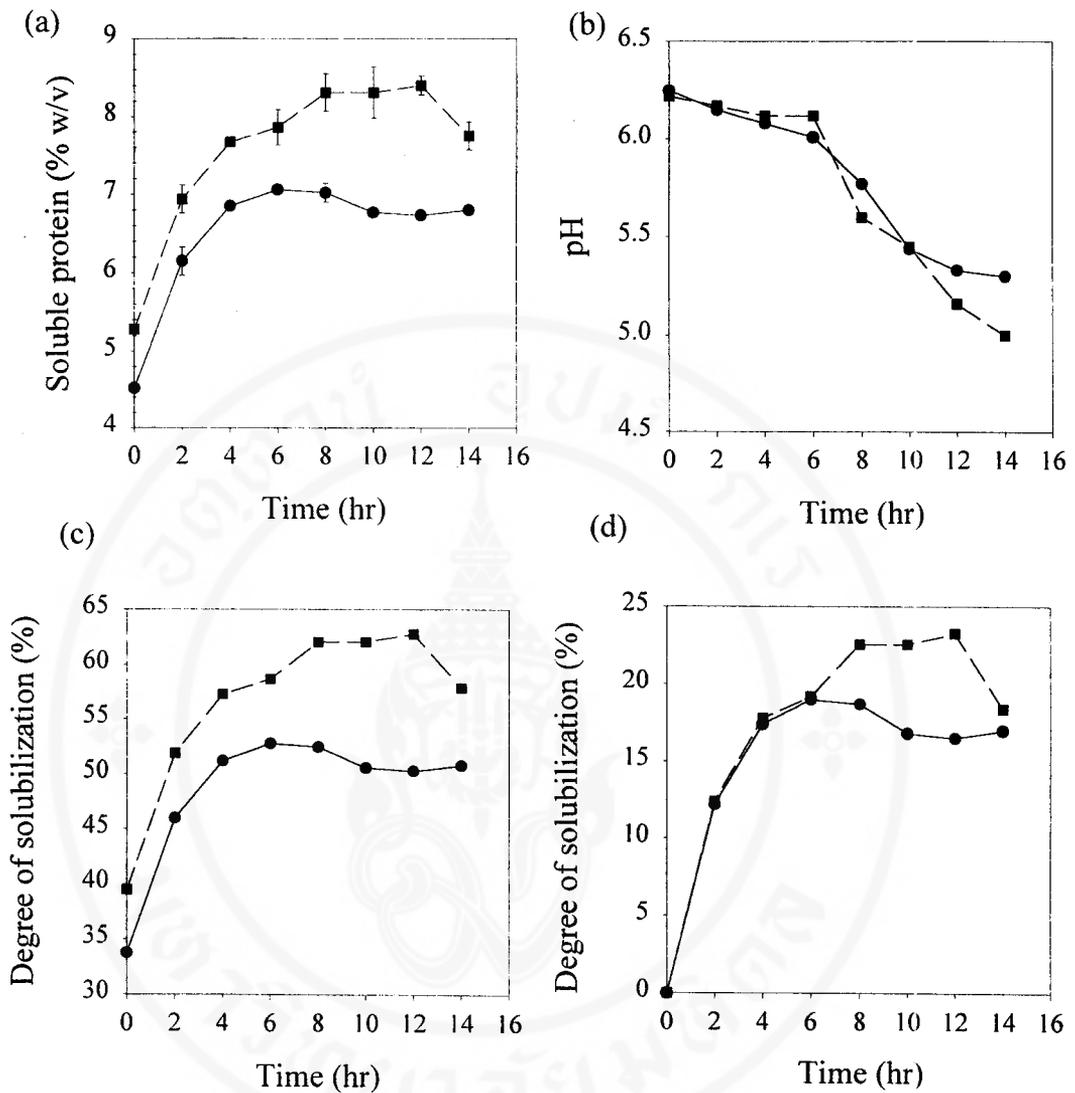


Figure 8 Effects of temperatures at 50 °C and 55 °C on hydrolysis of SBM.

One kg of SBM was mixed with 3.5 kg water to obtain total solid content of 22%. Then, Kojizyme™ was added at 2% levels based on protein content of SBM. The reaction mixtures were incubated at 50 °C (■) and 55 °C (●). At indicated time intervals, samples were taken and centrifuged at 12,000 rpm for 25 min. Each supernatant fraction was assayed for soluble protein (a) and pH (b). The efficiency of hydrolysis was calculated and expressed in term of degree of solubilization (c) and the increase in degree of solubilization from the beginning of hydrolysis (d).

The increase in degree of solubilization (Figure 8d) showed that the maximum increase in degree of solubilization of 50°C and 55°C were 23.3 and 19.0%, respectively. The data indicated that a temperature of 50°C gave 4% higher yield than at 55°C. Therefore, 50°C was optimal for the hydrolysis of SBM under the conditions outlined.

1.3 Effects of total water content on the hydrolysis of SBM

In the hydrolysis process, not only temperature and amount of Kojizyme™ will effect the efficiency of hydrolysis, but also water content. From a previous study (75), it was found that the optimal amount of water for hydrolysis of SBM was about 3.5 times of the substrate (w/v), i.e., a total solid content of 22 %. Therefore, in this study the reaction was run using water content at 3.5 and 5.0 times the amount of SBM, i.e., total solid contents of 22 % and 17 %, respectively. The increase in soluble protein and decrease in pH during the 14 hr incubation period were shown in Figures 9a and 9b, respectively. The percent soluble protein at 17 % and 22 % were about 2.2 and 4.0% at the beginning of hydrolysis, then increased to 5.2 and 9.4 % at the 12th hr of hydrolysis, respectively. There was no difference in the pattern of pH decrease in the solubilization mixture at 17 % or 22 %. The initial pH values were about 6.0 at the beginning and decreased continuously to the level of 5.2 to 5.3 at the 12th hr.

The time courses for production of SBH as expressed in term of degree of solubilization as shown in Figure 9c. It was found that 17 and 22% total solid content experiments had degree of hydrolysis about 23.3 and 29.8% at the beginning of hydrolysis and increase to about 56.1 and 70.1% at the 14th and 12th hr of hydrolysis,

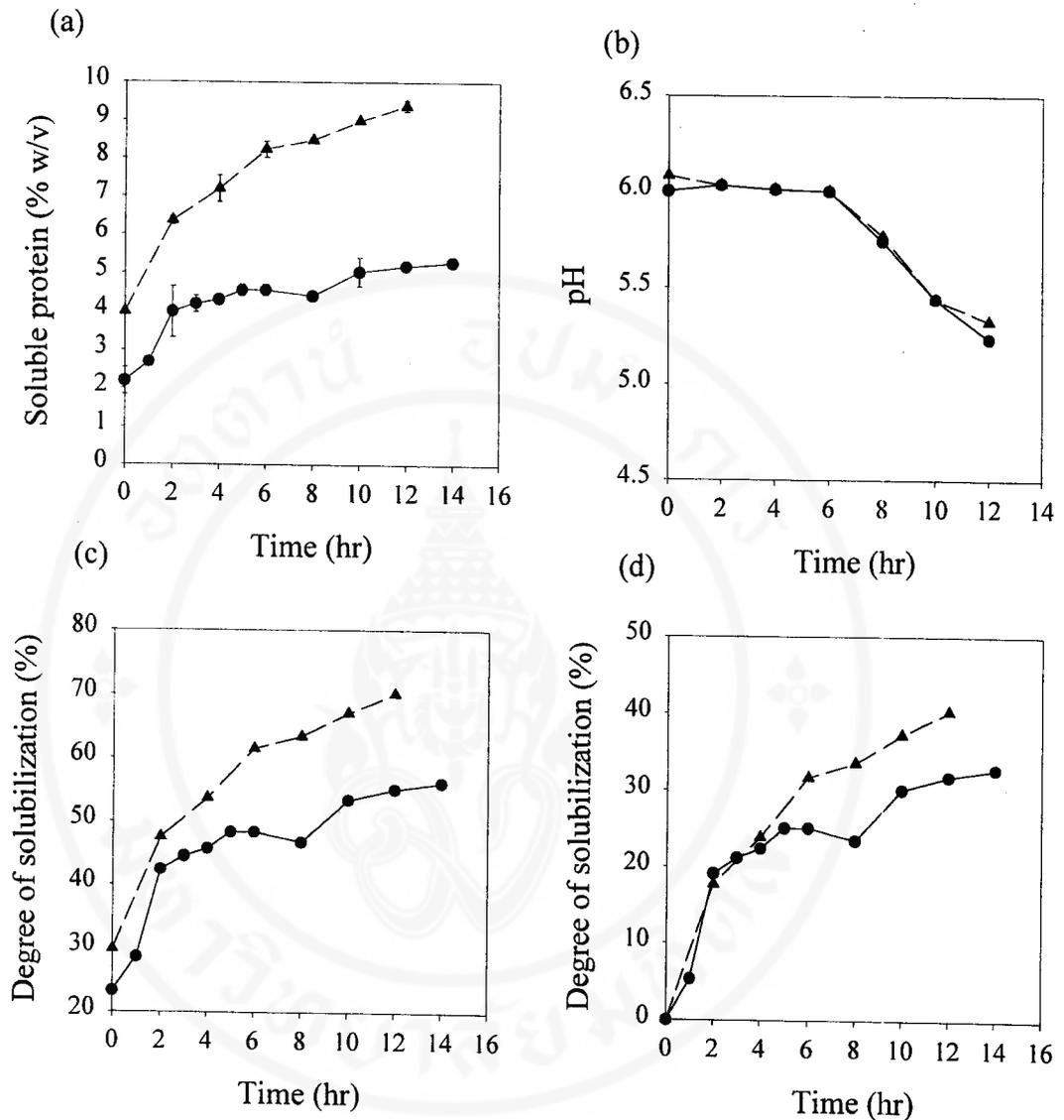


Figure 9 Effects of total solid contents at 17% and 22% on hydrolysis of SBM.

One kg of SBM was mixed with water at 3.5 and 5.0 kg to obtain total solid content of 17% (●) and 22% (▲). Then Kojizyme™ was added at 2% levels based on protein content of SBM. The reaction mixtures were incubated at 55 °C, and at indicated time intervals, samples were taken and centrifuged at 12,000 rpm for 25 min. Each supernatant fraction was assayed for soluble protein (a) and pH (b). The efficiency of hydrolysis was calculated and expressed in term of degree of solubilization (c) and the increase in degree of solubilization from the beginning of hydrolysis (d).

respectively. The increase in degree of solubilization (Figure 9d) showed that the maximum increase in degree of solubilization of 17 and 22% total solid content were 32.8 and 40.3%, respectively. The data indicated that a total solid content at 22% gave 7.5% higher yield than at 17% total solid content. Therefore, 22% total solid content was optimal for the hydrolysis of SBM under the conditions outlined.

In conclusion, the preferred conditions for SBM hydrolysis were Kojizyme™ at 2 % of SBM protein , total solid content of 22 % and temperature 50°C for 12 hr. Therefore, these conditions were used for the large scale production of SBH.

2. Pattern of soluble protein content, pH and bacterial contamination during the hydrolysis of SBM

Production of soluble protein from SBM was confirmed on the laboratory scale using Kojizyme™ at 2 % of SBM protein , 50°C and total solid content of 22 %. Data as shown in Figure 8 indicated the maximum level of soluble protein (8.3%) was obtained after 8 to 12 hrs of hydrolysis. It was also found that there was some reduction in the soluble protein content after the maximum level was obtained and attempts were made to clarify the nature of this reduction. When hydrolysis of SBM was undertaken as previously described and samples were taken at various intervals to determine the number of contaminating bacteria, it was found that there was a relation between percent soluble protein, pH and bacterial contamination as showed in Figure 10. From the 0 to 8th hr, the soluble protein and total bacterial count increased from 6×10^4 to 9.6×10^7 CFU/ml. After the 12th hr, the protein content decreased while the total bacterial count increased further until 20th and then decreased afterward. The pH

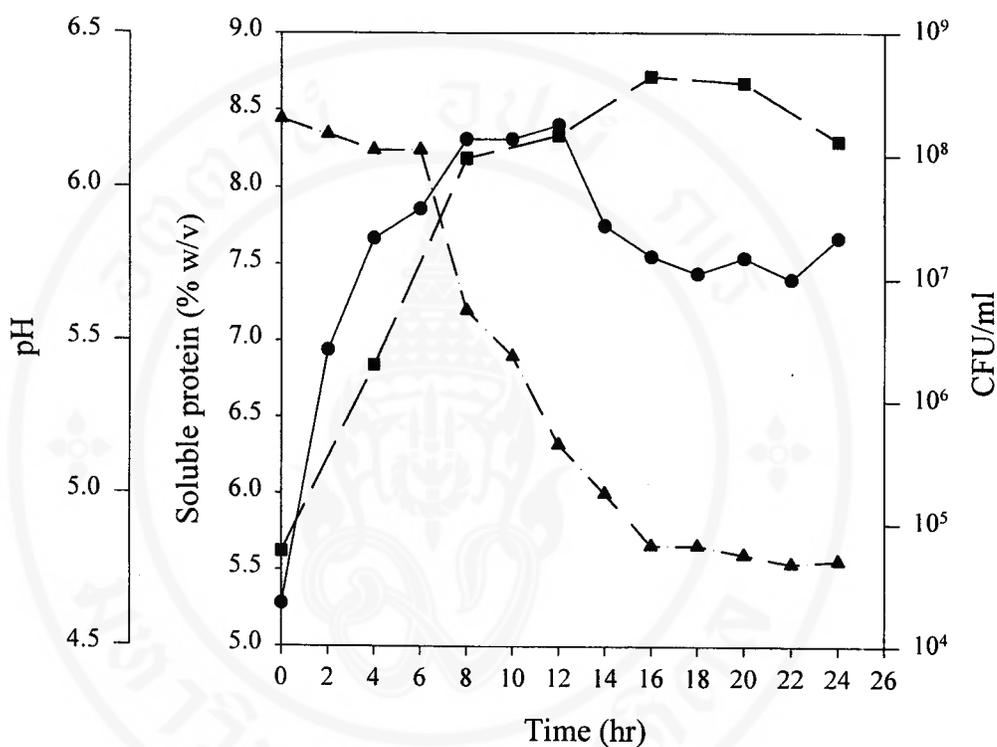


Figure 10 Pattern of soluble protein content, pH and bacterial contamination during the hydrolysis of SBM. SBM was hydrolyzed with conditions of total solid content of 22%, Kojizyme™ at 2 % of SBM protein and temperature at 50°C. The SBH samples were taken at the appropriate time intervals. The samples were tested for total bacterial count, CFU/ml (■). The SBH samples were then centrifuged at 12,000 rpm for 25 min to assay for soluble protein (●) and pH (▲).

of the hydrolysis products decreased slowly in the first 6 hr from 6.2 to 6.1 and then decreased rapidly to 4.8 at 16th hr of incubation.

3. Large scale production of SBH

Using similar conditions for hydrolysis of SBM at the laboratory scale as found in the previous experiments, attempts were made to produce SBH in large quantity.

The large scale production of SBH was carried out in a double jacket kettle by using 12.5 kg of SBM as the starting material mixed with 43.8 kg of water to provide a total solid content of 22 %. KojizymeTM at 106.3 g (equal to 2% of SBM protein) was added to the mixture. Reactions were controlled at 50°C for 12 hr. The detailed methods are described in the Materials and Methods section. Composition of raw materials that used in the large scale production are shown in Table 5.

For individual large scale experiments, each SBH sample was taken and centrifuged for collecting soluble fraction. The supernatant fractions were used for determining the soluble protein content and pH. Data of soluble protein content of five large scale productions of SBH at 12th hr were illustrated in Table 5 and these data were used for calculation of degree of solubilization. It was shown that degree of solubilization in large scale production of SBH was about 49.1% after 12 hr of hydrolysis.

The mean pH values in supernatant fractions of the five large scale batches are illustrated in Figure 11. The initial pH value was about 6.3 and decreased continuously to the level of 5.0 after about 12 hr of incubation. This pH profile was similar to that for laboratory scale hydrolysis.

Table 5 Compositions of raw materials used in the large scale production of SBH

Experiment	Soybean meal				Amount of water added (l)	Amount of water from soybean meal (kg) ³	Total water content (kg)	Soluble protein content after 12 th hr incubation (% w/v)	Total soluble protein (kg)	Degree of solubilization ⁵ (%)
	wet weight (kg)	Moisture content (%)	dry weight (kg)	% protein (w/w) ¹						
1 st	12.5	4.80	11.90	50.0	43.75	0.6	44.4	5.22 ± 0.47	2.3	38.3
2 nd	12.5	5.00	11.88	42.8	43.75	0.6	44.4	4.81 ± 0.35	2.1	41.2
3 rd	12.5	5.00	11.88	42.8	43.75	0.6	44.4	4.89 ± 0.17	2.2	43.1
4 th	12.5	4.28	11.97	43.7	43.75	0.5	44.3	7.49 ± 0.22	3.3	63.5
5 th	12.5	4.28	11.97	43.7	43.75	0.5	44.3	6.98 ± 0.26	3.1	59.6
Mean ⁶	12.5	4.67	11.92	44.6	43.75	0.6	44.4	5.88 ± 1.26	2.6	49.1

¹ % protein calculate base on dry basis of SBM

² total protein calculated from % protein (w/w) multiplied by dry weight of SBM that used in the reaction³

³ amount of water from moisture content in soybean meal

⁴ total soluble protein calculated from percent soluble protein content multiplied by total water content of the hydrolysate product

⁵ degree of solubilization calculated from ratio of total soluble protein to total protein and multiplied by 100

⁶ mean value of 5 experiments

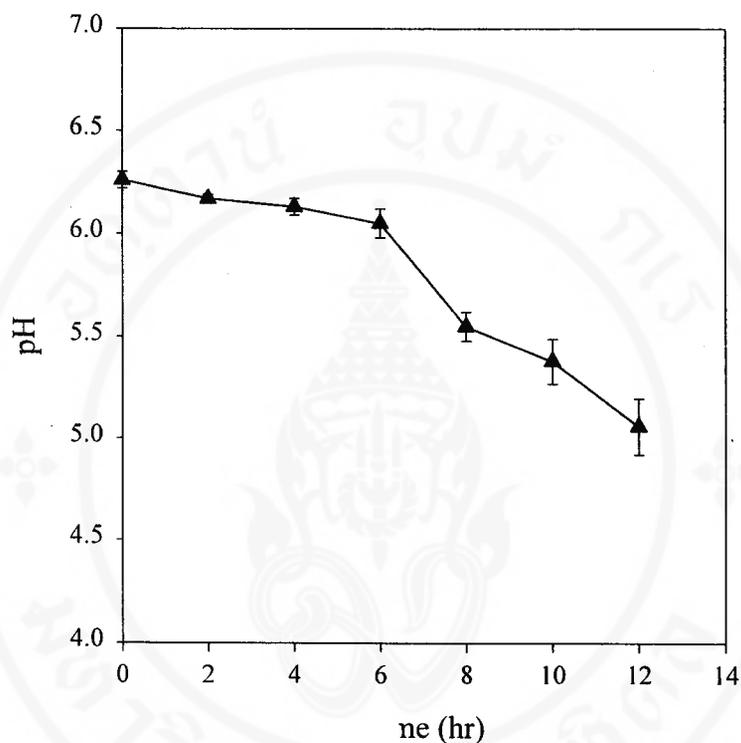


Figure 11 The pH* of SBH samples from large scale production.

Each reaction contained 12.5 kg SBM and 43.8 kg of water which provided a total solid content of 22 %. Kojizyme™ was added at 2 % of SBM protein. The reaction was performed at 50°C for 12 hr. Samples were taken at the indicated time intervals. Samples were centrifuged at 12,000 rpm for 25 min to collect the supernatant fraction for pH (▲) determination.

* the average value from 5 experiments

4. Enhancement of soluble protein content in traditional soy sauce product by addition of SBH

4.1 Chemical analysis of soluble protein enhanced soy sauce samples

Presently, there are several grades of Thai soy sauce products available in the market. The quality and prices differ substantially among these different grade soy sauce products. Quality is usually judged by the protein content which varies from 3% for the first grade to as low as 1.5% for the fourth grade. A good quality soy sauce usually contains 3 to 4% (w/w) protein. It might be possible to enhance the protein by adding an appropriate amount of SBH. Therefore, cSBH of 5.6% protein content was mixed with the finished soy sauce of 3.0% protein content at various ratio and the final salinity was adjusted by NaCl addition to obtain the final salinity of 22%. The results of chemical analysis of these products are shown in Table 6. The level of protein in the various mixtures was 3.04, 3.47, 3.85 and 4.93% w/v (i.e., mixtures containing 0, 0.5 part, 1 part and 1.5 parts of cSBH, respectively). This was equivalent to percent soluble protein increases of 0.43, 0.81 and 1.89% w/v, respectively. In the mixtures, reducing sugar and color decreased a little since the clarified SBH had lower values for these two parameters than typical soy sauce. The pH of the mixture did not show any significant difference from soy sauce and remained between 4.6 to 4.8. From the chemical analysis data, there was no difference in the typical and the mixed soy sauce products, except for the increase in the soluble protein content. However, since SBH might contain some components that cause off-flavor and off-odor in the product, it would be important that these products were further subjected to sensory evaluation in order to test for consumer acceptance and preference.

Table 6 Characteristics of traditional soy sauce and soluble protein enhanced soy sauce products

Product	% Protein (w/v)	% NaCl (w/v)	Reducing sugar (mg/ml)	pH	Color (OD ₄₂₀)
1. Typical soy sauce	3.04 ± 0.08	22.80 ± 0.00	9.63 ± 0.10	4.61 ± 0.02	3.99 ± 0.00
2. Mixture of SS ¹ with cSBH ² at the ratio of 1:0.5	3.47 ± 0.06	21.93 ± 0.00	7.86 ± 0.08	4.85 ± 0.00	3.84 ± 0.03
3. Mixture of SS with cSBH at the ratio of 1:1.0	3.85 ± 0.05	21.78 ± 0.26	7.54 ± 0.08	4.59 ± 0.02	3.63 ± 0.00
4. Mixture of SS with cSBH at the ratio of 1:1.5	4.93 ± 0.03	22.73 ± 0.32	6.51 ± 0.02	4.66 ± 0.00	3.37 ± 0.01

¹ SS = soy sauce² cSBH = clarified soybean meal hydrolysate

4.2 Sensory evaluation of SBH enhanced soy sauce samples

SBH enhanced soy sauce samples used in the sensory evaluation test are illustrated in Figure 12. The results are shown in Figures 13a and 13b and indicated no statistically significant differences ($P > 0.05$) in mean values for general appearance, overall acceptance, flavor, taste, salinity and off-flavor among the treatment groups. There was a statistically significant difference ($P < 0.05$) in mean values of odor among the treatment groups. As the amount of clarified SBH in the mixture increased, the sensory scores decreased. Thus, the ratio of typical soy sauce to clarified SBH at 1:0.5 was only acceptable ratio that still maintained odor of traditional soy sauce. In the mean scores of flavor and taste, there was no statistically significant difference among the treatment groups, but the mean scores decreased as the amount of clarified SBH increased in the mixture. There was a statistically significant difference ($P < 0.05$) in mean values of color among the treatment groups. The mean scores for color were lowest when clarified SBH was added to typical soy sauce at a ratio of 1:1.5.

The general appearance, overall acceptability and off-flavor were used as criteria to decide which mixture provided the best characteristics. Since there were no statistically significant differences among the treatment groups except for odor ($P < 0.05$) in the soy sauce samples that were mixed with clarified SBH at ratios higher than 1:0.5. Thus, at 1:0.5 the test subjects accepted that the mixture maintained the traditional odor of soy sauce. Therefore, the ratio of typical soy sauce to clarified SBH at 1:0.5 was only acceptable mixture for possible supplementation.



Figure 12 Appearance of soy sauce samples that were mixed with soluble protein from cSBH at various ratio's.

SS : soy sauce

cSBH : clarified soybean meal hydrolysate

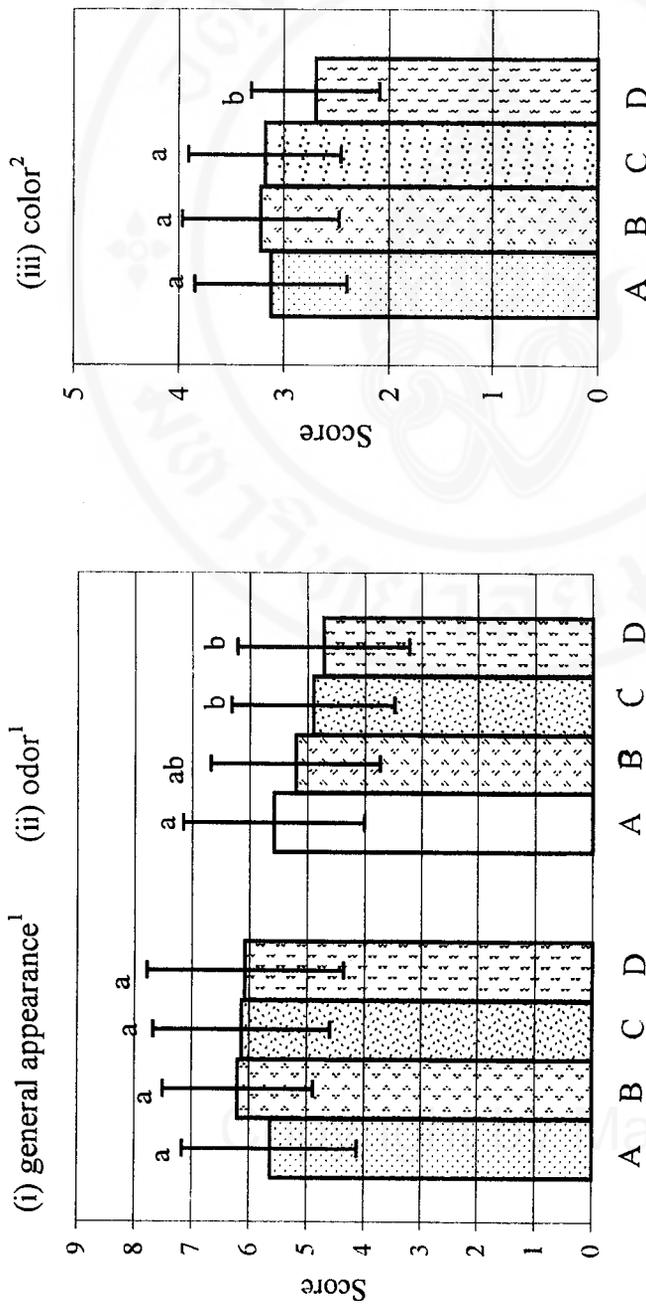


Figure 13a Sensory evaluation of traditional soy sauce samples mixed with various proportions of soluble protein from SBH.

The three different criteria for sensory evaluation were, general appearance (i), odor (ii) and color (iii).

The four soy sauce samples containing various level of clarified SBH (cSBH) were evaluated, namely no cSBH (A), 0.5 part cSBH added (B), 1.0 part cSBH added (C) and 1.5 parts cSBH added (D). Sensory evaluation scores are expressed as mean \pm standard deviation (SD) with n = 50. Means with the same letters within the same criteria were not significantly different ($P > 0.05$).

¹ nine-point hedonic scale, ² five-point hedonic scale

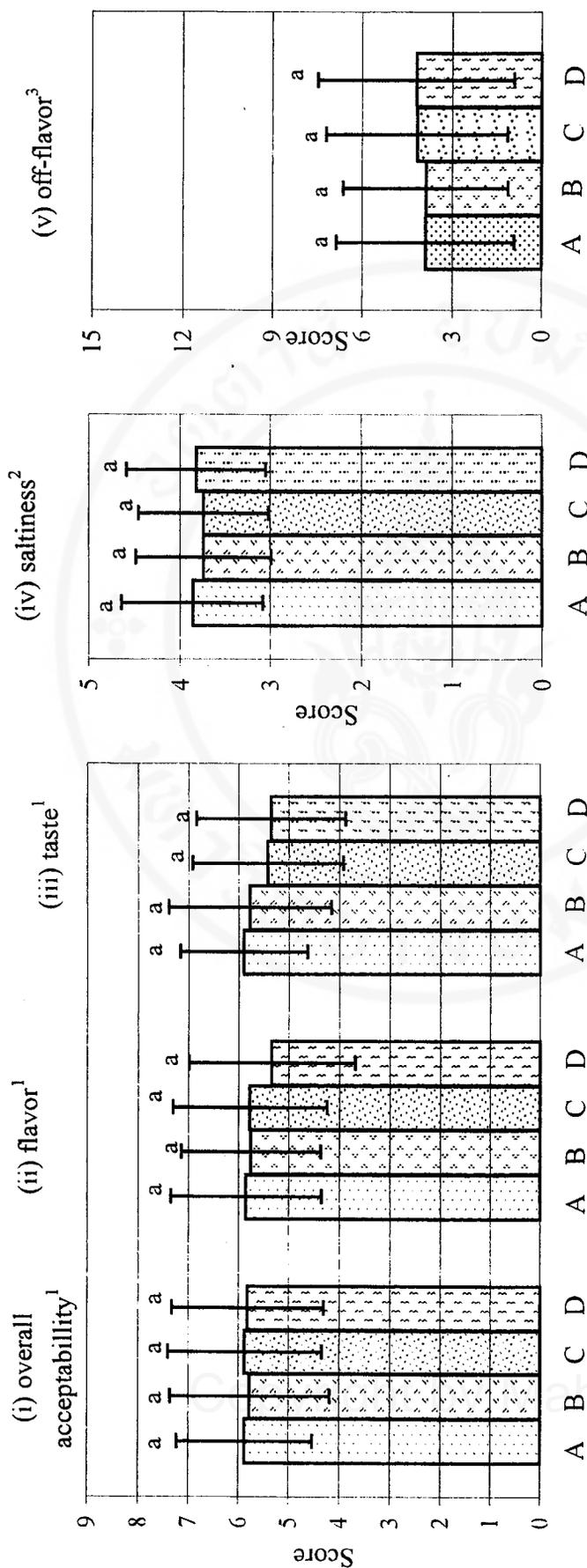


Figure 13b Sensory evaluation of traditional soy sauce samples mixed with various proportions of soluble protein from SBH.

The five difference criteria for sensory evaluation were, overall acceptability (i), flavor (ii), taste (iii), saltiness (iv), and off-flavor (v). The four soy sauce samples containing various level of clarified SBH (cSBH) were evaluated, namely no cSBH (A), 0.5 part cSBH added (B), 1.0 part cSBH added (C) and 1.5 parts cSBH added (D). Sensory evaluation scores are expressed as mean \pm standard deviation (SD) with $n = 50$. Means with the same letters within the same criteria were not significantly different ($P > 0.05$).

¹ nine-point hedonic scale, ² five-point hedonic scale, ³ fifteen cm line scale

5. Protein enrichment in primary moromi fermentation by addition of SBH or Kojizyme™

Since the addition of soluble protein from clarified SBH into the finished soy sauce gave a detectable off-odor at too high supplementation, another strategy was initiated to increase soluble protein in soy sauce by addition of SBH or Kojizyme™ to the moromi fermentation.

SBH or Kojizyme™ was added to the primary moromi fermentation at various times as described in the Materials and Methods section. The results of these analyses are described below.

5.1 Chemical analysis of modified primary moromi fermentations

5.1.1 Soluble protein from modified primary moromi fermentations

The amount of soluble protein in the various moromi mash treatments are shown in Figure 14. The soluble protein content of normal moromi (CSS₁) increased significantly by about 4% (from 1 to 5%) in the first week and then increased gradually thereafter reaching 9% at end of the fermentation period.

The soluble protein contents for the SBH treatments (ESS₁H₀, ESS₁H₃₄ and ESS₁H₄₉) were slightly lower than that for typical soy sauce (CSS₁). It was found that before SBH was added into the moromi mash at day 34 (ESS₁H₃₄) and day 49 (ESS₁H₄₉), the soluble protein content was about the same as that of CSS₁. However, after SBH addition, the soluble protein content decreased to about the same level as

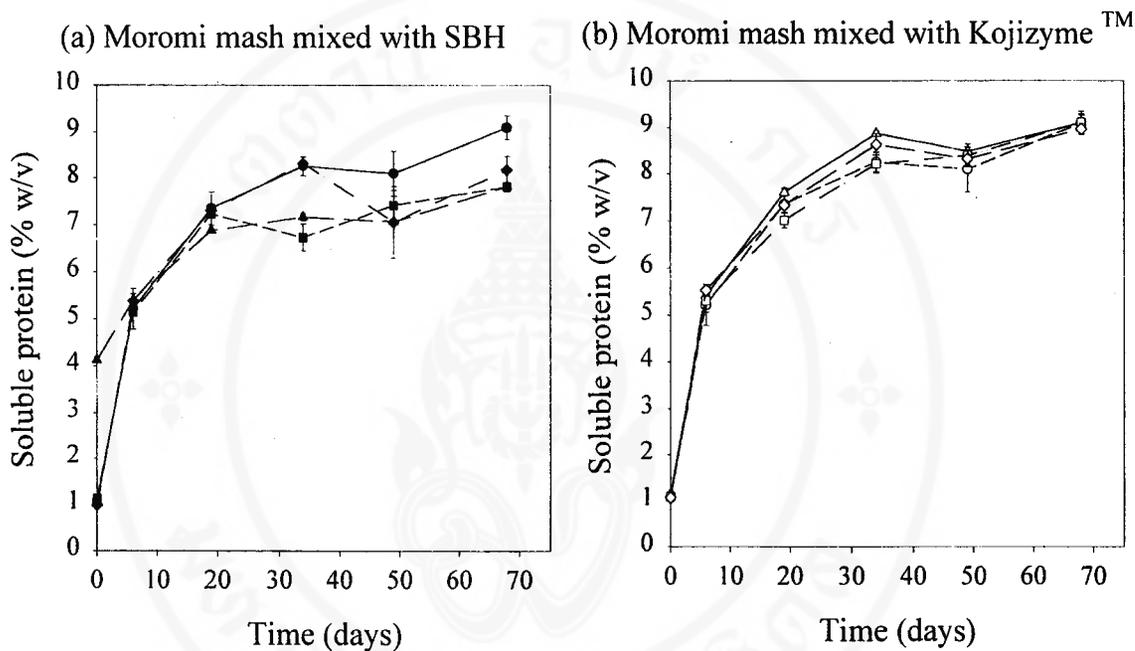


Figure 14 Soluble protein content during the primary moromi fermentation.

Moromi mash was prepared by mixing 17 kg of koji with 34 kg of saturated brine solution. One batch of moromi mash was divided into 2 equal parts and to one part was added 22 kg SBH (a). To the other batch was added with 80.3g of Kojizyme™ (b). SBH (●) was added to moromi mash at day 0 (▲), at day 34 (■) and at day 49 (◆). Similarly, Kojizyme™ (○) was added to moromi mash at day 0 (△), at day 34 (□) and at day 49 (◇). One moromi batch each without any addition was used as the control for the SBH (●) and Kojizyme™ (○) experiments.

that of ESS_1H_0 (Figure 14a). The slight decrease in soluble protein content after the SBH addition might have been due to dilution of protein by water contained in the SBH. The soluble protein in the enzyme treatments (ESS_1K_0 , ESS_1K_{34} and ESS_1K_{49}) was about the same as that of CSS_1 throughout the fermentation period (Figure 14b). Therefore, it was shown that Kojizyme™ did not accelerate the enzymatic degradation of soybean protein over that of the natural process.

Based on the above results, it was concluded that the addition of SBH and Kojizyme™ to the primary cycle moromi fermentation could not enhance the level of soluble protein in finished soy sauce.

5.1.2 Sodium chloride concentration from modified primary moromi fermentations

In all experiments, the initial sodium chloride concentration was adjusted to 24 to 27%. Saturated brine solution [31 % (w/v) or 23.8 °Baume] was used as the stock solution in most of the experiments excepted in SBH experiments where solid NaCl was added. Throughout the incubation period, the concentration of NaCl was controlled by addition of water to the jars if the volume decreased through evaporation. The NaCl concentration of samples fluctuated mostly between 24 and 27 % as shown in Figure 15. Figure 15a shows the NaCl concentration of CSS_1 and ESS_1H . In the first 6 days, moromi samples of ESS_1H_0 showed that the NaCl concentration increased from 22% to 26%, probably due to evaporation of water when exposed to direct sun. Subsequently, the NaCl concentration was held in the range of 24 to 27%. In ESS_1H_{34} , after the moromi mash was divided into two parts for SBH addition, solid NaCl was added incorrectly so that the final salinity rose to 31%, or about 4% higher than that desired.

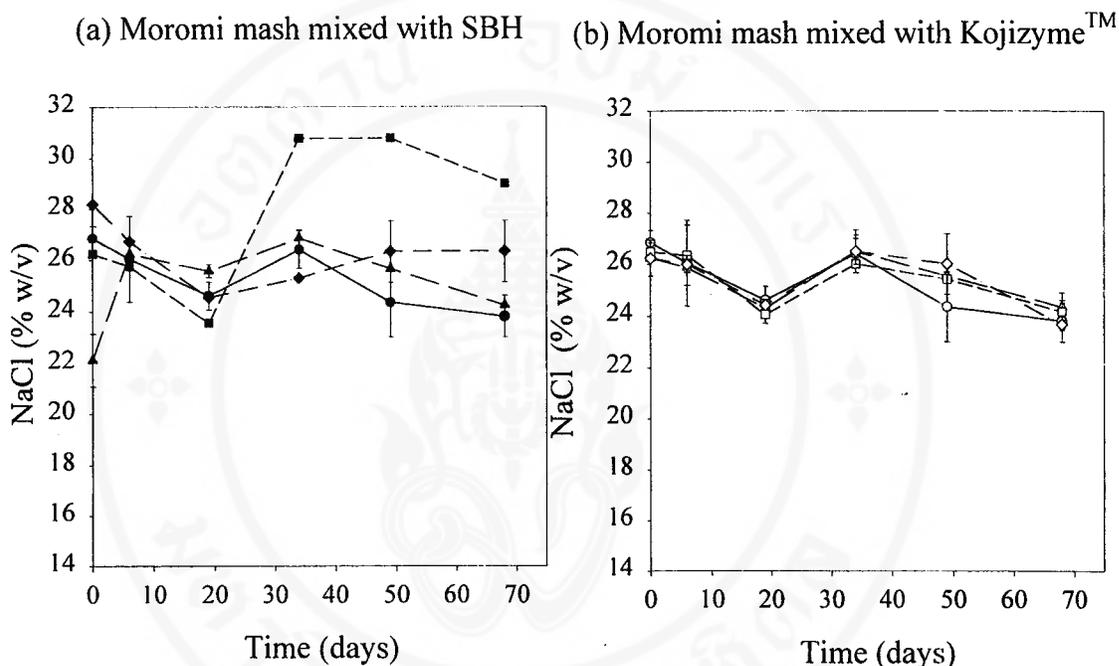


Figure 15 Sodium chloride concentration during the primary moromi fermentation.

Moromi mash was prepared by mixing 17 kg of koji with 34 kg of saturated brine solution. One batch of moromi mash was divided into 2 equal parts and to one part was added 22 kg SBH (a). To the other batch was added with 80.3g of KojizymeTM (b). SBH (●) was added to moromi mash at day 0 (▲ -), at day 34 (■ -) and at day 49 (◆ -). Similarly, KojizymeTM (◯) was added to moromi mash at day 0 (△ -), at day 34 (□ -) and at day 49 (◇ -). One moromi batch each without any addition was used as the control for the SBH (● -) and KojizymeTM (○ -) experiments.

5.1.3 Reducing sugar from modified primary moromi fermentations

The patterns for reducing sugar content of CSS_1 and ESS_1K (Figure 16b) were quite similar. Concentrations of reducing sugar increased significantly in the first week, then stabilized with small fluctuations throughout the rest of the fermentation period. The levels of reducing sugar were about 25 to 28 mg/ml at the end of fermentation period.

In comparing concentrations of reducing sugar in ESS_1H_0 and CSS_1 (Figure 16a), the level of reducing sugar of ESS_1H_0 was higher than that of CSS_1 at the beginning. However, in the 5 day old sample, the concentration of reducing sugar of CSS_1 became higher than that in ESS_1H_0 . In ESS_1H_{34} and ESS_1H_{49} addition of SBH resulted in a rapid decrease in the concentration of reducing sugar.

5.1.4 pH from modified primary moromi fermentations

In this study, the pH's was found to decrease in all experiments. The pH profiles of CSS_1 and ESS_1K were similar. Figure 17b showed that pH decreased rapidly from 5.8 to 5.3 at the first week, then decreased further to 4.8 throughout the 49th day. The pH of ESS_1H_0 was slightly lower than that of CSS_1 at the beginning, thereafter the pH profile was similar to that of CSS_1 (Figure 17a). In ESS_1H_{34} and ESS_1H_{49} , before adding SBH into the moromi mash, the pH's were similar to that of CSS_1 but after adding SBH, the pH decreased to 4.5 and 4.1, respectively probably due to the relatively low pH of SBH (4.7 to 4.8).

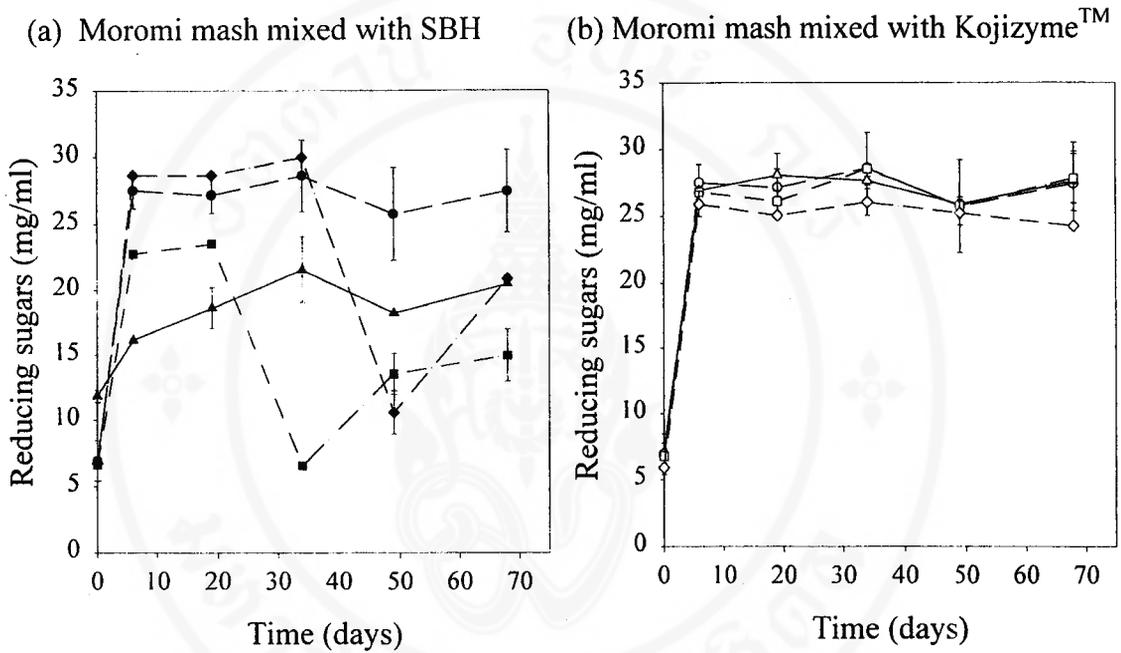


Figure 16 Reducing sugar content during the primary moromi fermentation.

Moromi mash was prepared by mixing 17 kg of koji with 34 kg of saturated brine solution. One batch of moromi mash was divided into 2 equal parts and to one part was added 22 kg SBH (a). To the other batch was added with 80.3g of Kojizyme™ (b). SBH (●) was added to moromi mash at day 0 (▲), at day 34 (■) and at day 49 (◆). Similarly, Kojizyme™ (○) was added to moromi mash at day 0 (▲), at day 34 (■) and at day 49 (◆). One moromi batch each without any addition was used as the control for the SBH (●) and Kojizyme™ (○) experiments.

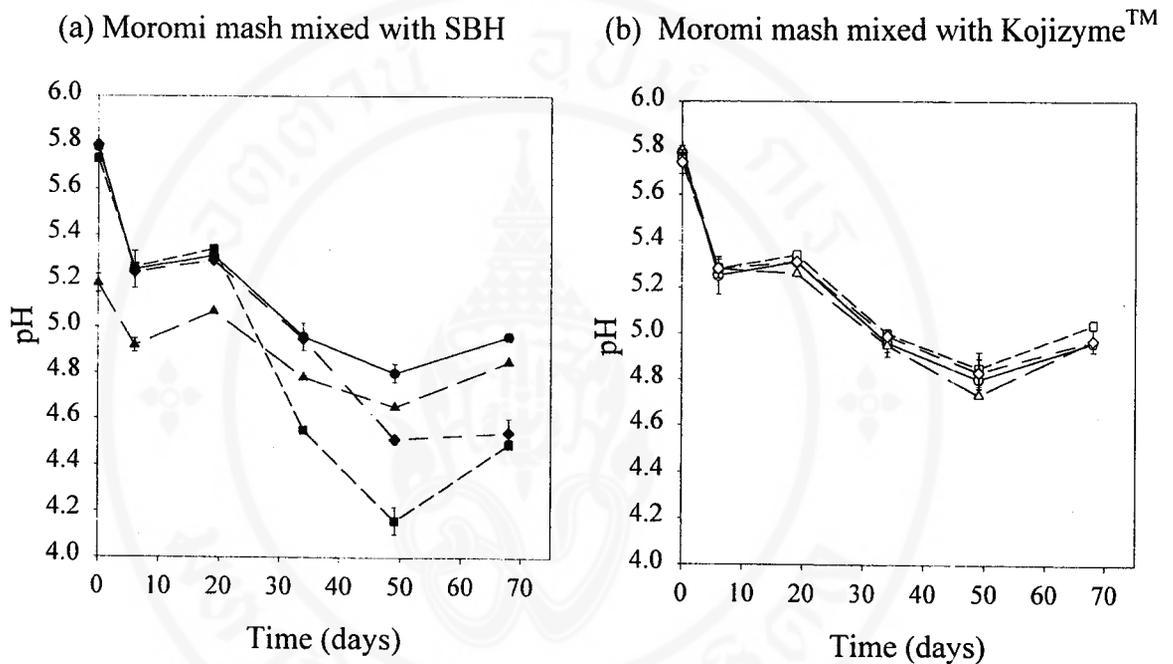


Figure 17 The pH profile during the primary moromi fermentation. Moromi mash was prepared by mixing 17 kg of koji with 34 kg of saturated brine solution. One batch of moromi mash was divided into 2 equal parts and to one part was added 22 kg SBH (a). To the other batch was added with 80.3g of Kojizyme™ (b). SBH (●) was added to moromi mash at day 0 (▲), at day 34 (■) and at day 49 (◆). Similarly, Kojizyme™ (○) was added to moromi mash at day 0 (△), at day 34 (◻) and day 49 (◇). One moromi batch each without any addition was used as the control for the SBH (●) and Kojizyme™ (○) experiments.

5.1.5 Color from modified primary moromi fermentations

Non-enzymatic browning or the Millard reaction occurs between amino groups and reducing compounds. In foods, the amino compounds are predominantly free amino acids or proteins and reducing compounds are primarily reducing sugar. This reaction leads to the development of a dark brown color which is measured in soy sauce by determining the optical density (OD) at absorbency of 420 nm.

The color profiles of CSS₁ and ESS₁K were similar (Figure 18b). The intensity of color increased rapidly during the first week. After the 19th day, the intensity increased linearly.

In ESS₁H₀ samples (Figure 18a), the color was more intense than those of CSS₁ at the beginning. Subsequently, the color intensity increased according to the length of fermentation period. In ESS₁H₃₄ and ESS₁H₄₉, before adding SBH into the moromi mash, the color intensities was similar to those of CSS₁. After adding SBH, the color intensity decreased approximately equal to those of ESS₁H₀.

5.2 Sensory evaluation of soy sauce from primary moromi fermentations

The soy sauce samples were first analyzed for chemical properties (Table 7). They were subsequently used in sensory evaluation tests as illustrated in Figure 19. The results of the sensory evaluation tests are illustrated in Figures 20a and 20b. There were no statistically significant differences at $P > 0.05$ in mean scores of general appearance, overall acceptability, flavor, taste and off-flavor among the treatment groups. The off-flavor scores were about 4.0 to 4.7. There was a relationship between flavor and off-flavor scores. The higher the flavor score, the

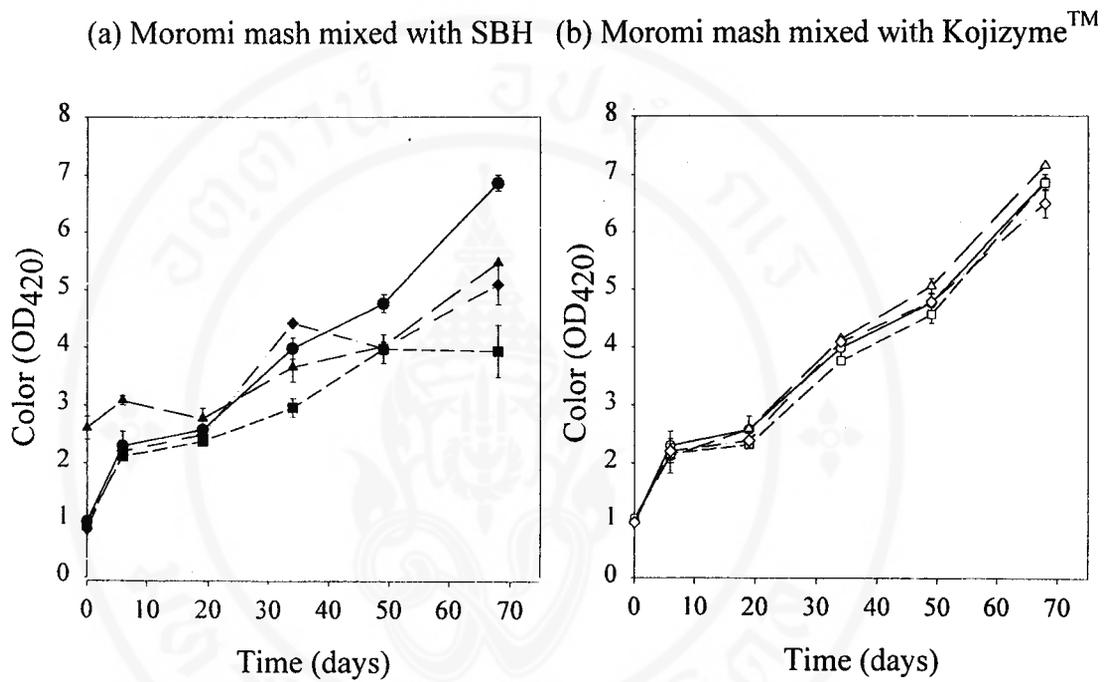


Figure 18 Color intensity during the primary moromi fermentation. Moromi mash was prepared by mixing 17 kg of koji with 34 kg of saturated brine solution. One batch of moromi mash was divided into 2 equal parts and to one part was added 22 kg SBH (a). To the other batch was added with 80.3g of Kojizyme™ (b). SBH (●) was added to moromi mash at day 0 (—▲—), at day 34 (—■—) and at day 49 (—◆—). Similarly, Kojizyme™ (○) was added to moromi mash at day 0 (—△—), at day 34 (—□—) and at day 49 (—◇—). One moromi batch each without any addition was used as the control for the SBH (—●—) and Kojizyme™ (—○—) experiments.



Table 7 Characteristics of soy sauce products obtained from traditional and modified primary moromi fermentation.

Product	% Protein (w/v)	% NaCl (w/v)	Reducing sugar (mg/ml)	pH	Color (OD ₄₂₀)	Yield ¹ of raw soy sauce (%)
1. CSS ₁ *	7.43 ± 0.04	19.73 ± 0.16	21.81 ± 0.07	5.13 ± 0.00	14.10 ± 0.02	35.0 ± 1.70
2. ESS ₁ H ₀ *	5.62 ± 0.00	18.94 ± 0.00	14.72 ± 0.28	5.03 ± 0.01	6.06 ± 0.06	39.0 ± 2.11
3. ESS ₁ H ₃₄	5.41 ± 0.06	20.74 ± 0.00	10.55 ± 0.07	4.67 ± 0.02	4.47 ± 0.05	35.0 ± 2.55
4. ESS ₁ H ₄₉ *	5.91 ± 0.19	19.39 ± 0.00	14.22 ± 0.57	4.73 ± 0.01	5.67 ± 0.03	30.6 ± 2.80
5. ESS ₁ K ₀ *	7.56 ± 0.15	20.52 ± 0.32	21.94 ± 0.04	5.15 ± 0.00	10.65 ± 0.01	35.0 ± 1.84
6. ESS ₁ K ₃₄	7.32 ± 0.12	20.74 ± 0.00	21.61 ± 0.28	5.10 ± 0.00	10.17 ± 0.01	45.8 ± 2.33
7. ESS ₁ K ₄₉	7.15 ± 0.12	19.62 ± 0.32	18.54 ± 0.07	5.13 ± 0.01	9.90 ± 0.03	56.7 ± 1.91

* These products were also used in sensory evaluation experiments.

¹ These values came from the harvesting raw soy sauce (l) divided by starting moromi mash (kg) and multiply by 100

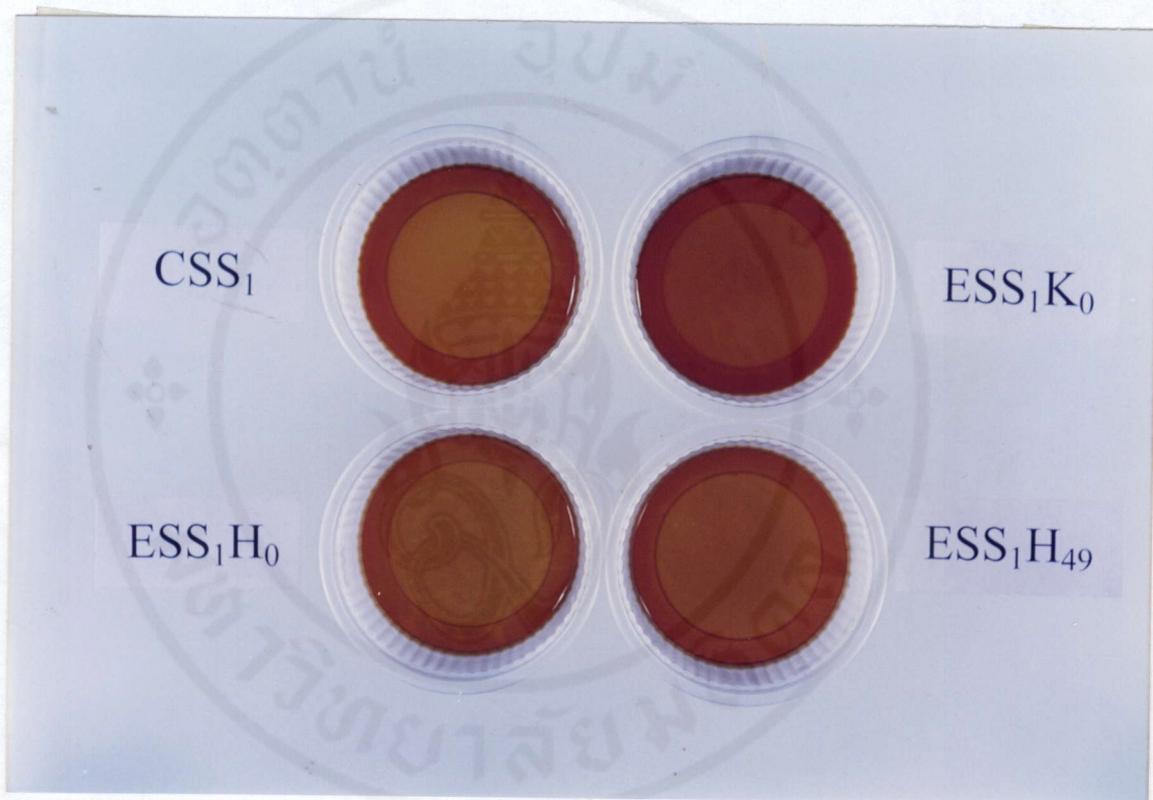


Figure 19 Appearance of soy sauce samples obtained from modified primary moromi fermentation.

CSS₁ : control soy sauce of primary moromi fermentation

ESS₁K₀ : Kojizyme™ added at day 0

ESS₁H₀ : SBH added at day 0

ESS₁H₄₉ : SBH added at day 49

lower the off-flavor score. The mean odor scores of samples CSS₁, ESS₁K₀, ESS₁H₀ and ESS₁H₄₉ decreased in that order. The mean odor scores of CSS₁ and ESS₁K₀ were not significantly different while mean scores of ESS₁H₀ and ESS₁H₄₉ were significantly different from the mean score of CSS₁ as shown in Figure 20a. From these result ESS₁K₀ was the only soy sauce sample that maintained the odor of traditional soy sauce (CSS₁). The mean scores of color and saltiness were significantly different ($P < 0.05$) among the treatment groups but they were not used for judging acceptability. The purpose of these two parameters was to determine the reliability of panelists by judging from the relationship between chemical analysis and sensory evaluation values for these two parameters. It was found that the higher the NaCl concentration, the higher the sensory score for saltiness and the higher the absorbency at 420 nm, the darker the color from sensory evaluation. Thus, the sensory evaluation scores from this group of panelists appeared to be relatively reliable.

In this group of panelists, there were about 12 factory's owners who had high experience in soy sauce sensory evaluation. The experience subjects also could not detect strong off-flavor characteristic (above 7.5 score which was 50% detectable of off-flavor) in these soy sauce samples and provided the similar results with those of general subjects. Therefore, it seem to be that this group of panelists relatively reliable.

From all of sensory evaluation results, it could be concluded that the finished soy sauce samples which added KojizymeTM at the beginning of the fermentation were acceptable and maintained the traditional flavor, aroma and taste of traditional soy sauce (CSS₁).

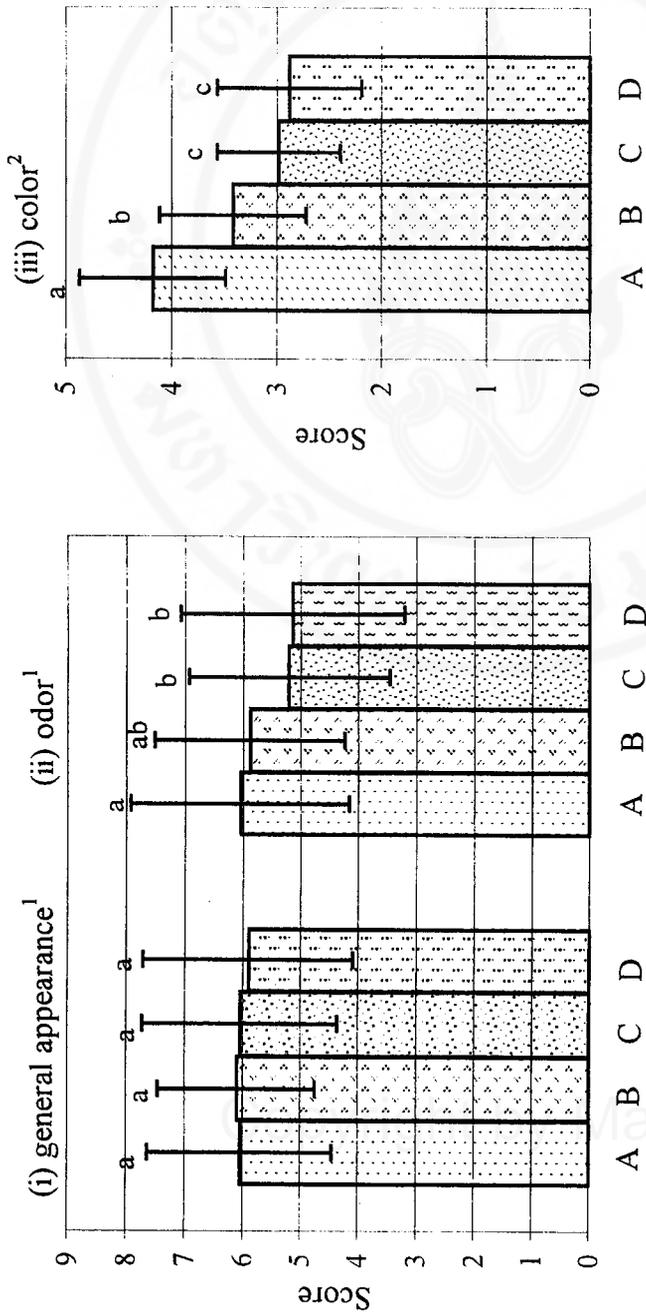


Figure 20a Sensory evaluation of soy sauce samples obtained from primary moromi fermentations.

The three different criteria for sensory evaluation were, general appearance (i), odor (ii) and color (iii).

The four soy sauce samples evaluated were CSS₁ (A), ESS₁K₀ (B), ESS₁H₀ (C) and ESS₁H₄₉ (D).

Sensory evaluation scores are expressed as mean \pm standard deviation (SD) with n = 50. Means with the same letters within the same criteria were not significantly different ($P > 0.05$).

¹ nine-point hedonic scale, ² five-point hedonic scale

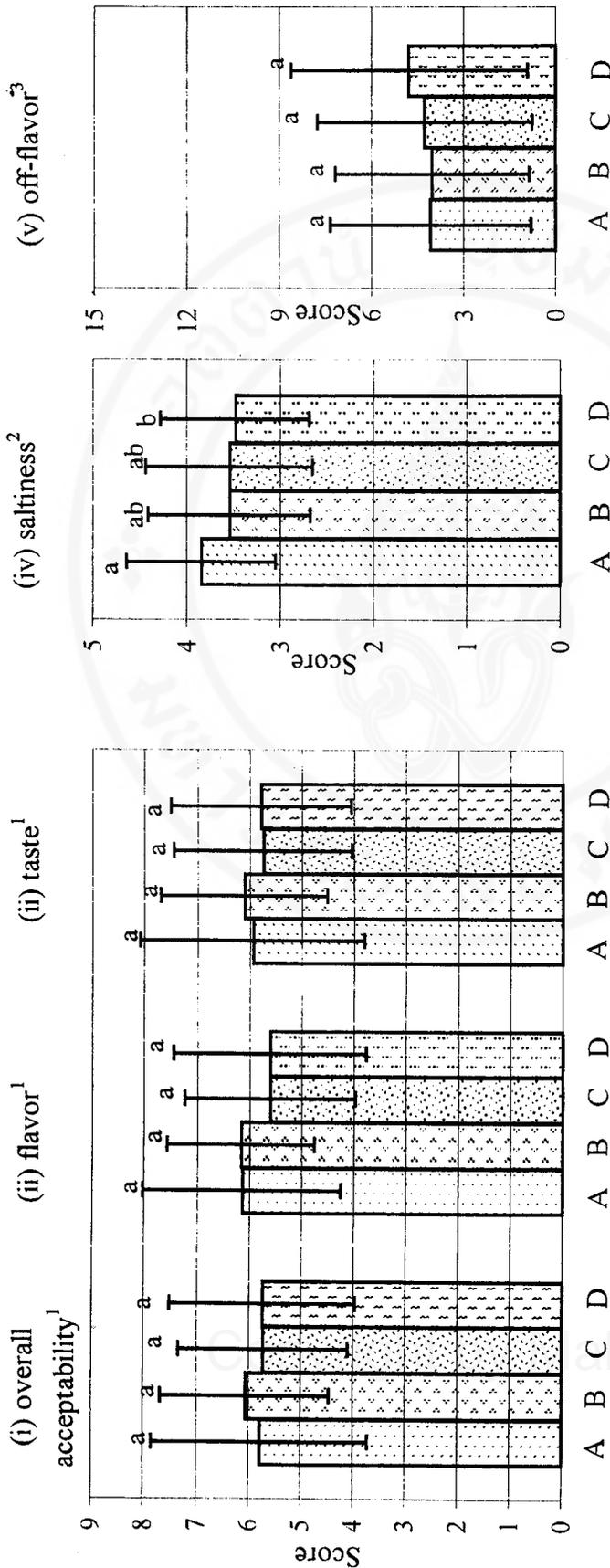


Figure 20b Sensory evaluation of soy sauce samples obtained from primary moromi fermentations.

The five difference criteria for sensory evaluation were, overall acceptability (i), flavor (ii), taste (iii), saltiness (iv) and off-flavor (v). The four soy sauce samples evaluated were CSS₁ (A), ESS₁K₀ (B), ESS₁H₀ (C)

and ESS₁H₄₉ (D). Sensory evaluation scores are expressed as mean ± standard deviation (SD) with n = 50.

Means with the same letters within same criteria were not significantly different ($P > 0.05$).

¹ nine-point hedonic scale, ² five-point hedonic scale, ³ fifteen cm line scale

5.3 Cost analysis for replacing koji protein with SBH protein in primary moromi fermentation

From the Table 3 in Materials and Methods section, it was shown that the preparation of protein enrichment in primary moromi fermentation could reduce the use of koji about half by replacing another half of protein with SBH which obtained the same amount of soluble protein content with the control soy sauce, e.g., ESS₁H₀ experiment. Therefore, the cost analysis for this experiment was performed. The raw material, utilities and labor cost for producing 1 kg of koji and SBH was about 10 and 9.5 Baht (see Table 10, page 114), respectively. It was found that the cost for using 22.6 kg SBH (215 Baht) instead of 8.5 kg koji (85 Baht) had higher cost than used koji about 2.5 times. Thus, the replacement of koji in primary moromi fermentation by using SBH was not appropriate for further application in of soy sauce production industrial scale.

6. Protein enrichment of secondary moromi fermentation by addition of SBH and SBM

A third strategy to enhance the soluble protein content in finished soy sauce was carried out by addition of SBH or soybean meal (SBM) into secondary moromi fermentations. The use of SBM was studied because it was the original raw material use for SBH production. If it could effectively increase the soy sauce protein content, it would be much easier to apply in industry.

In a secondary moromi fermentation, the starting material is the moromi mash residue (MMR) from a primary moromi fermentation. SBH or sterilized SBM was added to MMR in various ratio's as describe in the Materials and Methods. Brine

solution was then added at final NaCl concentrations of 22 to 25% and the contents were fermented for one month.

6.1 Chemical analysis of modified secondary moromi fermentations

6.1.1 Soluble protein from modified secondary moromi fermentations

The soluble protein content of final soy sauce samples from control soy sauce (CSS₂), experimental soy sauce with SBH (i.e., ESS₂H² and ESS₂H¹) and experimental soy sauce with SBM (i.e., ESS₂M² and ESS₂M¹) were about 0.72, 2.17, 1.51, 1.16 and 0.91% (w/v), respectively as shown in Figure 19. It was found that addition of SBH or SBM could enhance the soluble protein content of finished soy sauce when compared with control soy sauce. The soluble protein of final soy sauce of ESS₂H², ESS₂H¹, ESS₂M² and ESS₂M¹ were about 3.0, 2.1, 1.6 and 1.3 times higher than those of CSS₂, respectively (Figure 21). These results indicated that the soluble protein increased in proportion to the amount of SBH used. Similar results were obtained with SBM. Although SBH gave more significant increase than SBM.

6.1.2 Sodium chloride concentration from modified secondary moromi fermentations

Figure 22 showed that the sodium chloride concentrations in the jars were not significantly different and fluctuated between 22 to 25 %.

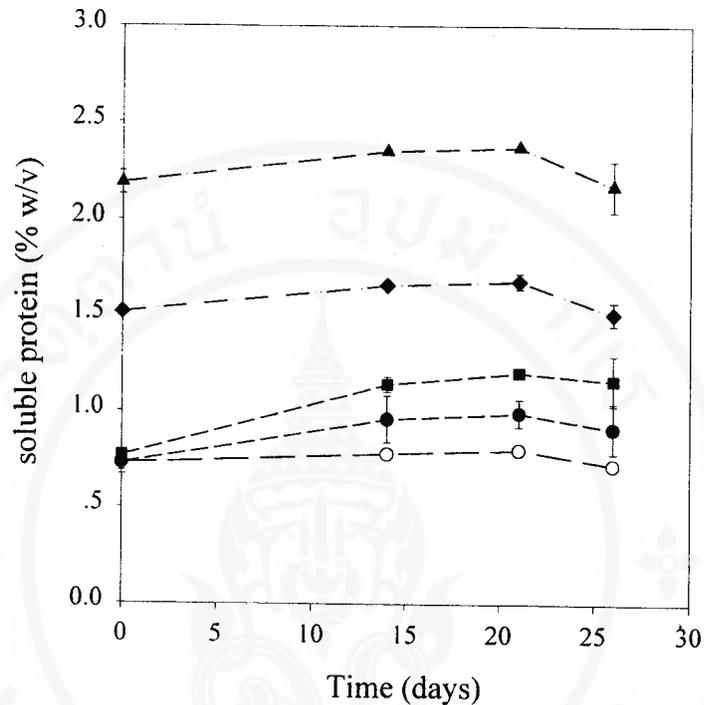


Figure 21 Soluble protein content of soy sauce samples from secondary moromi fermentations. The moromi mash residue (MMR) from a primary experiment was mixed with 3 times by weight of brine solution (the secondary moromi mash). SBH and SBM were taken added in various ratio at the beginning of fermentation and incubated for 26 days. At appropriate time intervals, samples were taken and centrifuged. The supernatant was assayed for soluble protein content. The symbols used in the figure are as follows, (○) CSS₂, (▲) ESS₂H², (◆) ESS₂H¹, (■) ESS₂M² and (●) ESS₂M¹.

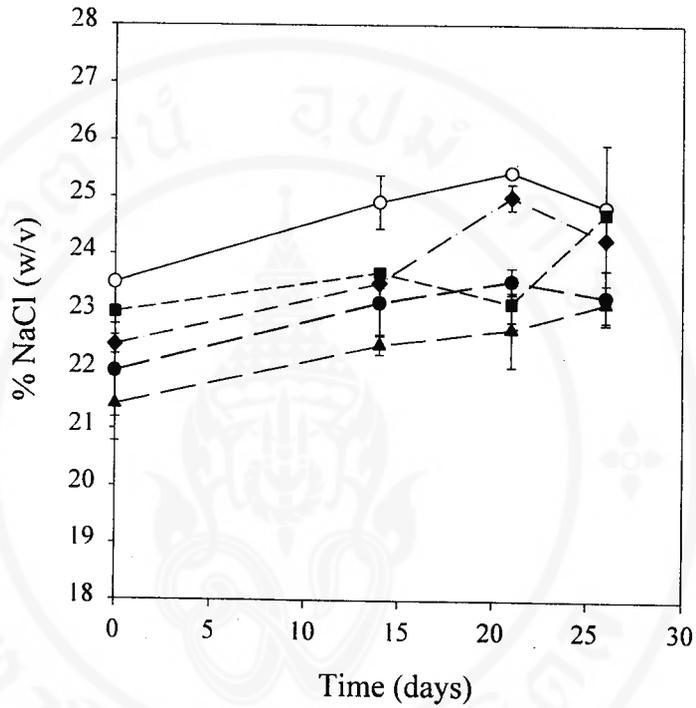


Figure 22 Sodium chloride concentration of soy sauce samples from secondary moromi fermentations. The moromi mash residue (MMR) from primary experiment was mixed with 3 times by weight of brine solution (the secondary moromi mash). SBH and SBM were then added in various ratio at the beginning of fermentation and incubated for 26 days. At the appropriate time intervals, samples were taken and centrifuged. The supernatant was assayed for soluble protein content. The symbols used in the figure are as follows, (○) CSS₂, (▲) ESS₂H², (◆) ESS₂H¹, (■) ESS₂M² and (●) ESS₂M¹.

6.1.3 Reducing sugar from modified secondary moromi fermentations

The level of reducing sugar in the secondary moromi fermentations was relatively low when compared with primary moromi fermentations. The results in Figure 23 show that reducing sugar contents were stable throughout the one month fermentation period, except in ESS_2M^2 where reducing sugar increased by about 1 mg/ml during the first 14 days of incubation. At the end of the fermentation period, the level of reducing sugar could be classified into 3 types. The first type, CSS_2 contained the lowest amount of reducing sugar (1 mg/ml). The second type, ESS_2H^1 and ESS_2M^1 contained about 2 mg/ml, and the third type, ESS_2H^2 and ESS_2M^2 contained about 3 mg/ml. These results indicated that SBH and SBM addition result in higher amounts of reducing sugars than in CSS_2 . The results also indicated that ESS_2H^1 and ESS_2M^1 which started with the same amount of SBM gave the same amount of reducing sugar. When the amount of SBM was increased the amount of reducing sugar increased.

6.1.4 pH from modified secondary moromi fermentations

The pH of secondary moromi fermentations were similar to those at the end of primary moromi fermentations (about 4.8 to 5.0). The pH was relatively constant with only small fluctuations (Figure 24) excepted in ESS_2M samples which started with pH 5.4 and decreased to 4.9 after one month incubation. At the end of the incubation period, the pH of all experiments decreased to between 4.6 to 4.9.

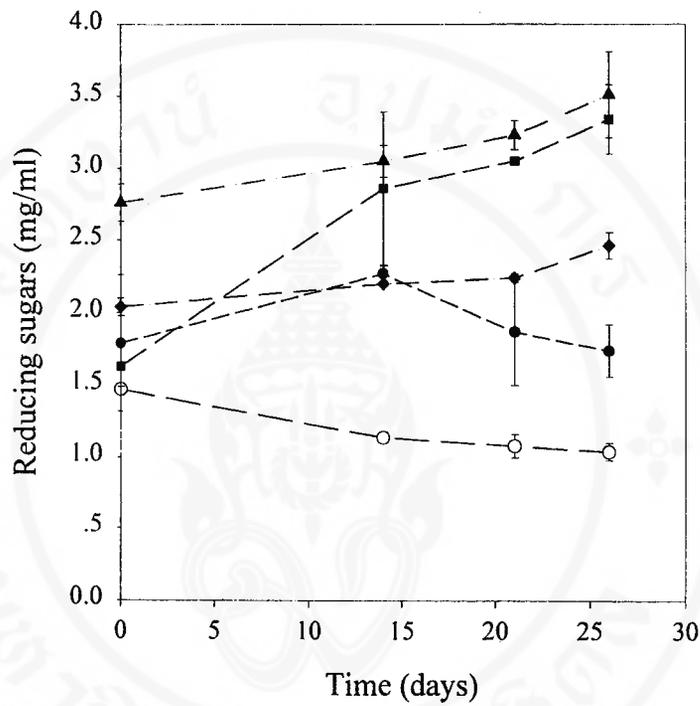


Figure 23 Reducing sugar content of soy sauce samples from secondary moromi fermentations. The moromi mash residue (MMR) from a primary experiment was mixed with 3 times by weight of brine solution (the secondary moromi mash). SBH and SBM were then added in various ratio at the beginning of fermentation and incubated for 26 days. At the appropriate time intervals, samples were taken and centrifuged. The supernatant was assayed for soluble protein content. The symbols used in the figure are as follows, (○) CSS₂, (▲) ESS₂H², (◆) ESS₂H¹, (■) ESS₂M² and (●) ESS₂M¹.

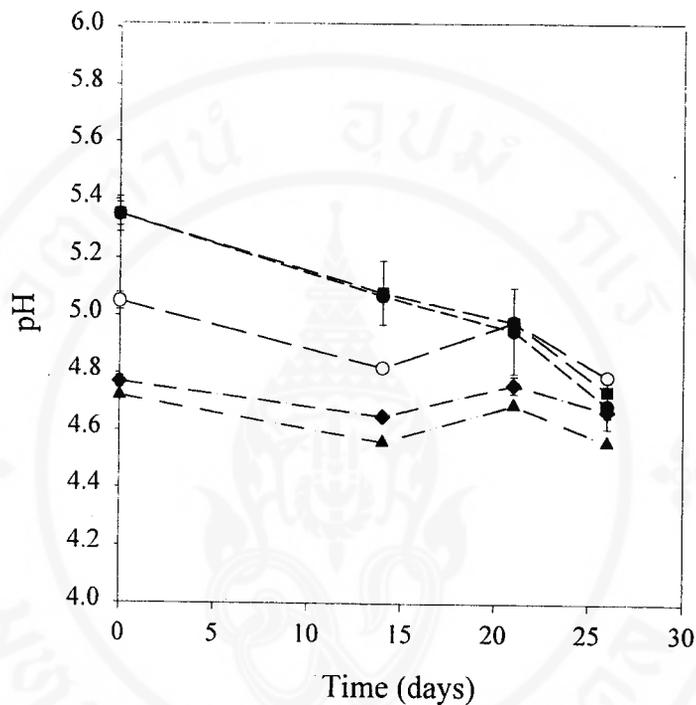


Figure 24 The pH profile of soy sauce samples from secondary moromi fermentations. The moromi mash residue (MMR) from primary experiment was mixed with 3 times by weight of brine solution (the secondary moromi mash). SBH and SBM were then added in various ratio at the beginning of fermentation and incubated for 26 days. At the appropriate time intervals, samples were taken and centrifuged. The supernatant was assayed for soluble protein content. The symbols used in the figure are as follows, (○) CSS₂, (▲) ESS₂RH², (◆) ESS₂RH¹, (■) ESS₂RM² and (●) ESS₂RM¹.

6.1.5 Color from modified secondary moromi fermentations

The color intensities of samples from secondary moromi fermentation were relatively constant throughout the one month fermentation period as shown in Figure 25. The increase in color intensity in CSS₂, ESS₂H or ESS₂M appeared to correlate to increased levels of soluble protein. The higher the protein content the higher the color intensity. Although, both protein and reducing sugar are involved in browning reactions. The reducing sugar appeared to be less important here.

6.2 Sensory evaluation of soy sauce samples from secondary moromi fermentations

The soy sauce samples from secondary moromi fermentation were first analyzed for chemical properties (Table 8). They subsequently used in sensory evaluation tests as illustrated in Figure 26. The results of the sensory evaluation tests are shown in Figures 27a and 27b. The results showed statistically significant differences ($P < 0.05$) in mean scores of general appearance, odor, color, overall acceptability, flavor, taste and saltiness among the treatment groups. The mean scores of all characteristics except saltiness increased in order in soy sauce samples CSS₂, ESS₂M², ESS₂H¹ and ESS₂H². The soy sauce samples of ESS₂M and ESS₂H possessed better flavor, aroma and taste than those of traditional soy sauce (CSS₂). ESS₂H² received the highest scores among the treatment groups. There was no statistically significant difference ($P > 0.05$) in mean scores of off-flavor among the treatment groups. The mean scores of off-flavor were about 3.0 to 3.5. There was a relationship between flavor and off-flavor. The higher the flavor score, the lower the off-flavor

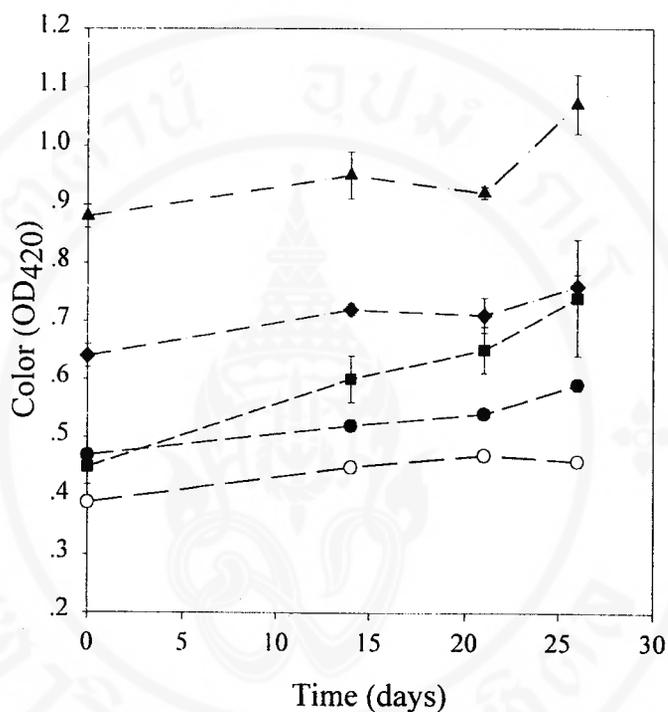


Figure 25 Color intensity of soy sauce samples from secondary moromi fermentations. The moromi mash residue (MMR) from primary experiment was mixed with 3 times by weight of brine solution (the secondary moromi mash). SBH and SBM were then added in various ratio at the beginning of fermentation and incubated for 26 days. At the appropriate time intervals, samples were taken and centrifuged. The supernatant was assayed for soluble protein content. The symbols used in the figure are as follows, (○) CSS₂, (▲) ESS₂H², (◆) ESS₂H¹, (■) ESS₂M² and (●) ESS₂M¹.

Table 8 Characteristics of soy sauce products obtained from traditional and modified secondary moromi fermentation.

	% Protein (w/v)	% NaCl (w/v)	Reducing sugar (mg/ml)	pH	Color (OD ₄₂₀)	Yield of raw soy sauce (%)
1. CSS ₂ *	0.73 ± 0.06	22.72 ± 0.64	1.12 ± 0.03	4.82 ± 0.00	0.63 ± 0.00	49.0 ± 4.03
2. ESS ₂ RH ² *	2.28 ± 0.03	22.08 ± 0.52	3.35 ± 0.06	4.47 ± 0.00	1.30 ± 0.01	47.1 ± 2.12
3. ESS ₂ RH ¹ *	1.53 ± 0.00	22.38 ± 0.77	2.20 ± 0.03	4.62 ± 0.01	0.94 ± 0.02	45.7 ± 0.85
4. ESS ₂ RM ² *	1.15 ± 0.00	22.10 ± 0.32	2.72 ± 0.06	4.71 ± 0.01	0.91 ± 0.00	46.8 ± 0.64
5. ESS ₂ RM ¹	0.96 ± 0.00	22.50 ± 0.64	1.75 ± 0.01	4.65 ± 0.02	0.79 ± 0.01	48.2 ± 0.99

* these soy sauce products were used in sensory evaluation experiment

¹ These values came from the harvesting raw soy sauce (l) divided by starting moromi mash (kg) and multiply by 100

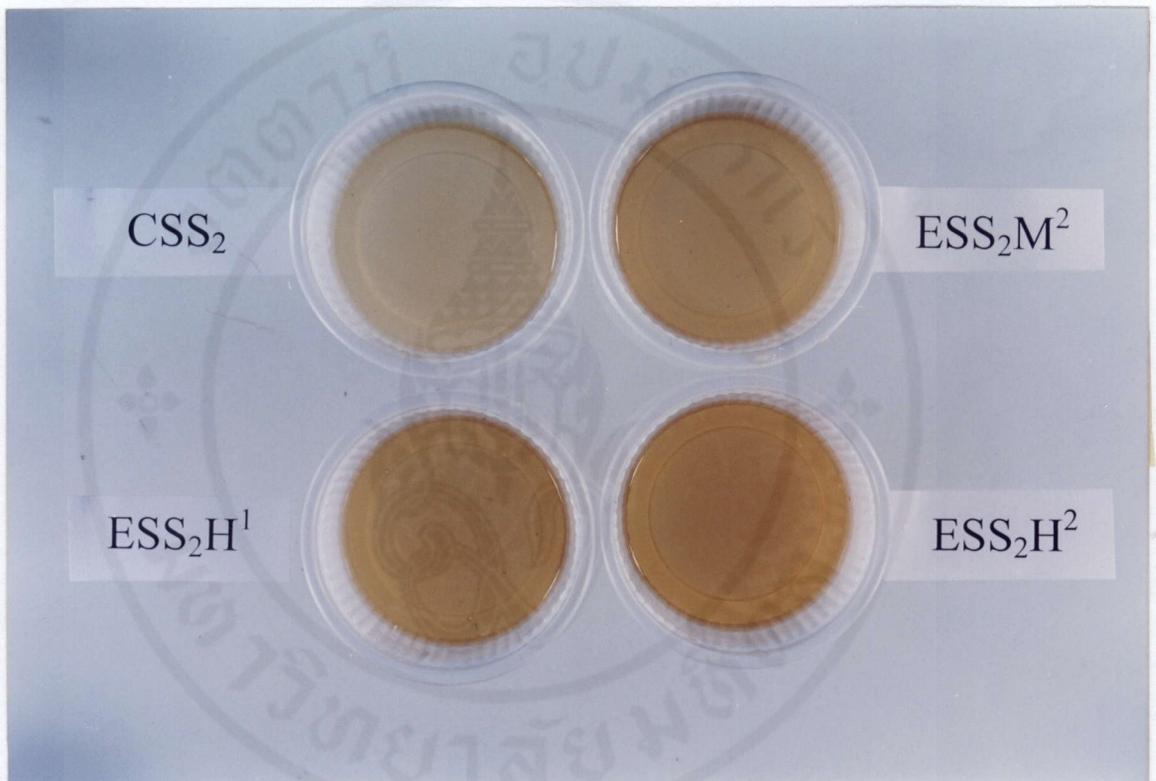


Figure 26 Appearance of soy sauce samples obtained from modified secondary moromi fermentation.

CSS₂ : control soy sauce of secondary moromi fermentation

ESS₂M² : MMR 20 kg : autoclaved-SBM 8.8 kg

ESS₂H¹ : MMR 20 kg : SBH 10 kg

ESS₂H² : MMR 20 kg : SBH 20 kg

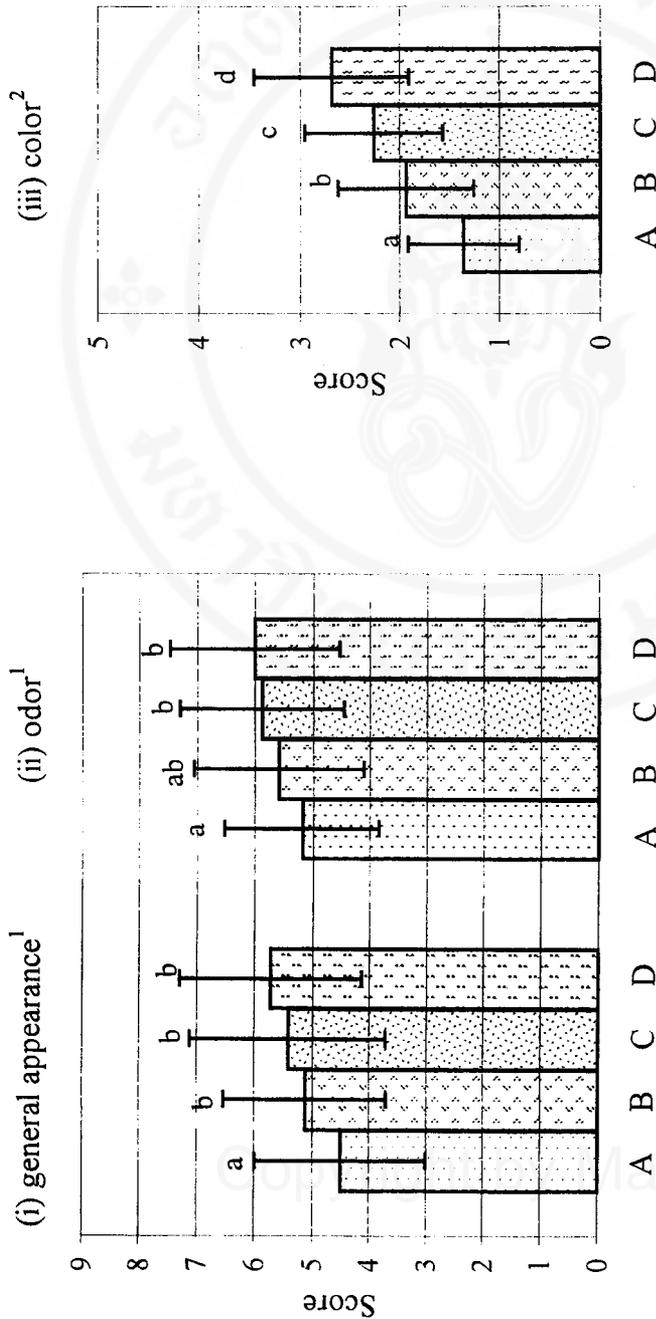


Figure 27a Sensory evaluation of soy sauce samples obtained from secondary moromi fermentations.

The three different criteria for sensory evaluation were, general appearance (i), odor (ii) and color (iii). The four soy sauce samples evaluated were CSS₂ (A), ESS₂M² (B), ESS₂H¹ (C) and ESS₂H² (D).

Sensory evaluation scores are expressed as mean ± standard deviation (SD) with n = 50. Means with the same letters within the same criteria were not significantly different ($P > 0.05$).

¹ nine-point hedonic scale, ² five-point hedonic scale

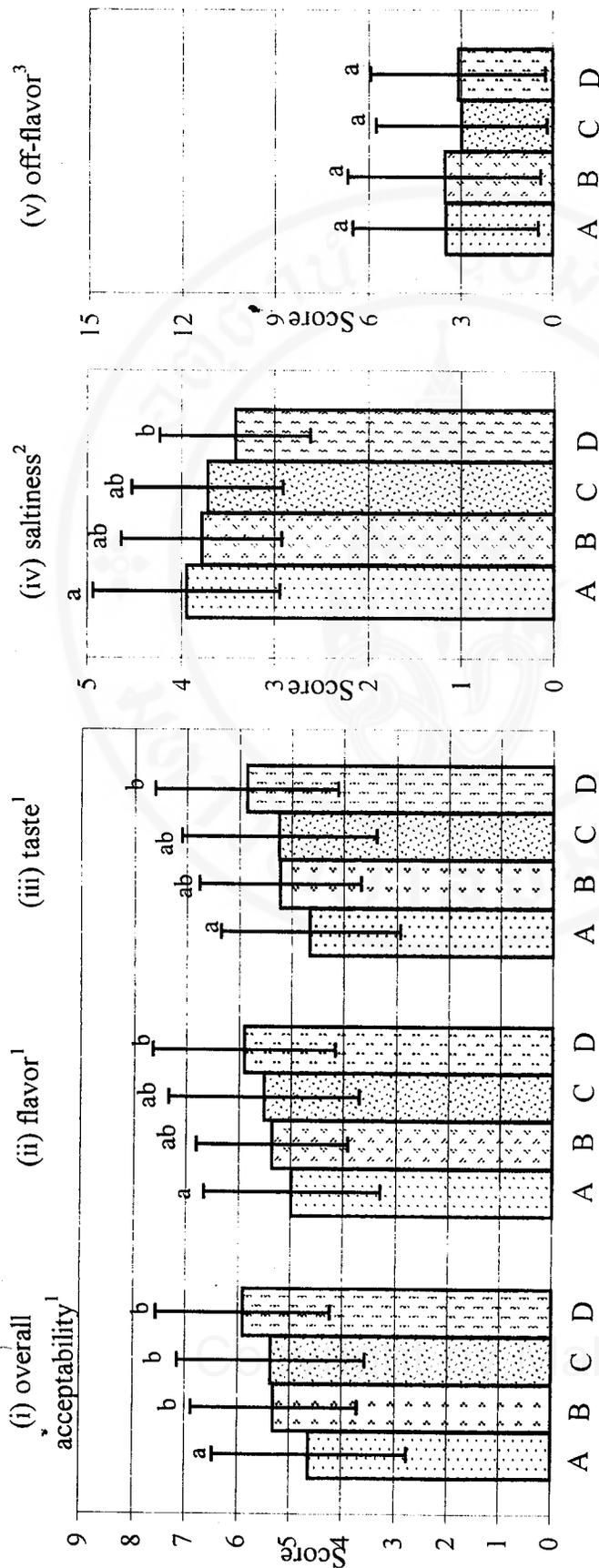


Figure 27b Sensory evaluation of soy sauce samples obtained from secondary moromi fermentations.

The five difference criteria for sensory evaluation were, overall acceptability (i), flavor (ii), taste (iii), saltiness (iv) and off-flavor (v). The four soy sauce samples evaluated were CSS₂ (A), ESS₂M² (B), ESS₂H¹ (C) and ESS₂H² (D). Sensory evaluation scores are expressed as mean ± standard deviation (SD) with n = 50. Meas with the same letters within the same criteria were not significantly different ($P > 0.05$).

¹ nine-point hedonic scale, ² five-point hedonic scale, ³ fifteen cm line scale

score in which ESS₂H² obtained highest score of flavor and lowest score of off-flavor. In this sensory evaluation test, experience subjects also provide the similar results with those of general subjects. Thus, ESS₂H¹ and ESS₂H² possessed better characteristics than CSS₂ and ESS₂M¹ and it can be concluded that the addition of either SBH or SBM into a secondary moromi can gave soy sauce with better characteristics (flavor, aroma and taste) than those of traditional soy sauce. Soy sauce samples derived from MMR with added SBH at 1:1(w/w) gave the best characteristics.

7. Cost analysis for addition of SBH in soy sauce

If the use of SBH is to be recommended to soy sauce manufacturers as a possible intervention to increase soluble protein content, one must consider the economic aspects for such intervention. When SBH was produced in a 56 kg batch, the be estimated cost of raw materials, utilities and labor for production of one kg of SBH with 9.6% (w/w) protein was 13.86 Baht (Table 9). From this one kg about 78% clarified SBH (cSBH) was obtained. Thus, the estimated cost for production of one kg of cSBH with 5.9% (w/v) soluble protein was 17.78 Baht. From this estimation, when the first grade soy sauce (3% (w/v) protein) with price of 45.5 Baht/L was mixed with cSBH (5%(w/v) protein) with the price of 17.78 Baht/kg at the ratio of 1: 0.5, the resulting product contained 4.0% (w/v) soluble protein and lowered the cost by 9.2 Baht/L. Furthermore, when the first grade soy sauce was mixed with 3% protein of cSBH which was diluted from 5.9% protein in the same ratio as mentioned above, the resulting product contained 3% soluble protein with the cost lowered by 12.1 Baht/L. From this initial calculation such a high reduction in cost for producing first grade soy sauce, the attempt was made to analyze cost of production plant for producing SBH

Table 9 Cost estimation for production of SBH

		Baht		
		Unit cost ^b	Subtotal	Total
1. Cost of raw material				
Soybean meal	12.5 kg @ 11 Baht/kg	137.50		
Kojizyme™	106.3 g @ 4.4 Baht/g	<u>467.72</u>		
	Sub-total		<u>605.22</u>	
2. Utilities				
Electricity ^a :	22.8 units @ 0.9 Baht/unit	20.52		
Water	: 0.04 m ³ @ 9 Baht/m ³	<u>0.36</u>		
	Sub-total		<u>20.88</u>	
3. Labor cost				
	One person at 150 Baht/ day		<u>150.00</u>	
	Total			<u>776.10^c</u>

^a Electricity required for the operation of kettle and water bath

^b Unit cost per 56 kg of SBH

^c This cost is for production of 56 kg of SBH. Therefore, it is about 13.86 Baht/kg.

and cSBH. It was assumed that this plant was an additional unit in an existing soy sauce factory, and only a few new machines for production of SBH and cSBH was required in addition to the existing ones such as retort and boiler. The production capacity for producing SBH was assumed to be about one ton per day. With the addition of a 2 ton capacity double jacket kettle, the cost for producing one kg of SBH or one kg of cSBH was about 9.5 (Table 10) or 13.52 Baht (Table 11), respectively. Thus, in order to produce 4.0% protein soy sauce, the cost of production was about 34.84 Baht/L which was much less than the present value of 45.5 Baht/L. To produce 3% protein modified soy sauce, the cost of production was about 32.63 Baht/L which was 12.87 Baht/L less than the present production cost. Therefore, it appeared that the addition of cSBH into finish soy sauce might be a possible practice for soy sauce manufacturer in order to increase soluble protein content in soy sauce product to lower production cost.

If SBH were used to supplement secondary moromi fermentation, the cost for one kg of SBH was about 9.5 Baht. This SBH was to be mixed with moromi mash residue from primary cycle moromi fermentation at the ratio of 1:1 (20 kg /20 kg). The soy sauce sample from modified secondary cycle moromi fermentation contained 2.0% soluble protein which was about three times higher than that of unmodified secondary moromi (0.7% soluble protein). For mixing raw soy sauce of 5% soluble protein soy sauce with soy sauce from secondary moromi fermentation in order to yield 3% soluble protein soy sauce product. It was found that the ratio of 5% soy sauce to modified soy sauce from secondary cycle moromi fermentation's soy sauce was 35:65 or 1:1.8 while in unmodified soy sauce from secondary moromi fermentation was 55:45 or 1:0.8. Therefore, it could reduce amount of 5% soy sauce

Table 10 Economic analysis for production of SBH

	Variable cost (Baht)	Fixed cost (Baht)
1) Raw materials	2,944,500	
2) Labor cost		
- Salary		52,000
- Wage	117,000	
3) Utilities		
- Electricity	44,928	
- Water	42,000	
- Fuel	130,000	
4) Maintenance		
- Equipment		6,000
- Building		1,000
5) Depreciation		
- Equipment		34,900
- Building		4,266
Total cost	3,240,628	98,166

Total cost for production of SBH per year = 3,338,794 Baht

The production of 1.35 ton of SBH / day × 260 days = 351 tons / year

The cost of production per year = 3,338,794 Baht for 351 tons

Therefore, the production cost per ton = 9,512.2 Baht

The production cost per kg = 9.5 Baht

Table 11 Economic analysis for production of cSBH

	Variable cost (Baht)	Fixed cost (Baht)
1) Raw materials	2,944,500	
2) Labor cost		
- Salary		52,000
- Wage	156,000	
3) Utilities		
- Electricity	80,496	
- Water	42,000	
- Fuel	130,000	
4) Maintenance		
- Equipment		9,000
- Building		1,000
5) Depreciation		
- Equipment		134,900
- Building		4,266
Total cost	3,315,196	201,166

Total cost for production of clarified SBH per year = 3,516,362 Baht

The production of 1 ton of cSBH / day × 260 days = 260 tons / year

The cost of production per year = 3,516,362 Baht for 260 tons

Therefore, the production cost per ton = 13,524.5 Baht

The production cost per kg = 13.52 Baht

used by as high as 200 ml/L. If the price of 5% raw soy sauce was 10 Baht/L, it could reduce cost by 2 Baht/L in mixing with modified secondary moromi fermentation soy sauce product to obtain 3% soy sauce product. It appeared that the addition of SBH into secondary moromi fermentation could enhance soluble protein content about three times of that in unmodified soy sauce and also reduce cost of producing 3% soy sauce sample when mixed with 5% raw soy sauce about 2 Baht/L. Therefore, the addition of SBH into the secondary moromi fermentation should be an appropriate strategy for soy sauce manufacturer to use for increase soluble protein content and it could also lead to reduction in production cost as well.

CHAPTER V

DISCUSSION

In Thailand, soy sauce is widely used as flavor enhancer. The major characteristic that used for classifying soy sauce product is the protein content (13). Production of good quality soy sauce usually requires a lengthy period of incubation during the moromi fermentation. Good quality soy sauce usually contains a high level of protein and has a very rich flavor and aroma. This study was initiated to examine the possibility of enhancing the protein content in soy sauce through supplementation with SBH.

Several approaches (10, 77, 78) have been used to increase the protein content in soy sauce. One approach was the selection of koji mold strains that produced high amounts of proteolytic enzymes (10). Another approach was to use high protease producing halophilic bacteria in the moromi fermentation (77). Muramatsu *et al.* (78) also studied the selection of seed koji to obtaining hydrolysates of high total-nitrogen content in a high-temperature autodigestion of soy sauce koji. They found that koji made by soy sauce koji seeds showed relatively high protease activities, but there was no substantial increase in the total nitrogen content of the hydrolysate.

In this study, we investigated the enhancement of protein content in soy sauce product by using SBH. Protein hydrolysate from SBM was carried out by using Kojizyme™ which is a commercial enzyme from Novo, Nordisk, Denmark.

Kojizyme™ is an exo-peptidase and endo-protease complex produced by fermentation of selected strains of *Aspergillus* and *Bacillus*. It is used in soy sauce fermentation for boosting the enzymatic degradation of raw materials (72). The method used for enriching protein content in soy sauce by addition of SBH into soy sauce might be more acceptable than other methods of enrichment. Other possible methods use for enrichment of protein in food products included the use of hydrolyzed vegetable protein (HPV) produced by acid hydrolysis of SBM. However, the use of HPV has not been popular among consumers who prefer to consume the natural process seasoning. Therefore, the protein enrichment in soy sauce with enzymatically produced SBH which produced by a natural process should be more acceptable to consumers.

1. Determination of optimal conditions for hydrolysis of SBM

In the preparation of SBM before hydrolyzing, the ground SBM was mixed with equal part (by weight) of water and autoclaved at 121°C for 15 min. The purpose was to denature the protein (28) and make it easily digested by the enzymes. In this study, the pH of the reaction mixture was not controlled because we wished to avoid the addition of any chemical substances such as NaOH or HCl to the product. The pH of the clarified hydrolysates in all experiments were about the same, ranging between 6.0 to 6.4 at the beginning of the hydrolysis and decreasing to about 5.0 to 5.3 at the 12th hr of hydrolysis. In hydrolysis of SBM by agarose-entrapped *Bacillus subtilis* cells which contained high protease, Khare *et al.* reported that the optimal pH for hydrolysis was between 7.0 to 7.5 at 55°C (79). Zhange *et al.*(34) also found that the optimal pH for hydrolysis of soybean protein by proteases from *A. oryzae* was at 7.0 at

35°C. In this study, the total solid content of reaction mixture at lower than 17% and higher than 22% also investigated (data not showed). It was found that at a total solid content lower than 17%, the mixture was too watery and too dilute for proper reaction. At a total solid content higher than 22%, the mixture was too viscose, so that the mixture could not mix well and homogeneously. Therefore, only 17 and 22% total solid content were investigated in this study.

In this study, it was found that the optimal conditions for hydrolysis of SBM were obtained when Kojizyme™ was used at 2% of SBM protein with a total solid content in the reaction mixture of 22%. The hydrolysis was carried out at 50°C for 12 hr giving a hydrolysate with 8.4% soluble protein and 62.8% degree of solubilization with 23.3% increased in degree of solubilization from the beginning of hydrolysis. This corresponded to the results reported by Martin and Porter (45). They reported that Alcalase® 2.4L a commercial enzyme produced from *B. licheniformis* (64) had the optimal temperature for hydrolysis of fish protein at 50°C. Olsen and Alder-Nissen (80) also found that the optimal condition of Alcalase® 0.6L for production of iso-electric soluble enzymatic hydrolysate of soya protein was 50 to 55°C and pH of 8 for 3 hr. In the continuous production of soy sauce in a bioreactor, Hamada *et al.* (81) hydrolyzed koji using proteases from continuous submerged koji mold culture at 40 to 50°C for 3 days. In the hydrolysis of soybean protein for lemon juice beverage, Dugimoto *et al.* (28) found that soybean protein hydrolyzed at 50°C for 8 to 10 hr gave solubilized nitrogen recovery of 88 to 90%. Ketsumpan (76) reported that the maximum degree of solubilization of SBM by *A. oryzae* protease was obtained upon hydrolysis at pH 10 and 50°C for 24 hr. Muramitsu *et al.* (78) also reported the

autodigestion of koji at 55°C for 48 hr. From all previous reports on either protease enzymes or soybean protein, the optimum temperature for hydrolysis was between 50 to 55°C but hydrolysis time varied widely.

2. Pattern of soluble protein content, pH and bacterial contamination during the hydrolysis of SBM

In the hydrolysis of SBM, it was found that the soluble protein content of hydrolysate product decreased after reaching a maximum level at 8 to 12 hr of hydrolysis. Therefore, an investigation was made to clarify this matter.

The results showed that the soluble protein content and bacterial contamination increased in the same pattern from the 12th hr. After that, the protein decreased, while the bacterial contamination continued to increase until the end of the hydrolysis period at 16 hr. The reduction in soluble protein probably resulted from bacterial growth. Subsequent reduction in bacterial contamination, it might have been due to the high acidity of the hydrolysate mixture. There is one previous report about inhibition of bacterial contamination in the process of yeast autolysis (82). Barrette et al. found that the growth of bacterial contamination decreased significantly when the pH was adjusted to 4.0. Therefore, it might be possible that low pH (high acidity) could inhibit the growth of bacterial cells. Although the hydrolysis of SBM at low pH might inhibit bacterial growth, low pH might not be suitable for SBH addition to the moromi mash, since the low pH might interfere with enzyme reactions. Furthermore, if SBH at pH 4 was added to the moromi mash, it might affect the function of protease and amylase enzymes activity and the growth of halo tolerant lactic acid bacteria and yeast. Therefore, if low pH were used to solve the bacterial contamination problem, it would

be necessary to study the effect on protease and amylase activities and the growth of microbe in the moromi fermentation.

This result also shown that there was very serious bacterial contamination in the SBH product (1.5×10^8 CFU/ml) after 12 hr of hydrolysis. This might have been due to some bacterial contamination originated from Kojizyme™ which contained about 5×10^4 /g of total viable count (72). In this reaction Kojizyme™ was used at 1% of SBM (2% of 50% protein in SBM). Therefore, it was about 10^2 /g of bacterial cells in the mixture as the starting culture and combined with some bacterial contamination from SBH and water, resulted in very high degree of bacterial contamination in the SBH product.

In order to reduce the degree of bacterial contamination in this study, the only possible approach was to reduce the chance of contamination by sterilizing equipment and raw materials used for hydrolysis as much as possible. Since the hydrolysis reaction was performed in an open system, it would be very important that the entire process for hydrolysis be carried out in the most hygienic way possible.

Another approach might be use for reduce degree of bacterial contamination was the addition of NaCl to the hydrolysis mixture. From the data of Kojizyme™ (72), it was shown that at 5% NaCl, Kojizyme™ still have a relatively high enzyme activity about 80%. Therefore, hydrolysis of SBM by adding 5% NaCl to it might be possible to inhibit some bacterial contamination and not interfere to the hydrolysate properties, and also could prolong shelf life of hydrolysate product.

3. Large scale production of SBH

For the large scale production of soybean hydrolysate, the optimal conditions determined on the laboratory scale were used. The raw material (SBM) was increased from 1 kg to 12.5 kg. The supernatant fractions from both processes were assayed for soluble protein content and pH and the analytical data from the large scale hydrolysis and the laboratory scale hydrolysis were compared.

The degree of solubilization in laboratory scale hydrolysis were about 53 to 70% in which the degree of solubilization of the reaction that hydrolyzed under condition of Kojizyme™ 2% of SBM protein, 22% total solid content, at 50°C for 12 hr was about 63%. The degree of solubilization in large scale hydrolysis with the condition that mention above was about 49% which lower than in laboratory scale about 14%. The lower yield in large scale hydrolysis than in laboratory scale hydrolysis might be due to a large volume of reaction mixture was added into the kettle and resulted in the vortex flow of the mixture. The others reason which might cause low yield of hydrolysis was the fluctuation of temperature in the kettle which might lead to the low efficiency of enzyme activity.

The supernatant fractions of laboratory scale and large scale soybean hydrolysate products had similar pH profiles (6.4 and 6.3 at the beginning and decreasing to 5.3 and 5.2 at the 12th hr of hydrolysis, respectively).

In future studies, attempts should be made to improve the degree of solubilization in the large scale SBH production through the use of better reaction design to allow for more complete mixing such as air bubble mixing instead of propeller and better temperature and pH control throughout the hydrolysis period.

4. Enhancement of soluble protein by addition of cSBH

This study showed that cSBH could not significantly enhance the soluble protein content in finished traditional soy sauce. The supplementation of cSBH increase the soluble protein content only about 0.43% when the cSBH was added to soy sauce in the ratio of soy sauce to cSBH 1:0.5. This ratio was the only one that provided acceptable product in terms of sensory evaluation tests when compared with traditional soy sauce. Although this strategy did not allowed for mixing large volumes of cSBH to produce high levels of soluble protein, it could lead to substantial reduction in production cost (see No. 7 of this section).

In the sensory evaluation of soy sauce products, it was shown that increasing the ratio of added cSBH increased the off-flavor score. It has been well documented that soya protein hydrolysate forms the bitter peptides and releases off-flavors (80). This off-flavor might come from components having a beany taste.

5. Protein enrichment in primary cycle moromi fermentation by addition of SBH or Kojizyme™

Chausa-ngavej *et al.* (77) indicated that total soluble protein in soy sauce fermentation broth increased steadily during the first two weeks of fermentation and then stabilized thereafter. When Kojizyme™ was added to accelerate the digestion of soy protein, it did not lead to substantial increases in protein content. This might have been due to the relatively high soluble protein content in the control sample which was about 9% when compared with Chausa-ngavej *et al.*'s (77) report which was about 7%. In addition, SBH also could not enhance soluble protein content in soy sauce product. This might have been due to the addition of SBH to primary moromi mash

was only to replace a half of protein content instead of koji which used in ordinary. Therefore, it did not surprise that SBH could not enhance soluble protein content in soy sauce product. Thus, it might call the addition of SBH to primary moromi fermentation as protein replacement instead of protein enrichment.

The sodium chloride concentration of the moromi mash was controlled in the range of 24 to 27% (w/v). Better control was difficult due to poor mixing. The NaCl concentrations in the experimental fermentations were relatively high when compared with commonly reported values of 18 to 22% (10). Therefore the KojizymeTM which functions well at low NaCl concentrations (not over than 20%) may not have functioned properly at such high NaCl concentrations.

In this study, reducing sugar increased rapidly during the first week and then remained rather stable with small fluctuations. These results are similar to those of Maungma (83), However, they are contrary to the theory that after the reducing sugar reaches a maximum level, the lactobacilli and yeasts will use the sugar as the substrate for producing lactic acid and ethanol for their respective fermentations (84). Perhaps there was such an exceptionally high reducing sugar amount (about 25 to 28 mg/ml) that there was only slight reduction.

There was a relationship between protein content, reducing sugar and brown color of soy sauce samples. The Millard reaction or non-enzymatic browning provided the brown color to the soy sauce product. The Millard reaction is a reaction between amino groups and reducing compounds. In food products, the amino acids are predominantly free amino acids or small peptides and reducing compounds are primarily reducing sugars (85). Therefore, when protein and reducing sugar contents increase, the color intensity of the soy sauce also increase.

In the first week of fermentation, the protein and reducing sugar contents increased rapidly, resulted in a sharp increase in color intensity. After that reducing sugar content stabilized but protein content still increased, so that color intensity increased gradually. In moromi samples with added SBH, the protein content and reducing sugar content were lower than those of the control. Therefore, it resulted in lower color intensity for the modified soy sauce samples.

In this study, the pH decreased from 5.8 in the beginning of fermentation and decreased to 4.8 by the 50th day of fermentation, after which it increased slightly. This result corresponded to the study of Muramatsu *et al.* (85). They found that the pH of moromi mash was about 5.8 at the beginning, decreased to 4.8 by the 60th day of fermentation and then stabilized throughout the fermentation. Lactic acid content increased from 0 at the beginning to 1 g/dl by the 60th day. The pH of moromi with added SBH was lower than that of the control and that with KojizymeTM added because the pH of SBH itself was relatively low at 5.2. Therefore, after SBH was added into the moromi mash, the pH decreased significantly.

In the preparation of soy sauce samples for sensory evaluation, the moromi mash was filtered and centrifuged to obtain a clear supernatant of raw soy sauce. Then, the raw soy sauce was heated to 100°C for 15 min for pasteurization. The pasteurization step is known to be very important for soy sauce production. There are many reasons for this, such as, improvement of flavor and aroma characteristics, development of a reddish brown color, improvement in clarity by precipitation of heat-coagulable substances, inactivation of most enzymes and inactivation of any undesirable microorganisms (1, 5, 7). Subsequently, the soy sauce was cooled and stored at 4°C until further used. In our experiments, preservative such as sodium

benzoate was not added including flavor enhancers such as sodium glutamate was not added since this might have affected the original taste and lead to errors in the sensory evaluation scores.

The sensory evaluation of soy sauce samples in this study revealed that addition of SBH to the moromi mash caused lower scores for odor, flavor and taste and higher scores for off-flavor when compared to the traditional soy sauce. This indicated that modified soy sauce was less preferable than traditional soy sauce. While in KojizymeTM added to soy sauce maintained the same characteristics as traditional soy sauce neither it nor SBH could enhance the soluble protein content in soy sauce samples when added to a primary moromi fermentation. This might be due to that fact that there was a relatively high soluble protein content (about 9%) in the unmodified sample. Such a high protein content is not found in typical soy sauce production processes. Perhaps, the high protein content resulted from the use of a relatively low amount of water in the moromi mash in these experiments.

From the cost analysis of this experiment, protein from SBH was added to replace half of protein content which ordinary came from koji and resulted in higher cost than original koji use. Therefore, the addition of SBH could not provide beneficial effect to the soy sauce product both in protein content and economic aspect. Thus addition of SBH was not suitable for supplementing soluble protein content in soy sauce product because amount of SBH added was about the same with protein content in koji which original use.

6. Protein enrichment of secondary moromi fermentations by addition of SBH or SBM

Repeated moromi fermentations after first grade soy sauce removal is commonly practiced by most soy sauce manufacturers (5). Thus SBH and SBM additions were tested for enhancement of the soluble protein content.

Results from this study showed that SBH and SBM could enhance the soluble protein of soy sauce samples significantly by about 1.3 to 3.0 times when compared to control sample. The higher amount of SBH and SBM, the higher protein content was obtained. The ratio of MMR to SBH of 1:1 rendered the highest protein content of about 2.17% which was about three times higher than that of the control sample.

Reducing sugar concentrations in secondary moromi mash were relatively low at the level of about 1 to 3 mg/ml when compared with those of primary moromi fermentation which were about 25 to 28 mg/ml. The low reducing sugar content together with the low soluble protein content (about 0.7 to 2.2%) resulted in the low browning reaction which provided the color to the soy sauce. Therefore in such a low amount of these parameter, the addition of SBH or SBM into the secondary cycle moromi fermentation could provide the significant increase of these values when compared with that of the control sample.

The amount of soluble protein, reducing sugar and intensity of brown color of all experiments were stabilized with slight fluctuation through one month incubation. This probably resulted from very little enzymatic degradation of protein or starch in the secondary moromi fermentation.

The finished soy sauce from the secondary moromi fermentation was heated at 100°C for 20 min, which was about 5 min longer than for first grade soy sauce. The

longer time of heating should provide for a darker reddish brown color. Because the color of this soy sauce was very pale, one might like to increase the color intensity in order to obtain a better characteristic soy sauce color.

The sensory evaluation of soy sauce products of this experiment revealed that the addition of SBH and SBM provided better characteristics than those of control soy sauce. Higher scores were obtained for flavor, taste and aroma and lower scores for off-flavor in which the soy sauce samples with the ratio of MMR: SBH at 1:1 (w/w) produced the best result. It also provided the highest soluble protein content.

The addition of cSBH to finish soy sauce and SBH to soy sauce production seen like add some more ingredient other than soybean, wheat/rice flour, salt and water to the soy sauce production. However, the regulation of soy sauce product from Thai Industrial Standards (13) indicated that soy sauce product could have some ingredient other than soybean, wheat/rice flour, salt and water that is hydrolyzed plant protein. The announcement of Ministry of Public Health (86) also indicated that seasoning sauce product which resulted from protein digestion of soybean could be process by digestion of soybean protein either by fermentation or other appropriate processes. Therefore, the addition of cSBH and SBH to soy sauce production should not have any problem of breaking the regulation of soy sauce production. Thus, the addition of cSBH and SBH to soy sauce production could be use for enhancing soluble protein content in soy sauce product.

7. Cost analysis for addition of SBH into soy sauce

For estimating the possibility and beneficial effect for adding SBH into soy sauce production, the calculation for production of SBH and cSBH in large scale (56 kg) and industrial scale (1 ton per day) was performed. It was found that the cost for production of one kg of SBH and cSBH at industrial scale level were lower than those of large scale level by about 4.36 and 4.26 Baht/kg, respectively. This reduction should be due to the cheaper price of Kojizyme™ when bought in the large quantities. The labor cost also contributed to the lower cost in the estimation cost for industrial scale production. When cSBH (5.9% protein) was used to mix with first grade soy sauce (3% protein) in the ratio of soy sauce to cSBH at 1:0.5, it could reduce the cost by 10.66 Baht/L. Furthermore this modified soy sauce contained a higher soluble protein content of 4.0%. Also, if cSBH was diluted to 3% protein and mixed with 3% soy sauce in the ratio of 1:2, it could reduce the cost by 12.87 Baht/L. Therefore, the addition of cSBH into soy sauce would be highly cost effective.

Attempts were also made to calculate the cost of using SBH in secondary moromi fermentations. When modified soy sauce from a secondary moromi fermentation containing 2% soluble protein was mixed with 5% raw soy sauce to produce a 3% soy sauce product, it could reduce the use of 5% raw soy sauce by 200 ml per one liter of product. The production cost for modified soy sauce could be reduced by 2 Baht/L if the price of 5% raw soy sauce was about 10 Baht/L. From this result, it appeared that modified soy sauce from secondary moromi fermentation could be beneficial both for production of higher protein content sauce (about 3 times of that in unmodified soy sauce) and for reduction of production cost. Therefore, addition of SBH to secondary moromi fermentation should be the most effective.

CHAPTER VI

CONCLUSION

The objective of this study was to enhance soluble protein content in traditional fermented soy sauce by using enzyme digested SBM. The first part of this study involved with production of SBH. The conditions used for hydrolysis were carried out by using KojizymeTM at 2% based on SBM protein with total solid content of 22%. The SBM was hydrolyzed at 50°C for 12 hr. Using these conditions in laboratory scale hydrolysis, the hydrolysate product contained 8.4% soluble protein with 62% for degree of solubilization. These conditions were also used for large scale production of SBH. The hydrolysate product from large scale production contained 5.9% soluble protein with 49% for degree of solubilization .

The second part of this study involved with supplementation of SBH in soy sauce product. There were three approaches employed to increase the amount of protein in soy sauce through the addition of SBH. In the first approach, cSBH was added into the finish soy sauce by using the ratio's of SS: cSBH at 1:0.5, 1:1.0 and 1:1.5. It was found that this approach could enhance the soluble protein content in soy sauce but it also lead to development of unpleasant characteristic if too much cSBH was used in the mixture. The only acceptable ratio of SS: cSBH which did not produce unpleasant characteristics was at the level of 1:0.5. In the second approach, SBH or KojizymeTM was added into the primary moromi fermentation at various stages of incubation. Chemical analysis of the modified soy sauce products indicated that neither SBH nor KojizymeTM could enhance the soluble protein content. Moreover,

judging from the sensory evaluation test, addition of SBH into the primary moromi fermentation rendered the modified soy sauce products to be less preferable in odor and off-flavor. The soy sauce sample which was added with Kojizyme™ at the beginning of fermentation could maintain traditional odor, flavor, aroma and taste of soy sauce, but this process did not lead to any enhancement of soluble protein content. The third approach was undertaken by adding SBH or SBM into the secondary moromi fermentation at the beginning of fermentation. The results showed that both SBH and SBM could enhance soluble protein content significantly. The most acceptable product was a modified soy sauce sample obtained from secondary moromi fermentation which was added with SBH at the ratio of MMR: SBH at 1:1. The addition of SBH led to the increase in soluble protein content about 3 times over that in control soy sauce sample. SBH and SBH also enhanced reducing sugar content and color intensity of the soy sauce product and rendered better characteristics such as higher scores of general appearance, odor, flavor and taste lower scores of off-flavor when compared with those of control soy sauce sample at the probability level of $P > 0.05$. Therefore, the addition of SBH into the secondary moromi fermentation appeared to be a potentially viable method to be used for enhancement of soluble protein content in soy sauce.

The cost for large scale production of 56 kg of SBH was about 13.86 Baht per kg which provided SBH product of 9.6% (w/w) protein content. This one kg SBH could provide 78% cSBH with 5.9% (w/v) soluble protein and cost about 17.78 Baht per kg. The estimation for industrial scale production also was performed. For one ton per day production capacity, the cost for producing SBH and cSBH was about 9.5 and 13.52 Baht/kg, respectively. The cSBH was used for mixing with first grade soy sauce

which contained 3% soluble protein with the price of 45.5 Baht/L. Such mixture could lower the cost by 10.66 Baht/L with the increasing soluble protein content from 3% to 4.0%. The price of the mixture could be lowered by 12.87 Baht/L for production of mixed soy sauce with 3% soluble protein. The cost for production of soy sauce using modified secondary cycle moromi fermentation was also found to be lowered than the non modified soy sauce. When the SBH which cost about 9.5 Baht/kg was added into secondary cycle moromi fermentation, it could produce soy sauce with 2% soluble protein which was about three folds higher than the typical one. When the modified and unmodified soy sauce were mixed with 5% soluble protein raw soy sauce to produce 3% soy sauce, it was found that the use of 5% raw soy sauce could be lowered by as much as 200 ml/L. Thus, the modified soy sauce could lead to substantial saving for the manufacture.

Therefore, it appeared that SBH could be used for supplementing into soy sauce for enhancing soluble protein content in the final product as well as for reduction in the cost of production.

There is a serious problem of bacterial contamination during the hydrolysis of SBM. Therefore, further study might be performed to reduce the degree of bacterial contamination. Another problem is lower degree of solubilization in large scale than in laboratory scale production. The better condition for hydrolysis should be investigated for degree of solubilization and more concentrate of SBH product for more effective application in soy sauce production. Finally, the strategy for enhancing of soluble protein content in soy sauce by addition of SBH to secondary moromi fermentation could introduce to soy sauce factory for improving the protein content in their soy sauce product.

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APPENDIX A

1. Mercuric nitrate solution, 0.2 M $\text{Hg}(\text{NO}_3)_2$

The solution of mercuric nitrate was performed by dissolving 68 g $\text{Hg}(\text{NO}_3)_2$ in 800 ml distilled water which containing 20 ml of 2 M HNO_3 and added more water to make 100 ml of solution. The mercuric nitrate solution was standardized with standard salt solution of 0.1 N NaCl.

2. Color indicator

0.1 % Diphenylcarbazone was performed by dissolving 0.1 g of diphenylcarbazone in 100 ml of 95% ethanol and stored in a dark bottle.

Mixed indicator was performed by dissolving 0.1 g each of Bromocresol green and Methyl red in 100 ml of 95% ethanol and stored in dark bottle.

3. Dinitrosalicylic acid (DNS) solution

The DNS solution was performed by dissolving 2.5 g of 3,5 Dinitrosalicylic acid in 50 ml of 2 N NaOH. Then 75 g of sodium potassium tartate (Rochelle salt) was added into the solution, stirred until well dissolve and added more water to make final volume of 250 ml. The solution was stored at room temperature in dark bottle.

APPENDIX B

แบบสำรวจผู้บริโภคทั่วไป

คำชี้แจง แบบสอบถามนี้เป็นส่วนหนึ่งของการศึกษา " การพัฒนาผลิตภัณฑ์จากชีอิ้วขาว " คำตอบของท่านในทุกคำถามจะเป็นประโยชน์อย่างยิ่งในการศึกษา จึงขอความร่วมมือมายังท่านในการกรอกแบบสอบถาม และขอขอบคุณมา ณ โอกาสนี้

วนิดา แซ่จิ่ง
นักศึกษาปริญญาโท
ภาควิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ ม.มหิดล

คำแนะนำในการกรอกแบบสอบถาม

โปรดกาเครื่องหมาย ลงในวงเล็บ () หน้าข้อความที่ตรงกับความจริง หรือความเห็นของท่าน โดยเลือกคำอธิบายที่พอใจที่สุดเพียงคำตอบเดียว นอกจากนี้จะได้ระบุเป็นอย่างอื่น

ตอนที่ 1 เกี่ยวกับผู้ตอบแบบสอบถาม

ชื่อ - นามสกุล _____

1. เพศ

() ชาย

() หญิง

2. อายุ

() ต่ำกว่า 20 ปี

() 20-25 ปี

() 26-30 ปี

() มากกว่า 30 ปีขึ้นไป

3. อาชีพ

() อาจารย์

() นักวิจัย / นักวิทยาศาสตร์

() นักศึกษา

() อื่นๆ (โปรดระบุ) _____

4. ระดับการศึกษา

() ต่ำกว่าปริญญาตรี

() ปริญญาตรี

() สูงกว่าปริญญาตรี

5. ถ้าขอความร่วมมือจากท่านในการทดสอบผลิตภัณฑ์ชีอิ้วขาว โดยการทดสอบทางประสาทสัมผัส ท่านจะยินดีหรือไม่

() ยินดี

() ไม่ยินดี

ตอนที่ 2 เกี่ยวกับผลิตภัณฑ์ชี้อีวขาว และการบริโภค

6. ท่านรู้จักผลิตภัณฑ์ชี้อีวขาวหรือไม่

รู้จัก

ไม่รู้จัก

7. ระหว่างชี้อีวขาวสูตร 1 และสูตร 2 ผลิตภัณฑ์ใดดีกว่ากัน

สูตร 1

สูตร 2

8. ท่านชอบบริโภคผลิตภัณฑ์ชี้อีวขาวหรือไม่

ไม่ชอบ เพราะ _____

ชอบ เพราะ _____

ถ้าชอบ ท่านทานบ่อยเพียงใด

มากกว่า 5 ครั้งต่อสัปดาห์

3 - 5 ครั้งต่อสัปดาห์

1 - 2 ครั้งต่อสัปดาห์

น้อยกว่า 1 ครั้งต่อสัปดาห์

9. ท่านบริโภคผลิตภัณฑ์ชี้อีวขาวในลักษณะใด (ตอบได้มากกว่า 1 ข้อ)

เครื่องจิ้ม

จิ้มกับเนื้อสัตว์ต้ม

จิ้มกับเนื้อสัตว์ทอด

เครื่องปรุงรส

ผัด

ปรุงรสในน้ำซุปร

ปรุงรสในข้าวต้ม โจ๊ก

ปรุงรสในการหมักเนื้อสัตว์

อื่นๆ (โปรดระบุ) _____

10. ในบรรดาผลิตภัณฑ์ชี้อีวขาว ในท้องตลาด ท่านบริโภคยี่ห้อใด (ตอบได้มากกว่า 1 ข้อ)

หยั่นหัวอ่อน (เด็กสมบูรณ์)

ง่วนเชียง (เรือ)

ภูเขาทอง

ฉลากทอง

อื่นๆ (โปรดระบุ) _____

11. ท่านคิดว่า ราคาของซีอิ๊วขาวสูตร 1 ขนาด 720 ซีซี ราคา 30-45 บาท ท่านยอมรับได้หรือไม่
- () ยอมรับได้
() ยอมรับไม่ได้ เพราะ
- () ราคาถูก () ราคาแพง
12. ในการเลือกซื้อซีอิ๊วขาว คุณพิจารณาปัจจัยใด (ตอบได้มากกว่า 1 ข้อ)
- () ลักษณะปรากฏ (สี ความใส)
() กลิ่น รสชาติ
() ราคา
() ผู้ผลิต ยี่ห้อ การโฆษณา
() คุณค่าทางอาหาร
() อื่นๆ (โปรดระบุ) _____
13. ปัญหาที่พบในการบริโภคผลิตภัณฑ์ซีอิ๊วขาว (ตอบได้มากกว่า 1 ข้อ)
- () มีกลิ่นผิดปกติ
() รสชาติไม่ดี
() สีผิดปกติ
() มีตะกอนที่ก้นขวด
() อื่นๆ (โปรดระบุ) _____

ตอนที่ 3 เกี่ยวกับผลิตภัณฑ์ใหม่จากซีอิ๊วขาว

14. ถ้าหากมีการพัฒนาผลิตภัณฑ์ใหม่จากซีอิ๊วขาว เช่น ซีอิ๊วขาวเสริมโปรตีน และซีอิ๊วขาวเสริมเกลือแร่ ท่านคิดว่าจะลองรับประทานหรือไม่
- () ลอง () ไม่ลอง () ไม่แน่ใจ
15. ปัจจัยในการเลือกซื้อผลิตภัณฑ์ดังกล่าวนี้ของท่าน คือ
- () ราคา () รสชาติ () องค์ประกอบที่มีประโยชน์ต่อร่างกาย
16. ท่านคิดว่าราคาผลิตภัณฑ์ซีอิ๊วขาวใหม่ดังกล่าวนี้ น่าจะเป็นเช่นไร
- () ถูกกว่า
() เท่ากัน
() แพงกว่า ท่านยอมรับได้หรือไม่ () ยอมรับได้
() ยอมรับไม่ได้

APPENDIX C

แบบสอบถาม ประเมินคุณภาพผลิตภัณฑ์ซีอิ๊วขาว

ชื่อ _____ เพศ _____ วัน/เดือน/ปี _____ เวลา _____

กรุณา พิจารณาลักษณะของตัวอย่างผลิตภัณฑ์ซีอิ๊วขาว โดยพิจารณาลักษณะปรากฏ คมกลิ่น และชิม ตามลำดับ จากนั้นให้กาเครื่องหมาย ✓ ในช่องที่ตรงกับความคิดเห็นของท่านมากที่สุด (โปรดเว้นปากและคืมน้ำทุกครั้งหลังจากท่านชิมแต่ละตัวอย่างเสร็จ)

ตัวอย่าง _____

กรุณาพิจารณาลักษณะปรากฏ และคมกลิ่นตัวอย่างผลิตภัณฑ์ก่อนชิม

ลักษณะปรากฏโดยรวม	สี	กลิ่น
_____ ชอบมากที่สุด	_____ เข้มมากเกินไป	_____ ชอบมากที่สุด
_____ ชอบมาก	_____ เข้มเกินไป	_____ ชอบมาก
_____ ชอบปานกลาง	_____ กำลังดี	_____ ชอบปานกลาง
_____ ชอบเล็กน้อย	_____ อ่อนเกินไป	_____ ชอบเล็กน้อย
_____ เฉย ๆ	_____ อ่อนมากเกินไป	_____ เฉย ๆ
_____ ไม่ชอบเล็กน้อย		_____ ไม่ชอบเล็กน้อย
_____ ไม่ชอบปานกลาง		_____ ไม่ชอบปานกลาง
_____ ไม่ชอบมาก		_____ ไม่ชอบมาก
_____ ไม่ชอบมากที่สุด		_____ ไม่ชอบมากที่สุด

กรุณาพิจารณาลักษณะต่าง ๆ เหล่านี้หลังจากชิมตัวอย่างผลิตภัณฑ์

การยอมรับโดยรวม	กลิ่นรส	รสชาติ	ความเค็ม
_____ ชอบมากที่สุด	_____ ชอบมากที่สุด	_____ ชอบมากที่สุด	_____ เค็มมากเกินไป
_____ ชอบมาก	_____ ชอบมาก	_____ ชอบมาก	_____ เค็มเกินไป
_____ ชอบปานกลาง	_____ ชอบปานกลาง	_____ ชอบปานกลาง	_____ เค็มกำลังดี
_____ ชอบเล็กน้อย	_____ ชอบเล็กน้อย	_____ ชอบเล็กน้อย	_____ เค็มน้อยไป
_____ เฉย ๆ	_____ เฉย ๆ	_____ เฉย ๆ	_____ เค็มน้อยเกินไป
_____ ไม่ชอบเล็กน้อย	_____ ไม่ชอบเล็กน้อย	_____ ไม่ชอบเล็กน้อย	
_____ ไม่ชอบปานกลาง	_____ ไม่ชอบปานกลาง	_____ ไม่ชอบปานกลาง	
_____ ไม่ชอบมาก	_____ ไม่ชอบมาก	_____ ไม่ชอบมาก	
_____ ไม่ชอบมากที่สุด	_____ ไม่ชอบมากที่สุด	_____ ไม่ชอบมากที่สุด	

หลังจากที่ท่านทดสอบตัวอย่างผลิตภัณฑ์นี้แล้ว กรุณาประเมินคุณภาพผลิตภัณฑ์ซีอิ๊วขาวนี้ ในด้านกลิ่นรส โดยขีดเครื่องหมาย " / " ตัดบนเส้นแนวนอน

ไม่มีกลิ่นผิดปกติ

มีกลิ่นผิดปกติ

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ท่านคิดว่า ผลิตภัณฑ์นี้มีคุณลักษณะทางประสาทสัมผัส (สี กลิ่น กลิ่นรส ความเค็ม) ที่ผิดปกติไปจากผลิตภัณฑ์ซีอิ๊วขาวอื่น ๆ โดยทั่วไป หรือไม่อย่างไร

APPENDIX D

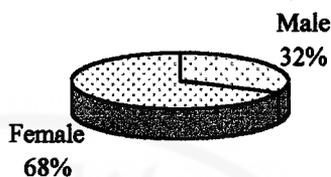
Survey of consumers attitude toward traditional soy sauce and new soy sauce product

To verify the attitude of consumers toward soy sauce product, the survey of consumer attitude toward soy sauce product either in traditional soy sauce or new soy sauce product was performed. The questionnaires that used in this survey was shown in Appendix B. The questions asked consumer in several aspect about soy sauce product. The results of this survey were described below.

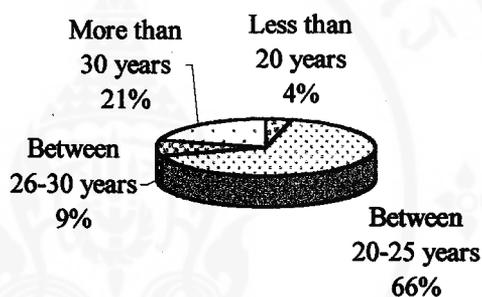
1. Personal information

In this surveying, about 100 subjects were asked to answer the questionnaires. Among these 100 subjects, 68 % are females and 32 % are males. Most of them (66 %) were between 20 to 25 years old, 21% were more than 30 years old, 9 % were between 26 to 30 years old and 4 % were less than 20 years old. About 71% of the subjects were students, 12 % were soy sauce factory owners, 9 % were university employees and 8 % were researchers. About 51% had bachelor degree qualifications, 36 % had educational qualifications higher than bachelor degree and 13 % had educational qualifications lower than bachelor degree. (Figure 28).

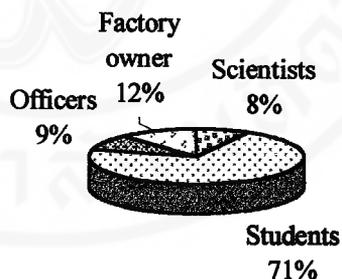
1. Sex



2. Age



3. Occupation



4. Educational background

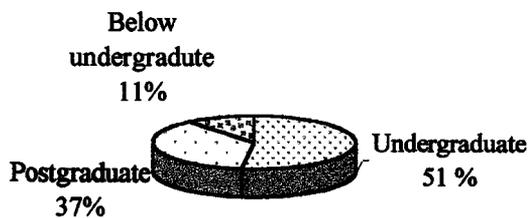


Figure 28 Personal information of the consumers that were asked for their attitude towards soy sauce and new soy sauce product

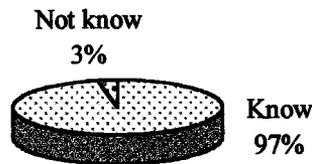
2. Attitude toward traditional soy sauce product

The majority of the subjects (97 %) knew soy sauce product (Figure 29).

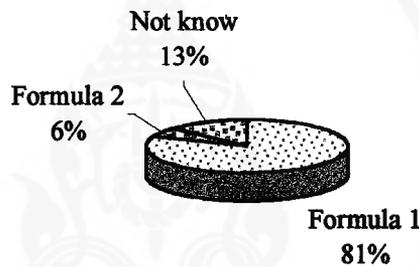
When the subjects were asked the question to test for the real understanding about soy sauce, it was found that about 81 % of the subjects thought that the soy sauce formula 1 was better than formula 2. About 6 % of the subjects thought that soy sauce formula 2 was better than formula 1 and about 13 % were not known that either formula 1 or formula 2 will be better. Among these subjects, most of them (95 %) liked to consume soy sauce. There were about 5 % did not like to consume soy sauce. Among the subjects that liked to consume soy sauce, 37 % of them consumed soy sauce more than 5 times per week, 28 % consumed soy sauce between 3 to 5 times per week, 24 % consumed soy sauce between 1 to 2 times per week and 11 % consumed soy sauce less than 1 time per week. About 56 % of the subjects used soy sauce as seasoning and 44 % as dipping sauce. When they used soy sauce as seasoning, they added soy sauce in the cooking processes of soup (27 %), rice porridge (26 %), marinated with meat (24 %) and stir fried (23 %). In dipping sauce, they used soy sauce to dip with boiled meat (72 %) and deep fried meat (28 %).

There were several brand names of soy sauce products in the markets. The subjects consumed Yan Wal Yun (34 %), Nguan Chiang (30 %), Golden mountain (18 %), Cha Lak Tong (6 %) and others (12 %). About 73 % of the subjects could accept the price of soy sauce formula 1 which had volume about 720 cc and price about 30-45 Baht. There were several factors that should be consider when choosing to purchase soy sauce products. The subjects thought that we should consider about flavor (30 %), general appearance (color, clearness)(21 %), price (20 %), nutritional value (18 %) and brand name (11 %). They also encountered with some problems when they

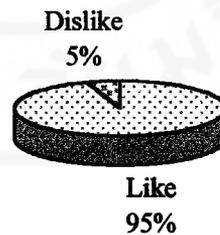
5. Do you know soy sauce



6. Do you know between formula 1 and 2 soy sauce, which formula have the better quality?



7. Do you like to consume soy sauce



How often to consume soy sauce?

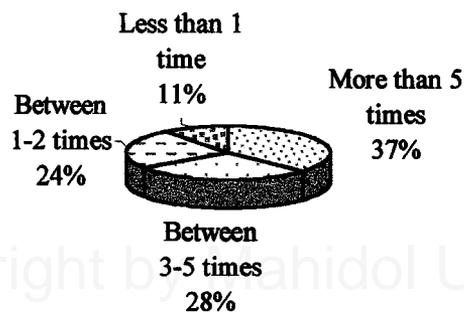
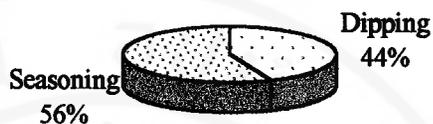
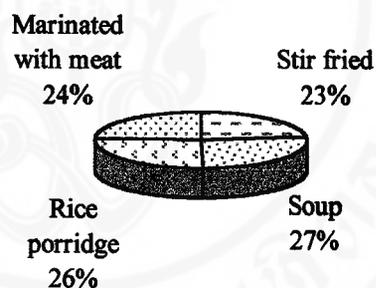


Figure 29 Consumer attitude towards knowing and consumption of soy sauce

8. Which purpose do you use soy sauce for?



9. Which cooking process that you will add soy sauce for seasoning purpose?



10. Which kind of food that you will dip with soy sauce?

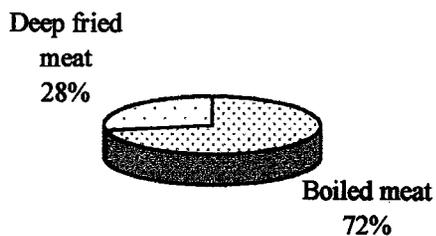
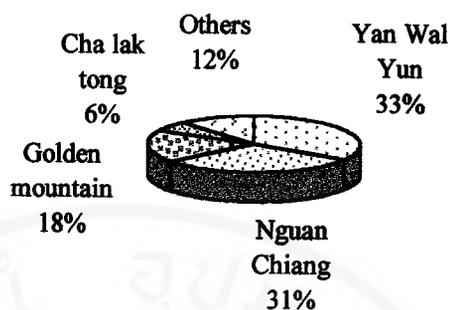
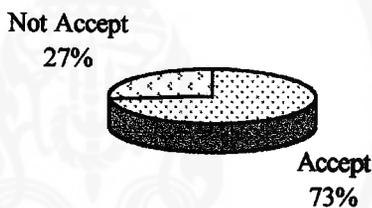


Figure 29 (continue)

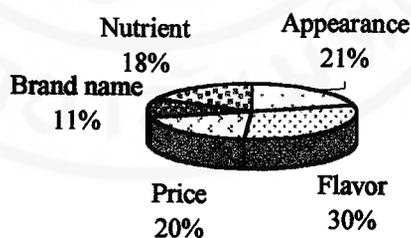
11. Which brand name of soy sauce that you usually use?



12. Can you accept the price of 30-45 Baht of soy sauce formula 1 at volume of 720 cc?



13. Which factor that you consider for purchasing of soy sauce?



14. Which problem that you encounter when you consume soy sauce?

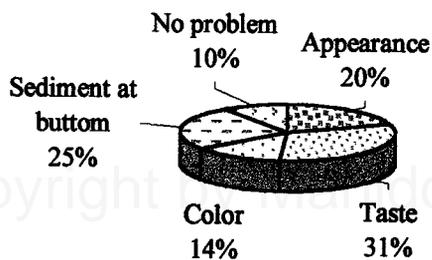


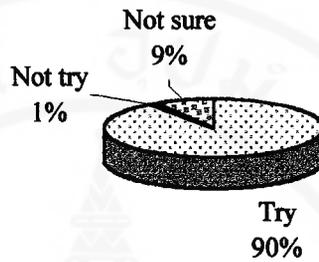
Figure 29 (continue)

consumed soy sauce. These problems comprised of off-taste (31 %), sediments at the bottom of the bottle (25 %), off-flavor (20 %), abnormal color (14 %) and about 10 % did not find any problem (Figure 29).

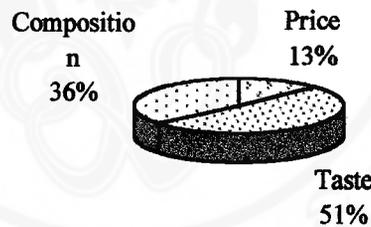
3. Attitude toward new soy sauce product

About 90 % of the subjects were interested in new soy sauce products that should be add some nutrient such as protein or minerals and would like to try it. Only 1 % did not try it. The rest 9 % were uncertain. The major factors that the subjects considered to purchase new soy sauce product were taste (51 %), its beneficial components such as protein or minerals (36 %) and price (13 %). About 53 % of the subjects though that the price of new product should be equal to the price of original product. About 31 % state that it should be more expensive than that of original soy sauce and about 16 % though that the price of new product should be cheaper than that of original product. In the group of subjects that state about the price of new product should be more expensive than that of the original product, 84 % of them could accept this higher price, the rest (16 %) could not accept as shown in Figure 30

15. If there is new soy sauce product eg. added protein or mineral, do you try it?



16. If you would like to buy new soy sauce product, which factor that you will concern?



17. What do you think about the price of new soy sauce product when compare with the price of original soy sauce product?

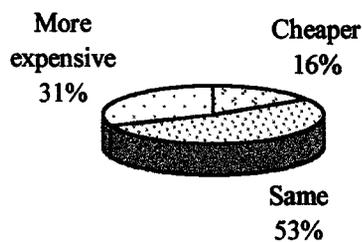


Figure 30 Consumer attitude towards consumption of new soy sauce product

BIOGRAPHY



NAME	Miss Wanida Saejung
DATE OF BIRTH	23 June 1974
PLACE OF BIRTH	Lampang, Thailand
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RESEARCH GRANT	Partially support by Thailand Research Fund

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