THE ROLE OF ENDOGENOUS PROTEOLYTIC ENZYMES IN THE PRODUCTION OF *BUDU*

by

NG YEE FEN

Thesis submitted in fulfillment of the requirements

for the degree of

Master of Science

JUNE 2011

ACKNOWLEDGEMENT

I would like to acknowledge Universiti Sains Malaysia for the Research Grant (1001/PTEKIND/811049). I would like to express my gratitude to Pn Wan Nadiah Wan Abdullah for supervising this project, Associate Professor Rosma Ahmad and Associate Professor Liong Min Tze for bundle of constructive ideas and advices. I am grateful to Universiti Sains Malaysia Fellowship for funding this research. Special thanks to Dr Norimah Bt. Yusof from Pusat Nuklear Malaysia, Bangi, Selangor for the use of gamma rays; and to Mr. Ismail Pengkalan Kubur, Kelantan, for the supply of anchovies in this study. Last but not least, to my family for their constant encouragement and support.

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LIST OF ABBREVIATIONS

AgCl	Silver chloride
$AgNO_3$	Silver nitrate
API	Analytical profile index
CFU	Colony forming unit
$CuSO_4 \cdot 5H_2O$	Copper (II) sulphate tetrahydrate
EDTA	Ethylediaminetetraacetic acid
FAME	Fatty Acid Methyl Ester
$FeCl_2.4H_2O$	Iron(II) chloride tetrahydrate
HCl	Hydrochloric acid
HNO_3	Nitric acid
KCl	Potassium chloride
KSCN	Potassium thiocyanate
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
$MnCl_2.4H_2O$	Manganese chloride tetrahydrate
NaCl	Sodium chloride
Na_2CO_3	sodium carbonate
PCR	Polymerase chain reaction
PMSF	Phenylmethanesulfonyl fluoride
TCA	Trichloroacetic acid
TLCK	Toysl-lysine chloromethyl ketone
TPCK	Toysl-phenylalanine chloromethyl ketone
VFA	Volatile fatty acid

PERANAN ENZIM PROTEOLITIK ENDOGEN DALAM PENGHASILAN BUDU

ABSTRAK

Objektif kajian ini adalah untuk menentukan samaada transformasi protein ikan semasa fermentasi budu disebabkab oleh enzim endogen, atau enzim eksogen yang dihasilkan oleh mikroorganisma yang hadir dalam visera dan otot ikan. Sos ikan Malaysia, budu, dihasilkan dengan mengeramkan campuran ikan bilis (*Encrasicholina punctifer*) dan garam dalam nisbah 3:1. Empat jenis pengolahan dilakukan pada ikan: keseluruhan ikan (tidak disiang) tanpa diiradiasi sebagai kawalan (budu C); keseluruhan ikan dan diiradiasi dengan 10 kGy radiasi gamma (budu R); ikan yang disiang dan tanpa diiradiasi (budu E), dan ikan disiang dan diiradiasi dengan 10 kGy radiasi gamma (budu A). Aktiviti proteolitik sampel budu daripada ikan keseluruhan yang tidak diiradiasi adalah lebih tinggi ($P<0.05$) dibandingkan aktiviti pada budu yang disediakan daripada ikan keseluruhan yang diiradiasi (R) . Tiada terdapat perbezaan yang signifikan ($P>0.05$) pada aktiviti proteolitik dalam budu yang dihasilkan daripada kedua-dua sampel ikan disiang dan diiradiasi (A) dan ikan disiang tetapi tidak diiradiasi (E). . Kajian perencatan menunjukkan protease dominan dalam budu dari ikan keseluruhan yang diiradiasi(R) dan ikan keseluruhan tanpa diiradiasi (C) ialah protease seolah-tripsin, daripada famili serine protease. Perencatan yang rendah oleh pepstatin A dan EDTA terhadap protease dalam budu menunjukkan bahawa protease aspartik dan metalloprotease kurang terlibat dalam penghasilan budu. Hitungan bakteria proteolitik yang rendah dalam semua sampel budu mencadangkan bahawa bakteria proteolitik

hanya memainkan peranan yang kecil dalam hidrolisis protein ikan. Dengan menggunakan sistem API, *Bacillus*, *Staphylococcus* and, *Micrococcus* telah dikenalpasti sebagai bakteria proteolitik dalam semua sampel budu. Kandungan protein dalam semua sampel memenuhi keperluan kandungan minimum untuk budu, yang ditetapkan oleh Akta Makanan Malaysia 1983 dan Peraturan Makanan 1985. Namun begitu, budu diperbuat dari ikan keseluruhan menunjukkan kualiti yang lebih baik dari segi jumlah nitrogen dan protein terlarut, untuk digunakan, dibandingkan dengan budu daripada ikan tersiang (P<0.05). Keputusan ini menunjukkan bahawa proteolisis bergantung pada enzim-enzim endogen daripada visera ikan, dan enzim bakteria, juga dari visera ikan memainkan peranan kecil dalam hidrolisis protein.

THE ROLE OF ENDOGENOUS PROTEOLYTIC ENZYMES IN THE PRODUCTION OF *BUDU*

ABSTRACT

The objective of this study was to determine whether the transformation of fish protein during *budu* fermentation was caused by endogenous enzymes or exogenous enzymes produced by microorganism present in the viscera and the muscles of the fish. Malaysian fish sauce, *budu* was produced by incubating a mixture of anchovies (*Encrasicholina punctifer*) and salt in a ratio of 3:1. Four types of treatment were carried out on the fish: i.e. whole, un-irradiated fish as the control (*budu* C); whole, 10 kGy gamma irradiated fish (*budu* R); eviscerated, un-irradiated fish (*budu* E), and eviscerated, 10 kGy gamma irradiated fish (*budu* A). Proteolytic activity was significantly higher in *budu* prepared from whole, un-eviscerated fish (C) compared to *budu* prepared from whole but irradiated fish (R). There was no difference (P>0.05) in proteolytic activity in *budu* prepared from both eviscerated, irradiated (A) and eviscerated and un-irradiated fish (E). Inhibition studies showed that the predominant proteases in *budu* prepared from whole and irradiated (R) fish and whole and un-irradiated (C) is trypsin-like protease belonging to the serine protease family. Low inhibition by pepstatin A and EDTA on protease in *budu* indicates the lack of involvement of aspartic protease and metalloprotease in the *budu* production. Low proteolytic bacterial counts in all *budu* suggested that proteolytic bacteria plays; only a minor role in the protein hydrolysis of fish. Using the API system, *Bacillus*, *Staphylococcus* and, *Micrococcus* were identified as the proteolytic bacteria in all *budu* samples. The protein level of all samples met the minimum requirement for *budu*, set by Malaysian Food Act 1983 and Food Regulations

1985. However, *budu* from un-eviscerated fish presented better quality of total nitrogen and soluble protein content for consumption, as compared to the eviscerated fish ($P<0.05$). These results suggest that proteolysis relies mainly on the endogenous enzymes from fish viscera, and bacterial enzymes associated with the fish viscera played a minor in protein hydrolysis.

CHAPTER 1

INTRODUCTION

1.1 Background

Fish is regarded as an excellent source of protein in terms of quality, supply, and cost. Unfortunately, fishes are easily perishable and became unpalatable due to improper handling and storage after capture. Furthermore, many types of fish are being rejected by the consumers and discarded as waste due to their textural qualities and unacceptable taste. Therefore, fish sauce processing appears to be an efficient technique to utilize small, less utilized pelagic fish. Additionally, the fermentation of fish is also a preservation method to turn underutilized fish species into value added by-products (Chayovan *et al.*, 1983).

Fermentation is a very old technique used in food preservation as it extends the shelf life of the product, and also enhances the flavour and nutritional quality of the food (Visessanguan *et al.*, 2004). Fermented food is widely consumed all over the world, especially the indigenous fermented fish and shrimps. These fermented foods are popular appetizers in Southeast Asia (Mardiana *et al.*, 2009) and contribute significantly to the diet of the populations in developing countries by increasing the protein intake (Alkolkar *et al.*, 2009). Fish sauce, with its characteristic flavour and aroma, is the most popular fermented fish products which is traditionally consumed in Southeast Asia and is becoming more popular in Europe and North America (Hjalmarsson *et al.*, 2007).

Small pelagic fish such as anchovies (*Stolephorus* spp.) and sardines have been widely used in the fish sauce production (Gildberg, 2001; Lopetcharat *et al.*, 2001). Traditionally, fish sauce preparation takes at least 6-18 months and involves the addition

of 20-30% of salt with whole un-eviscerated fish (Akolkar *et al.,* 2009). Many studies have been carried out to accelerate the process of fish sauce production in order to reduce production cost (Akolkar *et al*., 2009). Strategies adapted include the use of proteases and bacterial starter cultures (Yongsawatdigul *et al.,* 2004), the reduction of the salt content, as well as the addition of other exogeneous agents such as koji and plant enzymes (Akolkar *et al.,* 2009).

Proteolysis is one of the major biochemical processes occurring during the fermentation of fish sauce (Kilinc *et al.,* 2006). The proteolysis that takes place during fermentation was thought to be due to proteolytic activity of endogeneous proteinases in digestive tract and muscle of anchovy and that bacterial enzymes were not responsible in fish sauce production (Orejano and Liston, 1982). However, Kirschbaum *et al.* (2000) was of the opinion that proteases secreted by halophilic microorganisms that belong to the native microflora of fish also contributed in the proteolysis. The role of endogeneous proteinases in protein hydrolysis of fish was further reinforced by Siringan *et al.* (2006) who found that the endogeneous proteinases in Indian anchovy was stable at high salt concentrations. Thus, although it has been widely accepted that the enzymes required for protein hydrolysis are the endogeneous proteinases derived from the fish tissue, particularly the viscera; there was still the uncertainty on the role of microorganisms in this type fermentation. In addition, although the microflora diversity involved in the fish sauce fermentation has been demonstrated through several studies (Crisan and Sands, 1975; Ijong and Ohta, 1995; Sim *et al*., 2009; Guan *et al.,* 2010), the role of each type of microorganism in the maturation process of fish sauce is still poorly understood. There seems only little information available on the nature, origin and amounts of enzymes,

particularly protease, involved in fish sauce fermentation.

Traditionally, the process of fish sauce fermentation faces several obstacles which limit the expansion of fish sauce in the food industry. Complete fermentation to yield a good quality fish sauce needs at least 6 to 12 months. Therefore, the growth of fish sauce industry is limited by extremely long fermentation time and high capital investment of land (Yongsawatdigul *et al.,* 2007; Alkolkar *et al.,* 2009). In addition, the process is poorly controlled, resulting in inconsistent qualities of the product (Yongsawatdigul *et al.,* 2007). Apart from that, high salt content used in the fermentation limits the palatability and consumption of fish sauce (Xu *et al.,* 2007). Therefore, an understanding of the enzymatic reactions by endogenous proteinases of fish origin and microorganisms surviving during the fermentation would give a better clarification and understanding of the processes involved in fish sauce fermentation, so that the process can be brought under better technical control for optimal production of fish sauce.

1.2 Research objectives

This study seeks to elucidate the role of endogenous proteolytic enzymes involved in the production of the Malaysian fish sauce, *Budu*:

The specific objectives were:

i) To investigate the origin of proteolytic enzymes involved in the protein hydrolysis during production of *Budu* (fish sauce).

ii) To classify the proteolytic enzymes involved in *Budu* fermentation, into various protease groups.

iii) To isolate and identify the halotolerant bacteria and bacteria possessing proteolytic activity during the fermentation of *Budu* (fish sauce).

iv) To investigate the effect of endogenous protease on the chemical and physical properties of *Budu* (fish sauce).

CHAPTER 2

LITERATURE REVIEW

2.1 Fish sauce

Fish sauce, a dark brown to black condiment, is an indigenous fermented food obtained from protein hydrolysis of fish that occur during several months of storage of heavily salted fish at tropical temperatures (Gildberg , 2001; Rosma *et al.,* 2009). Fish sauce has various names depending on the country of origin: *Patis* in Philippines, *Shotturu* and *Ishiruin* in Japan, *Budu* in Malaysia, *Nampla* in Thailand, *Nuoc-nam* in Vietnam, *Ketjap-ikan* or *Bakasang* in Indonesia, *Yu lu* in China, and *Ngapi* in Myanmar (Fukami *et al.,* 2004). It is commonly used as a flavour enhancer or salt replacer in various food preparations (Lopetcharat *et al.*, 2001).

Budu, a Malaysian fish sauce, is manufactured in the two north-eastern states of Peninsular Malaysia, namely Kelantan and Terengganu and it is consumed mainly by people in these two states (Wan Daud, 1978; Lopetcharat *et al.*, 2001; Rosma *et al.*, 2009). *Budu* is normally produced from fish left over after drying of fish, and when the weather is not suitable for drying of fish (Lopetcharat *et al.*, 2001). Manufacturing of *Budu* is a backyard industry and, in 2009, there were only 12 registered *Budu* producers in Malaysia scattered all around in Kelantan and Terengganu (Rosma *et al*., 2009).

Fish sauce contains about 20g/l of nitrogen, of which 80% is in the form of amino acids; thus it is an important source of protein in the diet especially in certain social classes in Southeast Asia (Je *et al.*, 2004). Even though fish sauce contains a wide variety of nutrients, its nutritional values are limited due to its high salt content (Fukami *et al.*, 2004). Fish sauce is widely used as condiments in Southeast Asian countries

(Curtis, 2009). However, the consumption of fish sauce in Japan and Western countries are limited due to its characteristic and distinctive odour (Fukami *et al.*, 2004).

2.1.1 Nutritional and chemical composition

In Thailand and Vietnam, fish sauce is an important source of amino acids and many micronutrients, because it is rich in calcium, phosphorus, iron and vitamins B_{12} (Chaveesuk, 1991; Curtis, 2009). In other Southeast Asian countries like Thailand, Malaysia, Cambodia, Indonesia and Philippines, fermented fish products are a staple part of the diet as they contribute significantly to the protein intake of the populations due to their high amount of amino acids originating from fish proteins (Wood, 1998; Taira *et al.*, 2007). Scientific analysis revealed that consumption of 15-30 ml fish sauce contributes to approximately 7.5% of an individual's daily dietary protein requirement (Curtis, 2009). However, the protein level of the fish sauces differs. These variations in the protein content of fish sauce produced in different countries could be due to the variations in fish species, the level of salt and the period of fermentation of the sauce (Anihouvi *et al.*, 2006).

Fish sauce contains approximately 10 g amino acids (essential and non essential), per 100 ml (Curtis, 2009). The amino acid of fish sauce varies depending on the fish species and the enzymes supplemented. Fish sauces supplemented with squid hepatopancrease contains amino acids alanine, leucine, serine, lysine and, arginine (Raksakulthai and Haard, 1992). The predominant amino acids in *garum*, Roman fermented fish sauce, were lysine, leucine, isoleucine, valine, aspartic acid, glutamic acid, glycine, arginine and tyrosine (Aquerreta *et al*., 2001) while the most abundant

amino acids in *Bakasang* were alanine, isoleucine, glutamic acid and lysine (Ijong and Ohta, 1996).

2.1.2 Flavour

Fish sauce imparts both salty and umami taste (Curtis, 2009). The aroma might also be due to the salty taste of the fish sauce, but this salty taste tends to overpower other flavour in fish sauce (Sanceda *et al.*, 2003). Umami is the taste of foods that are rich in glutamic acid and 2 ribonucleotides, 5'-inosinate and 5'-guanylate (Curtis, 2009). The aroma and flavour of fish sauce are affected by the fish species, the process of fermentation and different enzymes used in the fermentation (Fukami *et al.*, 2004; Lopetcharat *et al.*, 2001). The aroma is due to the nonezymatic reactions of sugars, lipid and protein hydrolysate and reactions of endogeneous enzymes and bacteria that survives in the fermentation process (Fukami *et al.*, 2004). As fermentation progressed, proteins in fish decomposed into amino acids, oligopeptides and nitrogeneous bases (Taira *et al.*, 2007; Curtis, 2009), which contribute significantly to the sensory characteristic and flavour of fish sauce (Wood, 1998; Je *et al.*, 2004). These amino acids, peptides and soluble proteins contribute to the strong umami taste of the fish sauce (Taira *et al.*, 2007; Toyokawa *et al.*, 2010). It has been suggested that the amino acids produced through proteolysis served as substrates for the synthesis of flavour compounds by bacteria (Steinkraus, 1996).

The flavour of fish sauce can be classified into ammoniacal, cheesy and meaty notes (Fukami *et al.*, 2004). Ammonia and trimethylamine contribute to the ammoniacal taste of the fish sauce; whilst cheesy taste was due to low molecular weight fatty acids

(VFA) (Wood, 1998). Meaty aroma may be attributed to glutamic acid, nitrogen containing compounds such as pyridines, pyrimidines, amines and nitrile. These compounds then combine with aldehyde compounds to contribute to the meaty odour (Lopetcharat *et al.*, 2001). Therefore, combination of volatile fatty acids and non volatile fatty acids in fish sauce along with other biochemical reactions contribute to the ammonical, meaty and cheesy taste of fish sauce (Lopetcharat *et al.*, 2001; Curtis, 2009).

The most abundant flavouring constituents in fish sauce are volatile acids (Sanceda *et al.*, 2003). Glutamic acid contributes to the sour taste; arginine imparts a bitter taste while lysine has a sweet taste (Aquerreta *et al.*, 2001). Sometimes, unpleasant odour developed during the fermentation process limits the consumption of fish sauce. Development of an unpleasant odour during fermentation period is due to the formation of short chain-fatty acids and amines, such as trimethylamnie (Yoshikawa *et al.*, 2010).

2.2 Fish sauce fermentation

Fish sauce fermentation involves the physical, chemical and microbiological changes of the fish that occur at high salt concentration and low oxygen level (Lopetcharat *et al.*, 2001). These changes are induced by enzymes which break down protein and fat (Majumdar and Basu, 2010). Wood (1998) defined fermentation as the transformation of organic substances into simpler compounds by the action of microorganisms or enzymatic reactions.

The principal stages of salt fermentation of fish can be basically divided into two. During the first stage, salt diffuses into the fish and osmosis eliminates the water; proteolytic enzymes in the fish are released within the cells (Wood, 1998; Curtis, 2009; Majumdar and Basu, 2010). The second stage includes a slower ripening process whereby a series of complex biochemical processes such as proteolysis, lipolysis and lipid oxidation occur (Majumdar and Basu, 2010). As proteolytic enzymes attacked the membranes and muscle of the fish, the fish eventually loses its shape and begins to liquefy (Wood, 1998; Curtis, 2009). The dehydration bought about by osmotic effect reduces the moisture content of the fish and spoilage no longer occurs (Wood, 1998). The overall sensory qualities of the end products were determined by the physical and chemical changes occuring during the ripening stage (Majumdar and Basu, 2010).

The production of Malaysian fish sauce, *Budu* is represented in Figure 2.1. Generally, budu production begins with mixing of small pelagic un-eviscerated fish, usually anchovies with salt at a ratio of 3:1 (fish: salt) (w/w). Whole fish is mixed with coarse salt and the mixture is loaded on a concrete fermentation tank under roof (Wan Daud, 1978). Another layer of salt is spread on the top of the mixture. It is important to keep the fish immersed at all times, therefore weights may be placed to press the fish down in order to prevent the fish from becoming putrefied and to enhance osmotic dehydration (Lopetcharat *et al.*, 2001). After fermenting for 6 months under the sun, the mixture is filtered (Wan Daud, 1978). The unprocessed *Budu* is heated with tamarind juice, palm sugar, monosodium glutamate and, flavouring compounds (Rosma *et al.*, 2009). The *Budu* is then cooled and kept in a large earthenware containers before bottling (Wan Daud, 1978).

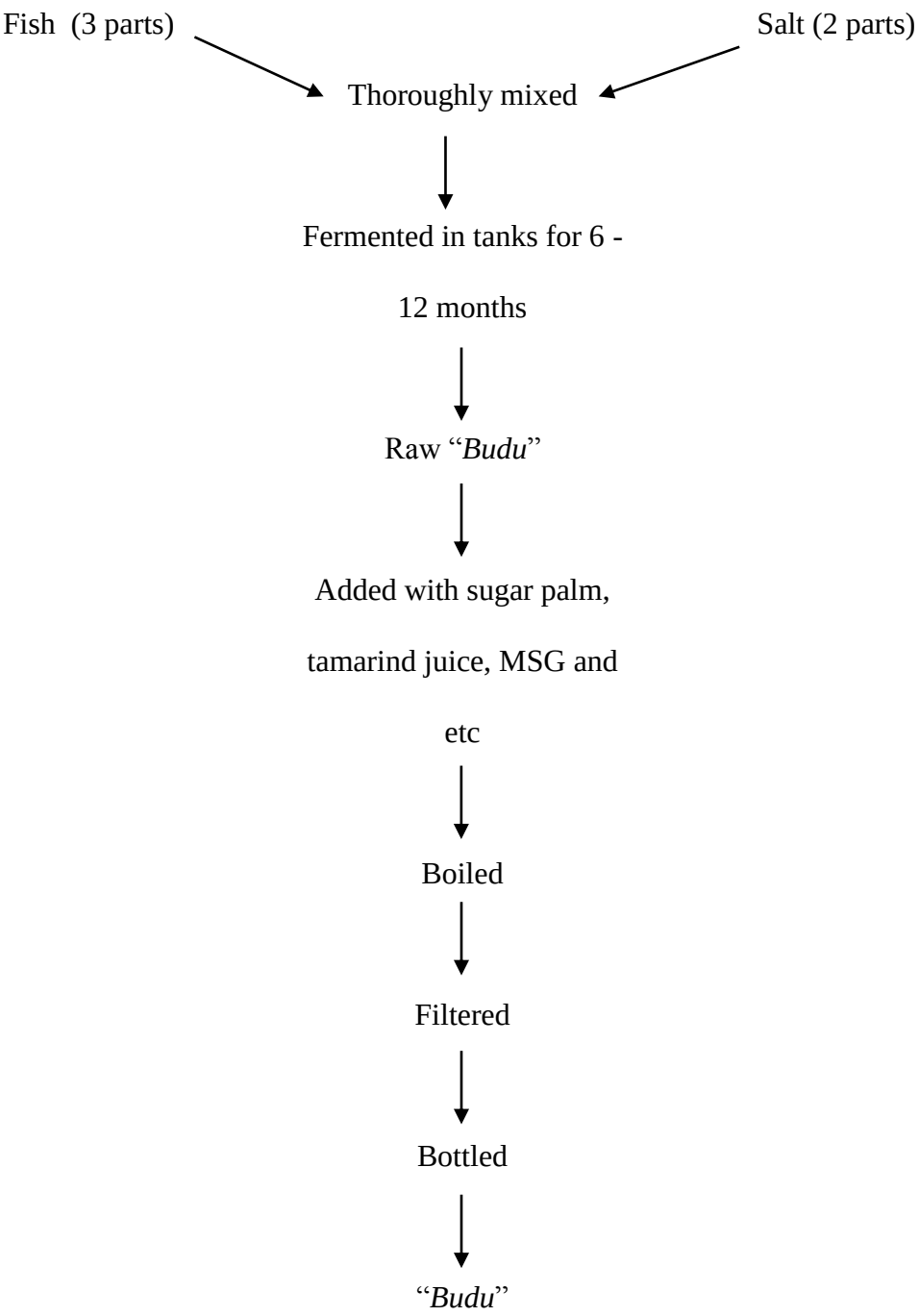

Figure 2.1 Flow chart for production of Malaysian "*Budu*" Adapted from Rosma *et al.* (2009)

The fermentation time of fish sauce varies; depending on the types of fish used and may range from a few months to 1 year or 18 months for larger fish (Steinkraus, 1996). A long fermentation time is needed to ensure complete hydrolysis of the fish as well as flavour and colour development of the fish sauce (Lopetcharat *et al.*, 2001). The protein breakdown during the production of *Budu* can be categorized into 3 stages. In the beginning 0-25 days of fermentation, osmosis takes place. Proteins are released during 80-120 days of fermentation while at 140-200 days; the distribution of nitrogen compounds in the liquid occurs (Lopetcharat *et al.*, 2001). At least 6 months is needed for the solubilisation of fish because the endogeneous proteolytic enzymes from the fish responsible in the breakdown of protein are likely to be less active at high salt concentration (Yongsawatdigul *et al.*, 2007).

2.2.1 Raw Materials

2.2.1.1 Fish

The primary raw materials in fish sauce processing are fish and salt. Fish sauce is a product that can be made cheaply from a great variety of fish which are not normally used for food as raw materials. Fish such as anchovies, sardines, mackerel and herring are commonly used as long as the proteolytic enzymes are sufficient for tissue or protein hydrolysis (Klomklao *et al.,* 2006). Fish that are un-eviscerated or viscera from large fish are normally used due to the high amounts of proteolytic enzymes in the viscera (Aquerreta *et al.,* 2001).

The nutritive and organoleptic quality of various fermented fish sauces varied depending on the fish species used due to wide variation in protein and fat content of the

fish (Chaveesuk, 1991). The amount of endogenous enzyme present in a tissue of fish species can be influenced by intra-specific factors such as biological age, diet, exercise, water salinity, hydrostatic pressure, season and feeding capacity at the time of capture (Makinodan *et al.*, 1984; Martinez and Gildberg, 1988; Wheaton and Lawson, 1985; Haard, 1992). The nutritional compositions of fish that are commonly used for fish sauce production are presented in Table 2.1.

Table 2.1 Nutritional compositions of three common species of fish used in fish sauce production

Nutrients	Fish species		
	Stolephorus spp. (Anchovy)	*Ristrelliger* spp. (Mackerel)	*Clupea* spp. (Herring)
Protein (g)	18.0	20.0	20.2
Fat (g)	0.3	6.7	4.3
Moisture (g)	80.5	72.0	74.4
Calcium (mg)	218	170	4.0
Phosphorus (mg)	211	60	175
Iron (mg)	1.7	11.9	2.0
Vitamin A (IU)	139	138	195
Vitamin B1 (mg)	0.02	0.03	0.12
Vitamin B2 (mg)	0.04	0.62	0.05
Niacin (mg)	0.60	9.20	3.00

All values are based on 100 g of sample. Adapted from Lopetcharat *et al.* (2001)

Fish that are commonly utilised for *Budu* production are *Stolephorus* spp., *Sardinella* spp. or *Decapterus macrosoma* (Rosma *et al.*, 2009). Anchovy (*Stolephorus* spp.*)* is one of the preferable species commonly used for fish sauce (*Budu*) production in Malaysia due to its high content of endogenous proteolytic enzymes, small size and lower content of poorly digestible connective tissue (Gildberg, 2001; Lopetcharat *et al*., 2001).

Anchovy are small pelagic saltwater fish preferring warmer waters around the world and have silvery blue-green backs, with a maximum length of 20 cm (Shiriskar *et al.,* 2008). The abundance of anchovies fluctuate widely from year to year associated with large scales in ocean temperature (Francisco *et al.,* 2003) and monsoons associated with heavy seas and high winds (Kirkley *et al.,* 2003).

Apart from the favourable characteristics highlighted by Gildberg (2001) and Lopetcharat *et al*. (2001), anchovies are also the most suitable type of fish used for fish sauce fermentation due to their chemical composition. The chemical composition of anchovies is 80.5% moisture, 0.3% fat, 18.0% protein, 0.1% fibre and no carbohydrate, which results in a product with pleasing aroma and taste (Sanchez, 2008). Pelagic fish with its small size and high protein content is susceptible to rapid autolytic degradation of the abdominal tissue by proteolytic enzymes from the digestive tract after capture if it is not stored and handled properly; all these factors limit the effective utilization of the fish (Martinez and Gildberg, 1988; Hariono *et al.,* 2005). Thus, pelagic fishes are commonly utilized in salted and dried form; only a very small quantity will sell in fresh in the market (Shiriskar *et al.,* 2008).

Due to a general decline in the marine fisheries, there is a growing interest to investigate the suitability of different raw materials for fish sauce production. Several workers had studied the utilization of different types of fish for fish sauce production, such as Gambusia (*Affinis affinis*) (Ibrahim, 2010); Capelin (Hjalmarsson *et al.,* 2007); rabbit fish (*Siganus fuscescens*) and fatty Japanese anchovy (*Engraulis japonica*) (Osako *et al.,* 2005). Fish sauce production has also been attempted from surimi waste with bromelin from pineaapple as a fermentation aid (Sangjindavong *et al.,* 2009).

The fish species used in this study was *Encrasicholina punctifer*, commonly known as Oceanic anchovy (Figure 2.2). *Encrasicholina punctifer* is a small pelagic schooling fish with a short life span of around a year, and is widely found in the Indian Ocean and the West Pacific waters (Maack and George, 1999).

Figure 2.2 *Encrasicholina punctifer*

2.2.1.2 Salt

Salt is the second important ingredient in fish sauce processing. Addition of salt during fish sauce fermentation increased the osmotic extraction of liquid from the fish tissue. The extracted liquid is rich in soluble protein (Chaveesuk, 1991).

Salt contributes to the water holding capacity, controls the microflora during fermentation, prevents microbial growth, facilitates the solubilisation of certain proteins and confers a typical salty taste by enhancing the flavour of processed meat products (Armenteros *et al.,* 2009). Salt also ensures the hygienic quality of the fish sauce by reducing the water activity, which then disrupts the osmotic balance in bacterial cells (Chaveesuk, 1991; Akolkar *et al.*, 2009; Majumdar and Basu, 2010).

The concentration of salt added to the fish during fish sauce fermentation must thus be at a level sufficient to prevent the growth of spoilage microorganism. Too high a

level of salt decreases the proteolytic activity, resulting in a slower rate of fermentation (Chaveesuk, 1991). In contrast, although a low level of salt accelerates the fermentation process by facilitating the growth of fermenting microorganisms and reducing the inhibition of protease activity, it also increases the development of ammonia, contributing to an undesirable flavour (Chaveesuk, 1991; Aquerreta *et al.*, 2001).

Three types of salt are commonly used in processing of foods: solar salt from the evaporation of surface salt water; rock salt and welled salt from subterranean salt deposits (Frazier, 1958). High purity salt is recommended for rapid hydrolysis in fish sauce manufacturing because low grade salt contains a large number of microorganisms and impurities like calcium, magnesium and sulphate ions that will slow down the penetration of salt into the fish muscle and thus permitting occurrence of spoilage (Priscilla, 2008). Besides, low grade salt also contains heavy metals that might enhance the oxidation of fat content of fish, resulting in over darkening of the fish product and development of undesirable colour and taste (Sanchez, 2008). Solar salt contains halophiles like *Halobacterium* sp. and *Serratia salinaria*. *Bacillus* sp. is the dominant bacteria found in solar salt while *Micrococcus* and *Sarcina* are found in low numbers (Frazier, 1958).

2.2.2 Browning and colour development of fish sauce

Colour is one of the quantitative parameters that determine the quality of fish sauce in Thailand and Korea (Lopetcharat *et al.*, 2001). However, the colour values of fish sauce are often determined subjectively because the colour of fish sauce can be adjusted using natural brown pigments, such as caramel (Lopetcharat *et al.*, 2001). Palm

sugar and tamarind juice are usually added in the production of fish sauce to promote browning and thus resulting in fish sauce with a dark brown colour.

The development of brown colour in fish sauce as fermentation progressed resulted in the intensity of the brown colour in fish sauce to develop gradually (Dissaraphong *et al.,* 2006). This development is due to non-enzymatic browning reaction (Lopetcharat and Park, 2002), which can be classified into 2 types: (1) Mailard reaction and (2) lipid-amino reaction (Chaveesuk, 1991).

Although the reducing sugar content present in fish sauce is very low, carbohydrate derivatives such as glucose- 6- phosphate can be a source of carbonyl compounds that initiate the Maillard reaction (Dissaraphong *et al.,* 2006). Apart from Maillard reaction, the browning in fish sauce is also due to the lipid-amino reaction. Lipids and lipid-degradation products (carbonyl compounds and peroxide) also act as a source of carbonyl group contributing to the non-enzymatic browning reaction in fish sauce (Chaveesuk, 1991).

2.3 Proteolytic enzymes in fish sauce

During fermentation, fish tissues are hydrolyzed by the proteolytic enzymes. Hydrolytic action of both endogeneous enzymes such as the intestinal proteases and proteases produced by halophilic microorganism surviving during the fish sauce fermentation, contributed to the hydrolysis of fish protein (Lopetcharat *et al.*, 2001; Fukami *et al.*, 2004).

The production of fish sauce is a slow process of protein autolysis due to the action of endogeneous proteolytic enzymes especially trypsin and pepsin from the

stomach and digestive tract (Curtis, 2009). These proteolytic enzymes, which vary with fish species, attack the fish tissues (Curtis, 2009).

2.3.1 Endogenous proteolytic enzymes in fish

Endogenous proteolytic enzymes refer to the proteolytic enzymes originating from the digestive tract, internal organs and muscle tissue of the fish (Chaveesuk, 1991). Endogenous proteolytic enzymes that have been widely investigated in fish include gastric proteinases, intestinal proteinases and hepatopancreases proteinases (Shahidi and Janak Kamil, 2001). Endogenous proteolytic enzymes in fish vary with fish species, fishing season and organs. Fish viscera or internal organs are an important source of proteolytic enzymes. Chong *et al.* (2002) reported that the proteolytic activity of intestine of discus fish (*Symphysodon aequifasciata*) was higher than in the stomach.

2.3.1.1 Proteolytic enzymes from the digestive system in fish

Fish digestive proteolytic enzymes that are commonly used in industry include trypsins and chymotrypsins secreted by pancreatic tissue or pyloric caeca, and pepsins, secreted by the gastric mucosal gland of the fish (Chaveesuk, 1991; Vecchi and Coppes, 1996). Intestinal proteases of fish are normally secreted from the pyloric caeca or pancreases (Shahidi and Janak Kamil, 2001). These digestive proteolytic enzymes showed a high activity in acidic regeion in the stomach and alkaline pH region in the intestine (Chong *et al*., 2002). The optimum temperature of fish digestive proteases is much higher than the habitat temperature of fish because the temperature inside the digestive tract is higher than the ambient temperature of sea water (Shahidi and Janak

Kamil, 2001). Of all digestive proteolytic enzymes in fish, pepsin, trypsin, trypsin-like, chymotrypsin, elastase and gastricsin are the most commonly studied digestive proteolytic enzymes from aquatic organism (Vecchi and Coppes, 1996). Table 2.2 shows the endogenous proteolytic enzymes of some fish species.

Table 2.2 Digestive proteolytic enzymes from fishes

Fish		**Habitat temperature**	**Enzymes**
Common name	**Species**		
Anchovy	*Engraulis encrasicholus*		Trypsin
	Engraulis japonica		Trypsin
Arctic fish capelin	*Mallotus villosus*	(-1)(-4)	Pepsin, Trypsin
Atlantic cod	*Gadus morhua*	0-5	Pepsin, Trypsin, Chymotrypsin, Elastase
Bonito	*Katsuonus pelamis*		Pepsin
Brook trout	*Salvelinus fontinalis**		Pepsin
Cunner	*Tautogolabrus adspersus*	>5	Trypsin
Dogfish	*Mustelus canis*		Pepsin
Eel	*Anguila japonica*	10	Elastase
Greenland cod	*Gadus ogac*	(-2)- 2	Pepsin, Trypsin
Hake	*Merluccius gayi*		Pepsin, Gastricsin
Mullet	*Mugil cephalus*	15	Trypsin
Polar cod	*Boreogadus saida*	<0	Pepsin
Rainbow trout	*Salmo gairdneri**	15	Pepsin,
	*Oncorhynchus mykiss**		Chymotrypsin
Salmon fish	*Oncorhynchus keta**		Pepsin,
Sardine	*Sardinos melanostica*		Pepsin, Trypsin
Seabass	*Yateolabrus*	10	Elastase
Spiny Pacific Dogfish	*Squalus acanthias*		Chymotrypsin
Tuna fish	*Thunnus albacores*	14-18	Pepsin,
Tuna- Bluefin tuna	*Thunnus thymus*		Elastase
Yellowtail	*Seriola quinqueradiata*		Elastase

*fresh water species

Adapted from Vecchi and Coppes (1996)

Anchovy (*Engraulis encrasicholus*) is susceptible to proteolytic degradation by digestive enzymes from pyloric caeca and intestines (Siringan *et al.,* 2006, 2007). The major proteinases found in the pyloric caeca and intestine of this type of anchovies were trypsin, chymotrypsin, elastase and aminopeptidase. Trypsin and chymotrypsin in *Engraulis encrasicholus* exhibited an optimal pH between 8 and 9 (Siringan *et al*., 2006).

Orejana and Liston (1981) studied the principal agents of proteolysis in *Patis,* using specific proteolytic enzyme inhibitor. They found that trypsin-like activity showed maximal activity during the initial first month of fermentation, then declined rapidly and remained at a relatively low level throughout the fermentation. They suggested that the decline in activity was due to the inhibition by end products such as amino acids and small peptides accumulated.

Choi *et al.* (2004) isolated and purified the proteolytic enzymes that played important roles in anchovy (*Engraulis japonica)* sauce fermentation. Three proteolytic enzymes such as trypsin, chymotrypsin and cathepsin L- like enzymes were investigated. Chymotrypsin and cathepsin L-like enzymes resulted in a higher hydrolysis of myofibrillar proteins of the anchovy than that by trypsin. Thus, during fermentation of fish sauce, cathepsin-L like enzyme and chymotrypsin were more important for the autolysis of muscle proteins compared to trypsin.

Klomklao *et al.* (2008) characterized the autolytic profile of sardine (*Sardinops melanostictus*) and found that the endogeneous enzyme, pepsin was the main enzyme responsible for the proteolysis during fish sauce fermentation. The enzyme's activity decreased with increasing NaCl concentration (0-30%).

The contribution of digestive enzymes during the ripening of fermented sardine with rice-bran was studied by Yatsunami and Takenaka (2000). They observed a higher activity of visceral proteolytic enzymes than that of muscle proteolytic enzymes, and concluded that the agents of hydrolysis were due to the serine proteases and the metal proteases from the viscera of sardines. These enzymes are trypsin-like, chymotrypsin-like and aminopeptidases-like enzymes. Similarly, Lopetcharat *et al.* (2001) concluded that the digestive enzymes that took part in autolysis of fish protein during fermentation were mainly trypsin and chymotrypsin.

2.3.1.2 Proteolytic enzymes from fish muscle tissues

Endogenous fish muscle proteolytic enzymes are located mainly in the muscle fibres and extracellular matrix of muscle. These enzymes may be found in the intercellular fluids and in the sarcoplasm or associated with different cellular organells (Shahidi and Kamil, 2001). Fish muscle tissue enzymes involved in fish sauce fermentation includes cathepsins, peptidases, transaminases, amidases, amino acid decarboxylases, glutamic dehydrogenases, calpain, neutral trypsin-like proteinases and alkaline proteinases (Chaveesuk, 1991; Yatsunami and Takenaka, 2000; Siringan *et al.*, 2006). Cysteine proteinases such as capthepsin B, H and L is believed to be the major muscle proteinases that degrade muscle proteins (Shahidi and Kamil, 2002). Neutral serine proteases, with an optimal pH at 7.5 were also purified in muscles of anchovy (*Engraulis japonica*) (Siringan *et al*., 2006).

2.3.2 Microbial proteolytic enzymes

Both bacterial and fish proteases are involved in protein hydrolysis of fish during fermentation process (Fukami *et al.*, 2004). However, fish endogenous proteolytic enzymes are more significant in proteolysis than microbial proteolytic enzymes due to the presence of high salt concentrations which results in a decreased in microbial counts during fermentation (Chaveesuk, 1991). According to Lopetcharat *et al.* (2001), bacteria involved in fish sauce fermentation can be classified into two main groups; the first group is the bacteria that produce proteolytic enzymes and the second group is the bacteria that relate to flavour and aroma development.

2.4 Proteolytic enzymes classification

Proteolytic enzymes refer to all enzymes that cleave peptide bonds. These enzymes are used in digestion, and include the proteinases, which catalyze the hydrolysis of protein molecule into large polypeptides fragments, and peptidases which hydrolyze polypeptide fragments to amino acids (Frazier, 1958).

Proteolytic enzymes can be classified based on different criteria, for instance: the origin of the enzyme (microbial, plant or animal), the pH range (acidic, neutral or alkaline), the substrate specificity or the catalytic mechanism (Aspmo *et al*, 2005). According to the International Union of Biochemistry, proteolytic enzymes can be classified into four major classes based on active site and mechanism of action (Neurath, 1989). These are cysteine, aspartic, serine and metalloproteases; and within these four classes, six families of protease are recognized (Neurath, 1989; Wong, 1995).

Proteolytic enzymes can also be classified into various groups based on their sensitivity to various inhibitors (Bougatef *et al.*, 2010). Proteolytic enzyme inhibitors are protein–based substances that are able to reduce the activity of proteolytic enzyme within the gastrointestinal tract of animals (López *et al.*, 1999). Table 2.3 shows this classification of proteolytic enzymes and the characteristic of the active site residues.

Table 2.3 Classification of proteolytic enzymes and the characteristic of their active site residues.

Family	Representative protease (s)	Characteristic active site residues
Serine protease I	**Chymotrypsin** Trypsin Elastase	Asp, Ser, His
Serine protease II	**Subtilisin**	Asp, Ser, His
Cysteine proteases	**Papain**	Cys, His, Asp
Aspartic proteases	**Penicillopepsin** Acid proteases Renin	Asp, Asp
Metallo-proteases I	**Bovine carboxypeptidase A**	Zn, Glu, Try
Metallo-proteases II	**Thermolysin**	Zn, Glu, His

Adapted from Neurath (1989)

2.4.1 Serine protease

Serine protease is a group of endopeptidases that have serine residue in their catalytic site (Disarapong, 2005). This class of proteolytic enzyme has been studied in more detail compared to other group of enzyme (Wong, 1995). Examples of serine proteases are trypsin, chymotrypsin and elastase (Wernersson *et al.*, 2006). Phenylmethanesulfonyl fluoride (PMSF) is the inhibitor for serine protease. PMSF

inhibits serine proteases by sulfonylating the hydroxyl group of serine residues at the active site (Gold, 1967).

Fish serine proteases exhibit their optimum activity under alkaline conditions. Trypsin and trypsin-like proteolytic enzymes have been purified from a variety of fish and this includes anchovy (Shahidi and Janak Kamil, 2001). Trypsin hydrolyses peptide bonds at the carboxyl terminus of lysine and arginine residues. It can be used as fermentation aid to accelerate the protein hydrolysis (Khantaphant and Benjakul, 2010). However, Gildberg (2001) reported that although trypsin enzymes an play essential role in the solubilisation of tissue during fish sauce fermentation, this contributes to inferior flavour of the final product.

Serine protease, e.g. trypsin and chymotrypsin have been purified and characterized from fish pyloric caeca (Shahidi and Janak Kamil, 2001), and are observed in the pancreatic and intestinal segments of Asian bony tongue *Scleropages* or known as Arowana in Asia (Natalia *et al*., 2004). Pyloric caeca has been reported to contain the highest amount of trypsin compared to other viscera (Khantaphant and Benjakul, 2010). This is in agreement with Vecchi and Coppes (1996) who found that trypsin is secreted by the pyloric caeca of many species of fish. Trypsin activities were generally higher compared to chymotrypsin for carnivorous fishes, while in omnivorous and herbivorous species, activities of chymotrypsin were higher (Chong *et al*., 2002).

Extracellular serine proteinases were extracted from *Virgibacillus* sp. isolated from fish sauce. This *Virgibacillus* sp. SK37 is a non motile Gram positive bacteria, with long rod and able to grow and hydrolyze anchovy at 25% NaCl (Sinsuwan *et al*.,

2007). In addition, *Halobacillus* sp. SR5-3 and *Filobacillus* sp. RF2-5 isolated from the fish sauce were also found to produce serine proteases (Sinsuwan *et al.*, 2007).

2.4.2 Cysteine protease

Cysteine protease is a group of endoproteinase that requires cysteine and histidine residues at the active site for hydrolysis (Sajid and McKerrow, 2002). Iodoacetamide or iodoacetic acid function as cysteine protease by inhibiting the thiol groups of cysteine protease (Aitken and Learmonth, 1996). Papain from the papaya fruit (*Carica papaya*) is the best-known cysteine protease. It is the first cysteine protease to be purified and characterized in 1879 (Sajid and McKerrow, 2002). Cathepsin also belongs to the cysteine protease group. Visceral and fish muscle cathepsins are responsible in the hydrolysis of fish protein (Yatsunami and Takenaka, 2000).

2.4.3 Aspartic protease

Aspartic proteases are characterized by high activity and are normally stable at acidic pH, but some enzymes such as rennin, are active under acidic conditions with optimum pH activity around 6-8 (Shahidi and Janak Kamil, 2001). Aspartic acid carboxyl groups are the functional groups of catalytic site of aspartic enzymes.

Aspartic proteases are secreted from the gastric mucosal glands of vertebrates including fish (Shahidi and Janak Kamil, 2001). Examples of aspartic proteases that have been isolated and characterized from the digestive tract of marine animals include pepsin, chymosin, gastricins. Pepsin is an example of aspartic proteases and is the major digestive protease present in the gastric juice of vertebrates.

2.4.4 Metalloprotease

Metalloprotease (metallopeptidases or metalloproteinases) is a proteolytic enzyme that cleaves peptide bonds by the action of a water molecule (Mansfeld, 2007)**.** The proteolytic action of metalloproteases is activated by complexing to bivalent metal ions. The metal ions can be zinc, manganese, cobalt, nickel or even copper ions which are complexes by three amino acids residues that can be Histidine, Asparagine, Glutamic acid and Lysine (Mansfeld, 2007). Metalloproteases are inhibited by chelating agents such as EDTA and 1,10-phenanthroline (Disarapong, 2005; Klomklao *et al.*, 2008). *Bacillus subtilis* JM-3 and *Halobacillus thailandensis* isolated from fish sauce were found to secrete metalloprotease (Sinsuwan *et al.*, 2007).

2.5 Microorganisms in fish sauce fermentation

Microorganisms play important roles in the degradation of protein and development of flavour and aroma of the end products in fish sauce fermentation (Lopetcharat *et al.,* 2001). *Nuoc nam* that is produced aseptically lacked the typical flavour of fish sauce; this suggested that microorganisms maybe contribute to the characteristic flavour of fish sauce (Thongthai and Gildberg, 2005). However, there is lack of research information on the role of these bacteria on the development of taste and odour of fish sauce.

During fermentation, a thick layer of salt is used to cover the top of the fish, and since oxygen is less soluble at high salt concentrations, this results in partial aerobic and anaerobic condition for microorganisms (Lopetcharat *et al.,* 2001). As the fermentation