Chapter 1

Introduction

Thailand is the largest fish sauce producer, where 390 fish sauce factories are located with the annual production of approximately 64,000 metric tons (Saisithi, 1994). Fish sauce is the liquid product developed during fermentation of heavily salted fish material in closed tanks at tropical temperatures (Amano, 1962). Fish sauce is mainly produced from anchovies (*Stolephorus* spp.), mackerel (*Rastrelliger* spp.) and herring (*Clupea* spp.) (Lopetcharat *et al.,* 2001). Traditionally, fish sauce is produced by mixing 1 part salt with 2 or 3 parts fish and fermenting at ambient temperature (30 to 40 ^{o}C) for 6 to 12 months or longer (Lopetcharat *et al.,* 2001). However, salt/fish ratio can be varied, depending on countries. During fermentation, protein hydrolysis is induced by endogenous proteinases in fish muscle and digestive tract as well as proteinases produced by halophilic bacteria (Gildberg and Thongthai, 2001). The volatile compounds contributing to flavor of fish sauce are produced by nonenzymatic reactions of

various components and enzymatic reactions by endogenous enzymes of fish origin and those of microorganisms surviving during fermentation (Fukami *et al.,* 2004). Fermentation process normally takes a long time to ensure the solubilization as well as the flavor and color development of fish sauce. The autolysis of fish proteins during fermentation was accelerated by the addition of fish internal organs or proteinases (trypsin and chymotrypsin) (Kim *et al.,* 1997; Morioka *et al.,* 1999) or the reduction of salt concentration (< 20%) (Morioka *et al.,* 1999). Gildberg (2001) reported that fish sauce from minced capelin was obtained after 6 months of fermentation with the addition of 5 - 10% enzyme-rich (trypsin and chymotrypsin) cod intestines.

Fish sauces are known to contain high levels of histamine depending upon fish type, handing condition as well as fermentation process. Histamine formation in fish and fishery products is related to free histdine content of fish muscle, the presence of bacterial histidine decarboxylase and environmental conditions to promote growth of histamine-forming bacteria (Lehane, 2000). Generally, histamine formation by bacteria is enhanced at elevated storage temperatures (Kim *et al.,* 2000) as well as high temperature abuse of post-harvested fish (Yongsawatdigul *et al.,* 2004).

Due to the tremendous amounts of solid wastes, especially internal organs in tuna industry, more attention has been given to those wastes as the raw material for value-added products. Those wastes contain a high amount of protein (Guérard *et al.,* 2001). Thus, tuna internal organs can be a potential starting material for fish sauce production owing to the abundant protein as well as high proteolytic activity (Klomklao *et al.,* 2004). Tuna internal organs can be hydrolyzed to form the liquid having the similar composition and characteristics to commercial fish sauce. After evisceration, internal organs may undergo the deterioration, particularly without the appropriate handling. Therefore, the properties and characteristics of fish sauce obtained might be determined by the quality of those wastes. Therefore, the use of tuna internal organs to produce fish sauce with the shortened fermentation time and low histamine/biogenic amines can be an alternative approach to fully utilize the tuna waste and obtain the novel value-added nutritive products.

Literature Review

1. Tuna / tuna wastes

Tuna and tuna-like species are important fish species due to their high global economic value and their prevalence in international trade for canning and sashimi. Tuna is the large pelagic fish that prevails in the tropics and subtropics (Al-Abdessalaam, 1995). They are commercially important in many countries and there is high demand from international markets. The average length and weight were 47.3 cm. and 1.74 kg, respectively (Mazorra *et al.,* 2000). Tuna is hard bone and can be classified to the family of scrombroidae, and the genus of Thunnidae (Saila and Norton, 1974). The tuna are sub-classified into 4 genera (*Thunnus, Euthynnus, Katsuwonus,* and *Auxis*) with 13 species all together (Figure 1) (FAO, 1997; Collette, 1986).

During processing, a large amount of wastes, both liquid and solid, is generated, causing the problem for disposal or management. Generally, head, bone as well as viscera are the major solid wastes produced and constitute 15-17% of body weight. Javeed and Mahendrakar. (1996) reported that fish internal organs constitute approximately 7.5% of body weight. As total world tuna catches are about 3 billion metric tons (FAO, 1997), the canned fish processing generates solid wastes that can be as high as 50□70% of the original raw material. Those wastes

were used for fish protein hydrolysates (FPH) production (Benkajul *et al.,* 1997; Shahidi *et al.,* 1995; Hoyle and Merrit, 1994). Fish internal organs and heads were converted to powdered fish flour used as animal feed (Stom and Eggum, 1981). Using fishery by–products for protease production is of great importance since low–cost proteases could promote new industrial application. Proteases can be used in leather processing, detergent industry, food industries, etc. (Kalisz, 1988). Tuna waste hydrolysate has been used traditionally for pet-food. (Guérard *et al.,* 2002).

The hydrolysates have a wide range of potential applications, e.g. as ingredients in animal feed (Faid *et al.,* 1997) or food (Frøkjaer 1994; Lahl and Braun 1994; Mahmoud 1994), as the peptone for microbial growth media (Vecht-Lifshitz *et al.,* 1990; Gildberg *et al.,* 1989), or as fertilizer (Kurbanoglu and Algur 2002). The term ‘‘peptone’’ is used for protein hydrolysates that are soluble in water and not heat coagulable. The market price of peptones is higher than those of the usual by-products from fish such as fish silage and fish meal. Vázquez *et al.* (2004) found that the hydrolysates from viscera waste derived from squid (*Logigo vulgaris*), yellowfin tuna (*Thunnus albacares*), swordfish (*xiphias gladius*), and rainbow trout (*Oncorhynchus mykiss*)

can substitute other peptones in the habitual formulation for culture of lactic acid bacteria, promoting biomass and bacteriocin production. Traditional fish hydrolysates including fish silage (Faid *et al.,* 1997; Arason 1994) or fish sauce (Gildberg 2001; Saisithi 1994) exploit the endogenous enzymes of the fish intestines.

Gelatin from fish by-products, such as skin and bone, has been paid attention increasingly as the replacement of mammalian resources (Gudmundsson, 2002). A few fish gelatins are available commercially, and are not commonly utilized because it is inferior to mammalian gelatin in theological properties (Choi and Regenstein, 2000). Cho *et al*. (2005) reported that the skin of yellowfin tuna can be a possible material for gelatin production, and the yellowfin tuna skin gelatin can be used in products requiring very high gel strength. If yellowfin tuna skin gelatin is modified by chemical and enzymatic methods to improve gelling and melting points, it may be used as the replacement for mammalian gelatins.

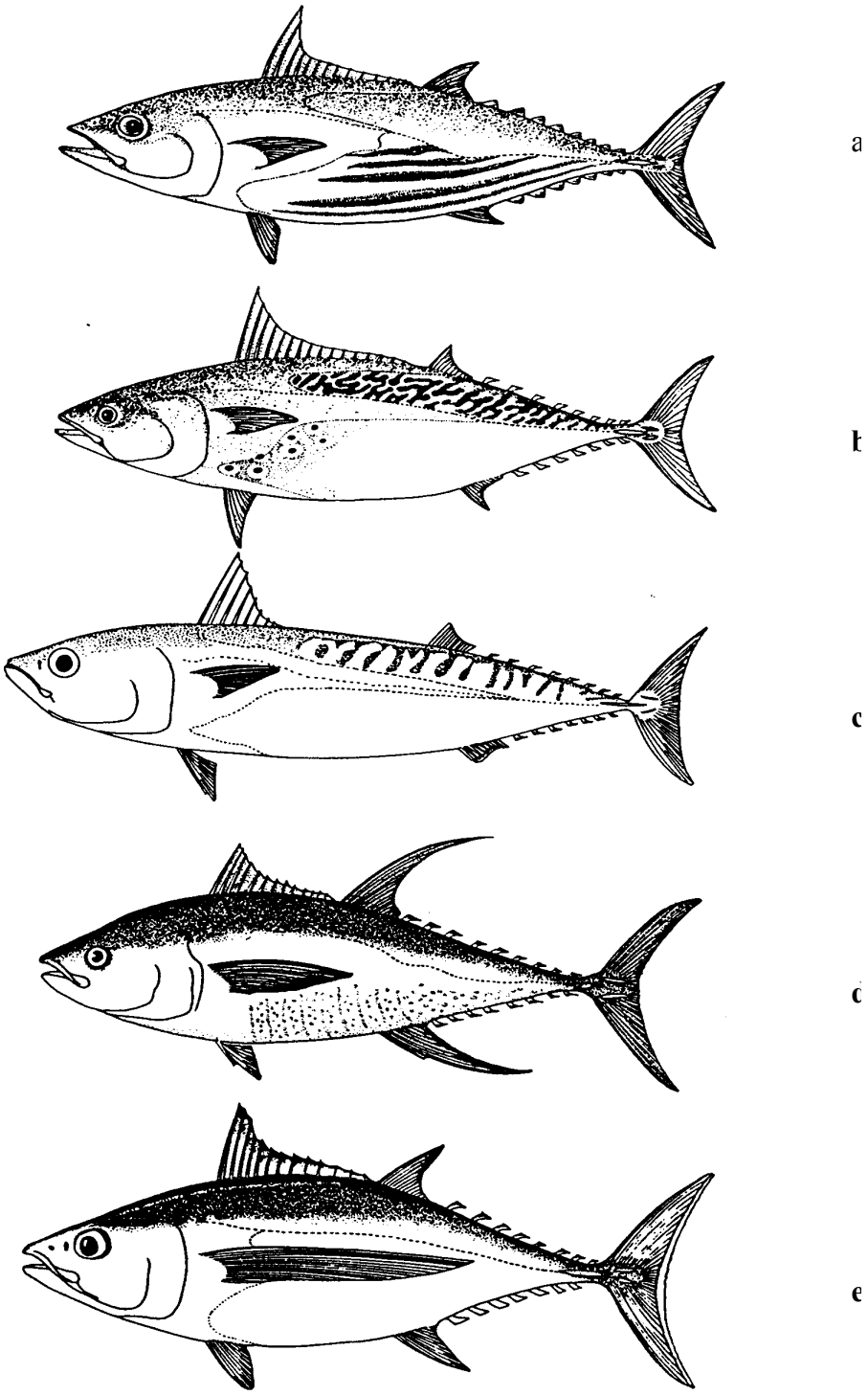

Figure 1 Tuna species: (a) skipjack tuna; (b) Eastern little tuna; (c) bullet tuna; (d) yellowfin tuna; (e) albacore tuna.

Source: Collette (1986)

2. Fish internal organs / proteolytic activity

Fish viscera or internal organs are the important source of enzymes, especially proteinases. Digestive proteinases from marine animals are produced by the digestive glands of marine animals. Likes the proteinases from plants, animals and microorganisms, digestive proteinases from marine animals are hydrolytic in their action, and catalyze the cleavage of peptide bonds with the participation of water molecules as reactants (Simpson, 2000). In addition to its adequate nutritional value, it contains high levels of digestive enzymes, making it a suitable source for recovering proteases for food application. Digestive proteinases have been studied in several species of fish (Vecchi and Coppes, 1998) and decapods (Garcia-Carreno and Haard, 1993). Proteinases found in the intestine of fish include trypsin, chymotrypsin, collagenase, elastase, carboxypeptidase and carboxyl esterase, and they are normally secreted from the pyloric caeca and pancreas (Haard, 1994). Pepsin and trypsin are two main groups of proteinases found in fish viscera. Pepsin is found in fish stomach and is active at acid conditions (Gildberg *et al.,* 1990), while trypsin is concentrated in pyloric caecum and active at neutral and alkaline condition

(Asgeirsson *et al.,* 1989). Pepsin and trypsin were also detected in the belly cavity adjacent to muscle of fresh fish (Siebert and Botkke, 1963). Gildberg (1992) suggested that the leakage of digestive proteinases into the belly cavity of fish can activate collagenases present in the connective tissue as well as directly initiate collagen degradation by digestive collagenase. Certain types of feed in the digestive tract can trigger post-mortem autolysis known as "feedy fish". Contaminated organ tissues can be a source of proteinase in minced fish and surimi, which causes the gel softening (modori), (Su *et al.,* 1981).

The distribution of proteinase varied, depending on species and organs. Torrissen (1984) reported that proteinase activity from intestine of rainbow trout (*Salmo gairdneri*) was higher than that of Atlantic salmon (*Salmo salar*). Pyloric caeca of Chinook salmon (*Oncorhynchus tschawytscha*) had a higher proteinase activity than that of rainbow trout (Dimes *et al.*, 1994). For discus fish (*Symphysodon aequifasciata*), proteinase activity in intestine was higher than that in stomach (Chong *et al.*, 2002). The varying distribution of protease activity in individual internal organ of three tuna species including skipjack tuna (*Katsuwonus pelamis)*,

yellowfin tuna (*Thunnus albacares*) and tongol tuna (*Thunnus tonggol*) commercially used in Thai tuna industries was reported by Klomklao *et al*. (2004).

2.1 Classification of digestive proteinases from marine animals

2.1.1 Acid/Aspartyl proteinases

The acid or aspartyl proteinases are a group of endopeptidases characterized by high activity and stability at acidic pH. They are referred to as "aspartyl" proteinases (or carboxyl proteinases) because their catalytic sites are composed of the carboxyl group of two aspartic acid residues (Whitaker, 1994). Based on the EC system, all the acid/aspartyl proteinases from marine animals have the first three digits in common as EC 3.4.23. Three common aspartyl proteinases that have been isolated and characterized from the stomach of marine animals are pepsin, chymosin, and gastricsin (Simpson, 2000).

Pepsin is assigned the number EC. 3.4.23.1. It has preferential specificity for the aromatic amino acids, phenylalanine, tyrosine, and tryptophan. In the EC system of classification, chymosin (formerly known as rennin) is assigned the number EC 3.4.23.4. Chymosin has

specificity for the aromatic amino acids, phenylalanine, tyrosine, and tryptophan, similar to pepsin. Gastricsin is assigned a code of EC 3.4.23.3. (Simpson, 2000).

Pepsin has an extracellular function as the major gastric proteinase. Pepsin, secreated as a zymogen (pepsinogen), is activated by the acid in stomach to an active form (Clarks *et al.,* 1985). Pepsin-like protease with an optimum pH value of 1.7 was reported to be predominant in the stomach of dover sole (Clarks *et al.,* 1985). Haard (1986) reported that the initial rate of hemoglobin digestion by Atlantic cod pepsin was maximal at 35°C, and pH 1.9. Fish pepsins were shown to hydrolyze haemoglobin much faster than casein (Gildberg and Raa, 1983). Guéraed and Le Gal (1987) reported that a hexapeptide is the smallest substrate to be hydrolized by fish pepsins. Most fish species contain two or three major pepsins with an optimum haemoglobin digestion at pH between 2 and 4 (Gildberg and Raa, 1983). Gildberg *et al.* (1990) found that the affinity of cod pepsin, especially pepsin I towards haemoglobin, was lower at pH 2 than at pH 3.5. Furthermore, pH optimum was highly dependent on substrate concentration. Pepsin I and II showed similar pH optima at pH 3.0 at high concentrations of hemoglobin, whereas pepsin I had a maximum activity at pH 3-4 with low substrate concentration.

2.1.2 Serine Proteinases

The serine proteinases have been described as a group of endoproteinases with a serine residue in their catalytic site. This family of proteinases is characterized by the presence of a serine residue, together with an imidazole group and aspartyl carboxyl group in their catalytic sites. The activity is inhibited by diisopropylphosphofluoridate (DFP), through reaction with the hydroxyl group of the active site serine residue (Simpson, 2000). The proteinases in serine subclass all have the same first three digits: EC 3.1.21. The three major serine proteinases purified and well characterized from the digestive glands of marine animals are trypsin, chymotrypsin, and elastase. Trypsin is assigned the code ED 3.4.21.4. Trypsin has a very narrow specificity for the peptide bonds on the carboxyl side of arginine and lysine. Chymotrypsin is assigned a code of EC 3.4.21.1 and it has a much broader specificity than trypsin. It cleaves peptide bonds involving amino acids with bulky side chains and nonpolar amino acids such as tyrosine, phenylalanine, tryptophan, and leucine. Elastase is designated as EC 3.4.21.11. Elastase exhibits preferential specificity for alanine, valine, and glycine (Simpson, 2000).

Serine proteinases, mainly trypsin and chymotrypsin, play a major role in protein digestion (Martinea and Serra, 1989). Fish trypsins are generally stable at alkaline pH. Purified trypsin from hybrid tilapia (*Tilapia nilotica/aurea*) intestines showed an optimal activity at pH 9.0 and 40°C (Shemy and Levin, 1997). Two trypsin-like enzymes, enzyme I and II, isolated from the gut of capelin had the optimum pH at 8.0-9.0 with the optimum temperature at 42°C (Hjelmeland and Raa, 1982). Simpson *et al.* (1990) reported that Atlantic cod trypsin was most active at pH 7.5 and 40°C. In dover sole, the activity at pH 7.0-8.0 was due to trypsin and chymotrypsin-like enzymes, while the optimal activity at pH 9.5-10.5 was due to elastase (Clarks *et al.,* 1985). The optimum pH for hydrolysis of casein by Greenland cod trypsin was 9.0-9.5. Hjelmeland and Raa (1982) found two trypsins from Arctic fish capelin with molecular weight about 28,000 Da. Greenland cod trypsins had the molecular weight of 23,500 Da (Simpson and Haard, 1984). Trypsin A and B from anchovy had molecular weights of 27,000 and 28,000 Da, respectively (Martinez *et al.,* 1988). Cohen *et al* (1981) reported that molecular weight of carp trypsin was 25,000 Da. A trypsin-like enzyme was reported to be the major form of protease in the digestive organs of Pacific whiting based on the molecular weight, the inhibition by TLCK

and the activity toward specific substrates (Cohen *et al.,* 1981). Guizani *et al.* (1991) reported that a trypsin from the pyloric caeca of mullet, *Mugil cephalus*, exhibited optimal activity at a pH of 8.0 and at a temperature of 55 °C. It was stable within a pH range of 7.5-9.0. Trypsin from the pyloric caeca of rainbow trout, *Oncorhynchus mykiss*, was purified and characterized by Kristjansson (1991). The isolated enzyme had an estimated molecular weight of 25,700 daltons. The enzyme was stable in the temperature range of 40 - 50°C and at a pH range of 5.4-8.0. However, the thermal stability was shown to be calcium-dependent. Hydrolysis of substrate was maximal at approximately 60°C. Qui□ones (2000) purified trypsin from pyloric caeca and intestinal tissues of the queen snapper, *Etelis occlatus*. The trypsin displayed optimal activity in a pH range of 8.0 - 9.0. The activity was highest at 50°C for pyloric caeca trypsin and 60°C for the intestinal tissues. This enzyme was inhibited by soybean trypsin inhibitor

The proteolytic activities in the gut of three carnivorous fish species, the deepwater redfish, *Sebaster mentella*, the turbot, *Scophthalmus maximus*, and the gilthead bream, *Sparus aurata*, showed optimal activity at a pH range of 9.5-10.0 with the temperature range of 35-40°C (Munilla-Mo□an and Saborrido-Rey, 1996). Sabapathy and Teo (1993) studied the

distribution of trypsin in rabbitfish, *Siganus canalicutus*, and sea bass, *Lates calcarifer*, digestive tract. Trypsin activity was higher in the rabbitfish, in which the enzyme was detected in all regions of the digestive tract. However, sea bass trypsin was confined to the intestine and pyloric caeca.

2.1.3 Thiol/Cysteine proteinases

The thiol or cysteine proteinases are a group of endoproteinases that have cysteine and histidine residues as the essential groups in their catalytic sites. These enzymes require the thiol (-SH) group furnished by the active site cysteine residue to be intact, hence this group is named “thiol” or “cysteine” proteinases. The thiol proteinases are inhibited by heavy metal ions and their derivatives, as well as by alkylating agents and oxidizing agents (Mihalyi, 1978). The first three digits common to thiol proteinases are EC 3.4.22. An example of a thiol proteinase from the digestive glands of marine animals is cathepsin B, which is designated as EC 3.4.22.1 (Simpson, 2000).

Sovik and Rustad (2006) reported that Cathepsin B from viscera had the maximum activity at 50°C in cod (*Gadus morhua*) and saithe (*Pollachius virens*), at 35°C in tusk (*Brosme brosme*) and ling (*Molva molva*) and at 20°C in haddock (*Melanogrammus aeglefinus*), while cathepsin B in liver had highest activity at 50°C in saithe (*Pollachius virens*) and tusk (*Brosme brosme*), and at 35°C in cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*). Cathepsin B from hepatopancreas (a digestive gland) of carp (*Cyprinus carpio*) had a temperature maximum of 45°C (pH 6) (Aranishi *et al.,* 1997).

2.1.4 Metalloproteinases

The metalloproteinases are hydrolytic enzymes whose activity depends on the presence of bound divalent cations. Chemical modification studies suggest that there may be at least one tyrosyl residue and one imidazole residue associated with the catalytic sites of metalloproteinases (Whitaker, 1994). The metalloproteinases are inhibited by chelating agents

such as 1, 10-phenanthroline, EDTA, and sometimes by the simple process of dialysis. Most of the metalloproteinases known are exopeptidases. They all have a common first three digit as EC 3.4.24. The metalloproteinases have been characterized from marine animals (e.g., rockfish, carp, and squid mantle) but have not been found in the digestive glands except in the muscle tissue (Simpson, 2000).

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3. Fish sauce

Fish sauce is a traditional fermented fish product and an important source of protein in Southeast Asia. It is widely used as a condiment and seasoning in most countries of Southeast Asia and becomes gradually popular worldwide (Saisithi, 1994). It has various names depending on the country in which it is produced: patis in the Philippines, shotturu and ishiruin in Japan, nouc-mam in Vietnam, ketjap-ikan or bakasang in Indonesia, yeesui in Chaina, ngapi in Myanmr, and nam-pla in Thailand (Lopetcharat *et al.,* 2001). Fish sauce is basically made from a mixture of fish and salt with a weight ratio of 3:1. This mixture is allowed to ferment for more than 6 months at 30 $^{\circ}$C to 35°C for complete hydrolysis and flavor development (Jay, 1996).

Protein hydrolysis is induced by endogenous proteinases and those produced by halophilic bacteria (Gildberg and Thongthai 2001; Saisithi 1994). Degradation of fish protein to free amino acids is primarily responsible for the delicious taste of fish sauce (Chayovan *et al.,* 1983). Various volatile compounds, including acids, carbonyls, nitrogen-containing compounds, and sulfur-containing compounds, are formed during fermentation and believed to be responsible for the distinct aroma of fish sauce (Fukami *et al.,* 2002). The final product is clear brown liquid that is separated by filtration or by decanting. The liquid has a high content of soluble nitrogenous compounds and a salt concentration in the range of 20-30% (Amano, 1962; Beddow, 1985). The remaining residue consists mainly of bones and scales and commonly has been used as a fertilizer (Van Veen, 1965). Fish sauce contains 20g/l nitrogen, of which 16 g/l is in the form of amino acids (Sanceda *et al.,* 2000).

Total nitrogen content is used as an indicator to determine the grade and price of fish sauce in Thailand. Products containing over 20 g/l total nitrogen are classified as Grade I and those with 15 to 20 g/l total nitrogen are graded as Grade II (TISI, 1983). In Thailand, fish sauce is classified by the Thai Public Health Ministry into three types based on the production process:

pure fish sauce, hydrolyzed fish sauce and dilute fish sauce (Lopetcharat *et al.,* 2001). Pure fish sauce is derived from fresh fish or fish residue obtained from the fish fermented with salt or brine. Hydrolyzed fish sauce can be obtained from the hydrolysates of fish or other kinds of animals, which are often treated with hydrochloric acid (HCl) or other hydrolyzing processes that are approved by the Thai Public Health Ministry. Diluted fish sauce is obtained from pure fish sauce or hydrolyzed fish sauce, but is diluted using approved additives or flavoring agents (Lopetcharat *et al.,* 2001).

3.1 Fish sauce production

Fish sauce results from the physical and microbiological changes of fish that occur at high salt concentration and low oxygen levels. Fish sauce production starts with cleaning fresh fish with cold water to remove impurities and to reduce the quantity of microorganisms in the raw materials (Lopetcharat *et al.,* 2001). Generally, cleaned fish are mixed with salt in a ratio of 2:1 or 3:1 (fish: salt) (w/w), depending on the area of production. The mixture is then transferred to concrete tanks built in to ground to maintain the temperature range of 37-40 ^{o}C, and

kept submerged under brine. The fermentation period takes from 6 to 12 months depending on the size of fish (Lopetcharat *et al.,* 2001). At the end of fermentation period, the supernatant liquid is drained off and filtered through sand beds. The filtrate may be sun-ripened from 1 to 3 months to improve the color and aroma of the sauce prior to packaging and distribution as a first grade fish sauce. The residue, which contains unhydrolyzed fish tissue, is further extracted with a saturated brine solution for up to 3 months and immediately bottled as a second grade fish sauce (Chaveesuk, 1993). BX water or Mikei water is applied to improve the quality of low grade or secondary fish sauce (Beddow *et al.,* 1979). BX water or Mikei water is the by-product of monosodium glutamate (MSG) production and is a rich source of glutamic acid, which improves the nitrogen content of low quality fish sauce in order to meet the requirements of the Thai Industrial Standard Institute. Caramel color and other additives, which are not harmful for consumers, are also added to improve color and flavor quantities of fish sauce (Lopetcharat *et al.,* 2001).

The most important single factor limiting the viability of the fish sauce industry is the long waiting (9-12 months) in its manufacture. It would be more advantageous if the fermentation

period could be shortened and the original quality should be controlled to yield a product acceptable to consumers (Lopetcharat *et al.,* 2001).

3.2 Role of proteinases in fish sauce production

The hydrolysis of the tissues appears to be mainly an autolytic process by endogenous fish enzymes (Beddows *et al,* 1979). Orejana and Liston (1981) reported that trypsin or trypsin-like enzymes are the principal agents of proteolysis in patis (a fish sauce) production. Bacteria or their enzymes are only of minor importance during the digestion, owing to the inhibitory effects of the high salt concentration (Orejana and Liston, 1981).

The endogenous hydrolytic fish enzymes are very much inhibited by the high salt concentration. The activity of pepsin from Atlantic salmon (*Samo sala*) was very low at 10% salt and completely inhibited by salt at very high concentration, but this inhibition is less pronounced and varies considerably with different species. Orejana and Liston (1981) showed that protein digestion during fish sauce fermentation was drastically reduced when tryptic enzymes were inhibited by addition of soybean trypsin inhibitor. This indicates that trypsin and

chymotrypsins are of vital importance for tissue solubilization and protein digestion, even if their activity is partly inhibited by the salt. Chymotrypsin most probably is more important than trypsin during fish sauce fermentation since chymotrypsin is more active at neutral and weak acid conditions (Heu *et al.,* 1995). Trypsin-like activity in patis fermentation increased and reached a maximum in the first month and then dramatically declined (Orejana and Liston, 1981). The decrease in trypsin-like activity in patis is thought to be caused by the accumulation of end products (amino acids and small peptides), inhibitors in fish blood or substances produced by bacteria (Lopetcharat *et al.,* 2001).

Trypsin and chymotrypsin are generally active in neutral condition and cathepsins are active in acid condition. The pH of fish sauce decreased from neutral pH (~7) to acidic pH (~5) during fermentation (Lopetcharat *et al.,* 2001). Therefore, during the first stage of fish sauce fermentation, trypsin and chymotrypsin are responsible for protein hydrolysis, but cathepsins involve in protein degradation in fish sauce fermentation when the pH drops to the acidic region. The decrease in cathepsins activity can be due to the decrease in high-molecular-weight proteins that serve as the substrate for the enzymes (Bersamin *et al.,* 1961).

Apart from trypsin and chymotrypsin, cathepsin A and D were found to be responsible for the protein hydrolysis in patis fermentation (Del Rosario *et al.,* 1984). However, cathepsin B and D showed the negligible effect on protein degradation in patis (Del Rosario *et al.,* 1984). In contrast, when Pacific whiting and its surimi by-products, after being mixed with high salt concentrations up to the level of 25%, were subjected to autolysis at 50°C, cathepsin L-like enzymes and metalloproteases played a significant role in hydrolyzing proteins (Lopetcharat *et al.,* 2001).

In traditional fish sauce fermentation, the rate of production depends on the activity of enzymes in the fish. The fermentation normally takes a long time until the desired product is obtained. Lopetcharat and Park (2002) found that the enzymes in Pacific whiting (whole fish and surimi by – product) are functioning at 25 % salt and high temperature. Tungkawachara *et al* (2003) found that the activities of cathepsin H -like enzymes were extremely low during fish sauce fermentation of Pacific whiting, while the cathepsin B-like enzyme was most active at the incubation temperature of 30°C. An *et al*. (1994) reported that cathepsin L has

highest activity at 55°C, while cathepsin B has maximal activity at 20 to 37°C, and cathepsin H has highest activity at 20°C.

To activate the proteolytic activity of fish, some activators were added. Yoshinaka *et al.* (1983) showed that calcium not only protected trypsin against self-digestion, but it also slightly increased its proteolytic activity. Furthermore, Sipos and Markel (1970) concluded that calcium promotes the formation of a calcium-trypsin complex from a reversible inactive form. Delaage and Lazdunski (1968) reported the existence of a specific calcium binding site on trypsinogen. The binding of calcium to trypsinogen induces a conformational change, which is associated with the formation of active form. Klomklao *et al.* (2004) found that proteinase activities of splenic extracts from three species of tuna increased with the addition of calcium chloride. At a concentration lower than 1 μM, calcium had no influence on the activity of spleen extracts for all species. When the concentration of calcium was increased from 1 μM to 1 mM, proteinase activity apparently increased. However, Gildberg (2001) reported that addition of 2mM calcium chloride had no effect on fish sauce produced from male Arctic capelin and Atlantic cod intestines.

3.3 Rapid fermentation of fish sauce

In traditional fish sauce fermentation, the rate of production depends on the activity of enzymes in the fish. The fermentation normally takes a long time until desired product is obtained. Many approaches have been studied to shorten the fermentation of fish sauce (Choi *et al.,* 1999; Kim *et al.,* 1999; Sanceda *et al.,* 1996). The first is to raise the initial temperature. A couple of weeks at 45 ^{o}C in the initial phase reduce the total production time from 1 year to 2 months (Gildberg *et al.,* 1984). The second is addition of acid combined with reduced salt content. The third is initial alkalization at low salt concentration (Gildberg *et al.,* 1984) and the fourth is addition of enzyme (Gildberg *et al.,* 2001).

3.3.1 Increasing temperature

In fish sauce production, increasing temperature (45 ^{o}C) and reducing salt concentration can also reduce fermentation time (Lopetcharat *et al.,* 2001). Yongsawatdigul *et al.* (2004) found that total nitrogen content of fish sauce fermented at 40 ^{o}C increased at a faster rate

compared with those fermented at room temperature. In general, the optimum temperature for fish sauce fermentation is between 35 and 45 °C (Lopetcharat *et al.,* 2001). Lopetcharat and Park (2002) reported that fish sauce fermented at 50°C had the higher total nitrogen content than at 35°C. Therefore, increasing fermentation temperature could result in high degree of hydrolysis, which could in turn lead to acceleration of fermentation process. Korean fish sauce is usually fermented at 20-25 °C in order to maintain taste and flavor (Lopetcharat *et al.,* 2001). Nevertheless, fermentation at higher temperature (50°C to 65°C) resulted in a cooked flavor, which was objectionable (Yongsawatdigul *et al.,* 2004).

3.3.2 Acid hydrolysis

Acid digestion has been suggested as a possible means to accelerate the processing of fish sauce (Beddows and Ardeshir, 1979a, b). The disadvantage of this process is the lack of aroma and flavor. Gildberg *et al.* (1984) found that fish sauce hydrolysis could be accelerated considerably by autolysis at pH 4 and low salt concentrations, followed by subsequent neutralization and salt addition. The final product, although initially lacking in flavor, developed

flavor characteristics similar to those of traditionally produced fish sauce during storage (Gildberg *et al.,* 1984).

Acid hydrolysis of mixed-minced fish in the pH range from 3.6 to 3.8 at room temperature with the addition of 0, 10 and 15% salt showed the varying hydrolysis. At pH 3.6 to 3.8, protein conversion as high as 25.2% was obtained in 6 days in the absence of salt, compared to 10.8 and 7.2% when salt concentrations of 10 and 15%, were used respectively. However, at low pH (3.6), only hydrochloric acid could not prevent fish spoilage (Poosaran, 1986).

3.3.3 Alkaline hydrolysis

The use of alkaline, primarily sodium hydroxide, to hydrolyze protein often results in poor functionality and more importantly can adversely affect the nutritive value of the hydrolysate. Tannenbaum *et al* (1970) reported that the alkaline treatment can aid in modifying the properties of insoluble fish protein concentrates and its applications. A small-scale batch process that utilized high pH (12.5) and 95°C for 20 min was developed and the resulting product consisted of large peptides (Tannenbaum *et al.,* 1970).

The fish by-products male Arctic capelin and Atlantic cod intestines can be utilized as raw materials for the production of high value fish sauce. Initial alkaline treatment of minced capelin supplemented with 5-10% enzyme-rich cod pyloric caeca accelerated the tissue solubilization and gave a better fish sauce recovery without the adverse effect on the final product (Gildberg, 2001).

3.3.4 Enzyme hydrolysis

Enzymes are biological catalysts capable of speeding up chemical reaction and have been used as the biological tools for improving food quality or food processing operations. Use of an enzyme as a processing aid has a number of advantages over the use of chemicals, including high specificity, efficiency of catalysis at moderate temperatures, and being environmentally friendly. The enzymes recovered from fish have been successfully used as seafood processing aids including the accelerating fermentation of fish sauce. Fish sauce fermentation can be accelerated by the addition of enzymes (usually papain, bromelain, or

bacterial proteases) (Baddows and Ardeshir, 1979a). Addition of bromelain, ficin and papain could shorten fermentation time of fish sauce. (Choi *et al.,* 1999).

Fish hydrolysates manufactured by the use of plant proteolytic enzymes can be added into the traditionally produced fish sauce without affecting the nutritional quality (Beddows and Ardeshir, 1979a). However, on their own, the artificially produced fish hydrolysates are inadequate in term of quality since they lack of aroma, and flavor, which are the most important factors for consumer acceptability of the traditional fermented fish sauce (Beddows, 1985). Baddows and Ardeshir (1979a) found that the plant enzymes, including bromelain, ficin and papain could digest fish tissues in a short period, thus producing hydrolysates with distribution and concentration of nitrogenous compounds similar to that of fish sauce, but lacking in aroma. The addition of papain accelerated proteolysis but the typical aroma of traditionally produced fish sauce was not observed (Ooshiro *et al.,* 1971)

Raksakulthai *et al.* (1986) reported that a good quality fish sauce could be prepared from capelin (*Mallotus villosus*) with the help of squid hepatopancreas. Addition of proteinases such as fungal pronase, trypsin, and chymotrypsin to mince generally increased the

initial rate of protein hydrolysis, but did not yield a product with a much higher content of free amino acid in comparison with sauces without added enzymes (Raksakulthai *et al.,* 1986). Chaveesuk *et al*. (1993) studied the effect of addition of trypsin and chymotrypsin (0.3% w/w) at various proportions (100:0, 50:50 and 0:100) on the acceleration of fish sauce fermentation using herring as raw material. The supplementation with trypsin and chymotrypsin significantly increased protein hydrolysis. Fish sauces prepared from herring with enzyme supplementation contained significantly more total nitrogen, soluble protein, free amino acid content and total amino acid content, compared to fish sauce with no added enzyme. An acceptable fish sauce product could be produced from herring and the addition of enzymes has the potential to reduce the fermentation time to ~2 months.

4. Microbiology of fish sauce

Fish sauce has very high concentration of salt (25-30%). Thus microorganisms found during fish sauce production are generally classified as halophilic. The important roles of bacteria in fish sauce are protein degradation and flavor-aroma development. Consequently, when

fish sauce is produced under aseptic conditions, typical fish sauce aroma is not developed (Beddow *et al.,* 1979a). Bacteria involved in fish sauce can be classified into two major groups: bacteria that produce proteolytic enzymes and bacteria that contribute to flavor and aroma development.

Bacteria, which produce proteolytic enzymes, include *Bacillus sp., Pseudomonas sp., Micrococcus* sp*., Staphylococcus sp., Halococcus sp., Halobacterium salinarium., Halobacterium cutirubrum.* Highly concentrated NaCl (25%) does not have any effect on the proteolytic activity of enzymes from *H. salinarium and H. cutirubrum*; A chelating agent such as EDTA inactivates these enzymes completely. Fukami *et al.* (2004) reported that bacterium, which was isolated from fish sauce mush (moromi) of frigate mackerel and identified as *Staphylococcus xylosus*, could change notes of an odor in fish sauce made in Thailand. Volatile compounds of the fish sauce after incubation at 32°C for 24 days with the cultured bacterium were produced. *Bacillus, Micrococcus, Streptococcus, Pediococcus*, and other halophilic bacteria that produce lactic acid are found in fish sauce, including nampla, shotturu, bakasang, and nouc-mam (Ijon and Ohta 1996; Tanasupawat and Komagata 1995). However, it is unclear how these bacteria act

on the production of the characteristic taste and odor of fish sauce during fermentation. Three significant microorganisms identified in all fish sauce from Pacific whiting include *Staphylococcus*, *Bacillus*, and *Micrococcus*. Bacillus type bacteria and *Stapphylococcus* strain 109 were isolated from fish sauce and produced a measurable amount of volatile acids (Saisithi *et al.,* 1966). Futhermore, *Micrococcus*, *Coryneform*, and *Streptococcus* are commonly found in anchovy fish sauce (Ijong and Ohta 1996; Saisithi *et al.,* 1966). In addition of flavor development, *Staphylococcus*, *Bacillus*, and *Micrococcus* also produced proteolytic enzymes that are active in the presence of high salt concentration. Lopetcharat and Park (2002) reported that microorganisms should play an important role in the later stage of fermentation and the ripening stage. Protein degradation by those microorganisms leads to the production of volatile compounds from amino acid and small peptides.

5. Changes during fermentation of fish sauce

During fermentation, protein hydrolysis is induced by endogenous proteinases in fish muscle and digestive tract as well as proteinases produced by halophilic bacteria (Gildberg and

Thongthai, 2001). The volatile compounds contributing to flavor of fish sauce are produced by nonenzymatic reactions of various components and enzymatic reactions by endogenous enzymes of fish origin and those of microorganisms surviving during fermentation (Fukami *et al.,* 2004).

5.1 Chemical and biological changes

5.1.1 Changes in nitrogenous compounds

The soluble nitrogen components including proteins, peptides, and amino acids were generated by the activities of proteolytic enzyme. Nitrogenous compounds in fish sauce are composed of protein and nonprotein nitrogenous (NPN) compounds such as free amino acid, nucleotides, peptide, ammonia, urea and TMAO (Shahidi, 1994; Finne, 1992). Total nitrogen content is used as an indicator to determine the grade and price of fish sauce in Thailand. Products containing total nitrogen content over 20 g/L are classified as Grade I and 15 to 20 g/L as Grade II (Thai Industrial Standard, 1983). Total nitrogen contents from Pacific whiting fish sauce increased from 6.40 to 15.7 g N/L during the first 10 day of fermentation (Lopetcharat and Park, 2002). Tungkawachara *et al*. (2003) reported that total nitrogen content in fish sauce increased

during fermentation. At the early stage of fermentation (1 and 3 month), fish sauce produced from Pacific whiting mixed with byproduct (WB) had higher total nitrogen content than fish sauce produced from Pacific whiting (W), possibly due to the greater degree of hydrolysis. W and WB fish sauce had total nitrogen contents of 1.28 and 1.36%, respectively. Fish sauce made from mackerel, sardine, and squid at 12 months of fermentation had total nitrogen contents of 1.89, 1.52, and 1.48 %, respectively (Funatsu *et al.,* 2000). Brillantes *et al.* (2002) reported that total nitrogen content produced from fresh anchovy ranged from 3.0 to 4.9 g/L, increased dramatically during the fermentation period especially in the first 4 months, then remained relatively constant after 6 month. Fish sauce produced from low quality fish contained the high total nitrogen levels (4 to 9 g/L) in the first-week of fermentation, increased significantly until 7 months and then remained relatively constant with final value of 18 to 22 g/L at the end of 12 months. Yongsawatdigul *et al.* (2004) found that total nitrogen content of fish sauce prepared from Indian anchovy (*Stolephorus indicus*) increased to reach the plateau of 2.1 to 2.3 g N/100 ml within 25 weeks and remained relatively constant until 52 weeks.

The increase in formol nitrogen content suggested the increased hydrolysis of peptide (Tungkawachara *et al.,* 2003) caused by the endogenous or microbial proteinases. Not only free amino acid or peptide but also ammonia was produced during the extended fermentation time. Ammonia might be formed by deamination process (Lopetcharat *et al.,* 2001). Lopetcharat and Park (2002) reported that the ammoniacal nitrogen reached a maximum level at day 10 and then decreased to a minimum at day 30. The increased ammoniacal nitrogen content during the first 15 day could be due to fish enzymes that were active during early stage of fermentation (Beddow *et al.,* 1980). However, the diminishing of ammonia in the fish sauce might be caused by the slow dissipation into the air. Another tentative explanation is the formation of Schiff base in the reaction of amine with aldehyde or ketone group. (Wade, 1991).

Amino nitrogen is usually used as an indicator for degree of fermentation (Lopetcharat *et al.,* 2001). The amino nitrogen content represents the amount of amino groups in fish sauce. An increase in amino nitrogen concentration is related to the degradation of the polypeptide (Tungkawachara *et al.,* 2003). The longer fermentation time allowed more breakdown of soluble protein and peptide into free amino acids and volatile nitrogen (Chaveesuk

et al., 1993). The increased amino nitrogen contents could be due to hydrolytic activity of fish enzymes that was active during the early storage of fermentation (Beddows *et al.,* 1980). Tungkawachara *et al.* (2003) reported that amino nitrogen content were about 50 to 52% of the total nitrogen in Pacific whiting fish sauce after 3 months of fermentation.

5.1.2 Changes in salt content

Salt content in fish sauce is normally constant, especially with increasing fermentation time. Yongsawatdigul *et al.* (2004) reported that salt content of fish sauce prepared from Indian anchovy (*Stolephorus indicus*) was approximately 25-26%. Lopetcharat and Park (2002) reported that salt content in the fish sauce from Pacific whiting waste increased at day 5. Thereafter, it remained constant at about 25-30% during 60 days of fermentation. This could be due to the salt concentration having reached equilibrium (Chayovan *et al.,* 1983). Wilaipan (1990) found that salt content in commercial fish sauce produced in Thailand was 25%. Chin and Koehler (1983) reported that the very high concentration of salt used in the mixture during

fermentation might inhibit the growth of microorganisms that could decarboxylate free histidine to histamine.

5.1.3 Changes in histamine content

Histamine in foods is mainly formed by amino acid decarboxylases of bacteria (Rodtong *et al.,* 2005). During storage, the protein breakdown products, peptides and amino acids, represent precursors for amine formation used by spoilage microorganisms (Straub *et al.,* 1995; Straub *et al.,* 1994). The freshness of fish greatly influenced the formation of histamine during the fermentation process in the manufacture of fish sauce and the very high concentration of salt also inhibited the growth of microorganisms that could decarboxylate histidine to form histamine (Sanceda *et al.,* 1999). Generally, fish sauce produced from high fresh quality raw materials had lower histamine values than that produced from low quality raw materials (Brillantes *et al.,* 2002). Yongsawatdigul *et al.* (2004) reported that histamine was the predominant amine and gradually increased from 0.3 mg/100mL to 0.9 mg/100 mL in 52 weeks of fermentation at 40°C. However, spermine, spermidine, and tryptamine remained less than 10

mg/100 ml throughout the fermentation period. A small increase in histamine of fish sauce might be a consequence of the activity of histidine decarboxylase secreted by histamine-forming bacteria before the fermentation, rather than from halophilic bacteria during fermentation (Yongsawatdigul *et al.,* 2004).

5.1.4 Changes in pH

Due to different protein hydrolysis, the difference in pH probably resulted from the differences in free hydrogen ions, free amino acids, and amino acid of oligopeptides. The decrease in pH might be associated with organic acids, such as lactic acid, acetic acid produced by some microorganisms, especially lactic acid bacteria during fermentation. Conversely, the increase in pH might be caused by the formation of basic compounds. Ijon and Ohta (1996) reported that pH of bakasang (Indonesian fermented fish sauce) ranged from 5.95 to 6.50, whereas Aquerreta *et al.* (2001) found a pH range of 4.90-5.42 in garum (Greece fermented fish sauce). The pH of fish sauce from the fresh Pacific whiting fish flesh decreased gradually during

fermentation and reached 5.6 after 9 months (Tungkawachara *et al*., 2003). Lopetcharat and Park (2002) reported that the pH of Pacific whiting fish sauce fermented for 40 days ranged from 6.1 to 6.3. Chaveesuk *et al.* (1993) found that the final pH value of fish sauce produced from Atlantic herring (*Clupea harengus*) ranged from 5.82 to 5.85. The pH of garum increased during the fermentation process, probably as a consequence of the accumulation of basic compounds (Aquerreta *et al.,* 2001). Shih *et al.* (2003) found that the rise of pH was probably due to the increase of alkaline TVB during the fermentation period.

5.2 Physical changes

The Maillard reaction produced a variety of intermediate products and finally brown pigments (melanoidins) are formed (Van Boekel, 1998). Lertsiri *et al.* (2003) reported that free amino acids and reducing sugar generated during fish sauce fermentation contributed to the development of brown pigment via Maillard reaction. Fluorescent compounds are possible precursors of brown pigments (Labuza and Baisier, 1992). Lopetcharat and Park (2002) reported that the brown color of fish sauce made from Pacific whiting and surimi by-products was caused

by nonenzymatic browning reaction. Fish sauce produced from whole fish was darker and brown color development was faster than fish sauce produced from by-produced. Most of the nitrogenous compounds in fish sauce are free amino acids and small peptides, which contribute to brown color development. Even though reducing sugar content in fish is low, carbohydrate derivatives, such as glucose-6-phosphate and other substances present in the metabolic pathways, can also act as reactants to initiate the Maillard reaction (Kawashima and Yamanaka, 1996). Fish and soy sauce became darker with melanoidine produced by the Maillard reaction during storage (Lee *et al.,* 1997). Kim *et al.* (2004) found that the color L* value of salted and fermented anchovy sauce decreased during storage.

5.3 Sensorial changes

Aroma is a prime indicator of the quality of fish sauce (Shih *et al.,* 2003). Nitrogenous compounds from the supernatant liquor could have an effect on consumer acceptability: Other nitrogenous compounds are equally important in terms of the flavor and nutritional values.

The volatile acids were the most abundant group of volatile compound in fish sauce (Sanceda *et al.,* 2001). In general, fish spoilage increased the amount of volatile acids in fish liquid during fermentation. Volatile acid contents in the aerobically fermented fish sauce were significantly higher than in the anaerobically fermented fish sauce (Sanceda *et al.,* 2001). The differences in the aroma of fish sauce were thought to be due to the differences in the level of concentrations of the major acids (Shih *et al.,* 2003). Differences in the aroma of the commonly used fish sauce from different countries were attributed to the aroma characteristics of the major as well as the minor volatile compounds (Shih *et al.,* 2003).

Degradation of fish protein to free amino acid is primarily responsible for the delicious taste of fish sauce (Chayovan *et al.,* 1983). Various volatile compounds, including acids, carbonyls, nitrogen-containing compounds, and sulfur-containing compounds, are formed during fermentation and believed to be responsible for the distinct aroma of fish sauce (Fukami *et al.,* 2002).

Fish sauce produced from different raw materials and protocols might posses different sensorial characteristics. Tungkawachara *et al.* (2003) reported no significant difference

in overall sensory acceptance and flavor acceptance for all fish sauce produced from Pacific whiting (*Merluccius productus*) and Pacific whiting mixed with surimi by-product. However, fish sauce made from Pacific whiting mixed with surimi byproduct has lower color acceptance scores than commercial anchovy fish sauce. No significant difference in color acceptance was detected between Pacific whiting fish sauce and commercial anchovy fish sauce. Chaveesuk *et al.* (1993) found no difference in the color, aroma and flavor scores between the commercial fish sauce and fish sauce produced from Atlantic herring (*Clupea harengus*) with enzyme supplement. However, there was a significant difference between the color score of the enzyme supplemented fish sauce and the commercial fish sauce. Panelists preferred the clear, light brown color of the enzyme supplemented herring fish sauce than the darker color of commercial fish sauce. Nevertheless, commercial fish sauce had a slightly stronger flavor and aroma than the enzyme supplemented fish sauce.

5.4 Microbiological changes

Microorganisms play an essential role in fish sauce fermentation. Thongthai and Siriwongpairat (1990) demonstrated the presence of at least two bacterial populations. The major

population consisted of red extremely halophilic bacteria, identified as halobacteria. These reached maximum density in the liquor after 3 weeks and persisted throughout the fermentation period, whereas the other minor bacterial population was heterogenous and halotolerant or moderately halophilic.

Microbial counts decreased continuously when the fermentation time increased, possibly caused by high concentrations of salt and reduced pH (Jay 1996). Lopetcharat *et al.* (2001) reported that an increase in the number of halophilic microorganisms was observed at day 10 during Pacific whiting fish sauce fermentation and then decreased rapidly to an undetectable level at day 20. Three significant microorganism genera were identified in fish sauce from Pacific whiting*: Staphyloccus*, *Bacillus*, and *Micrococcus* (Lopetcharat and Park 2002). *Bacillus* type bacteria and *Staphyloccus* strain 109 were isolated from fish sauce and produced a measurable amount of volatile acids (Ijong and Otha 1996). Furthermore, *Staphyloccus*, *Micrococcus,* and *Coryneform* are commonly found in anchovy fish sauce (Sands and Crisan 1974). The microorganism should play an important role in the later stage of fermentation and the ripening

stage. Protein degradation by these microorganism leads to the production of volatile compounds from amino acids and small peptides (Lopetcharat *et al.,* 2001).

6. Factors affecting the quality of fish sauce

There are four major factors influencing fish sauce quality: fish species, salt, temperature, and time (Lopetcharat *et al.,* 2001). A certain aspect of fish sauce quality is also dependent on specific consumers. For example, budu has dark color and is preferred by Malaysian consumer, but not by those in Thailand (Lopetcharat and Park 2002).

6.1 Fish species

Fish sauce is mainly produced from anchovy (*Stolephorus spp*), mackerel (*Ristrelliger spp*.), and herring (*Clupea spp*.) (Wilaipan 1990). The type of fish used in manufacturing fish sauce, which varies from country to country, affects the nutritional quality of fish sauce, especially its nitrogen content. Thus, the different protein contents of anchovies and

sand lance are reflected by the different protein content of their respective fish sauce (Choi *et al.,* 1999). Minerals and vitamins present in fish contribute to the nutritive value of fish sauce. The nutrition composition of some fish used for fish sauce production is listed Table 2. Fish species also affects the type of proteins that serve as nutrients for microorganism and substrate for enzymes, both of which hydrolyze into small peptides and amino acid (Lopetcharat, 1999). Tungkawachara *et al.* (2003) found that fish sauce made from Pacific whiting whole fish and a mixture (1:1) of its byproducts showed a nonsignificant difference in overall acceptance, compared with commercial anchovy fish sauce.

Fish sauce can be made cheaply from various fish, which are not normally used for food (Gildberg, 2001). The capelin (*Mallotus villosus*) caught in the winter with a low content of digestive enzymes is not suitable for fish sauce production unless it is supplemented with proteolytic enzymes (Gildberg, 1988). Fish by–products, Arctic capelin and Atlantic cod intestines, can be utilized as raw materials for the production of high value fish sauce for human consumption (Gildberg, 2001). The proteases present in cod pyloric caeca are cold adapted enzymes, which accelerate tissue solubilization and give high fish sauce recovery. Oetterer *et al*.

(2003) reported that fish sauce can be produced from whole sardine. Raksakulthaiet *et al.* (1986) found that fish sauce can be made from male winter capelin if it is supplemented with 2.5% squid (*Illex illecebrosus*) pancreas containing a high content of cystein proteinases (Raksakulthai and Haard, 1992). Soyiri *et al.* (2003) used tuna processing waste (bone, fins scales and solf tissues) as raw materials for the production of fish sauce. The sauce produced was dark brown in color and had 16.0% sodium chloride, 18.0% crude protein, 36.6 % free amino nitrogen and pH was 4.4

Table1. Nutritional composition of three species of fish used for fish sauce production.

Nutrients	Unit	Different species of raw materials		
		Stolephorus spp. (Anchovy)	*Ristrelliger spp.* (Mackerel)	*Clupea spp.* (Herring)
Protein	g/100g	18.0	20.0	20.2
Fat	g/100g	0.3	6.7	4.3
Moisture	g/100g	80.5	72.0	74.4
Calcium	mg/100g	21.8	170	4.0
Phosphorus	mg/100g	21.1	60	175
Iron	mg/100g	1.7	11.9	2.0
Vitamin A	IU/100g	139	138	195
Vitamin B_1	mg/100g	0.02	0.03	0.12
Vitamin B_2	mg/100g	0.04	0.62	0.05
Niacin	mg/100g	0.6	9.2	3.00

Source: Wilaipan (1990).

6.2 Salt content

Salt is the second main ingredient in the fish sauce production. Salt controls the type of microorganisms and retards or kills some pathogenic microorganisms during fermentation. Sea salt is usually used by the fish sauce industry because of its availability. Both sea salt and rock salt are mainly composed of sodium chloride (NaCl). For Thai sea salt, sodium chloride is 88.26 ± 2.79%, while salt from other countries has a high NaCl content (97%). Mg^{2+}, Ca^{2+}, SO_4^{2-}, and other impurities, which retard the diffusion of NaCl in to the fish flesh (Wilaipan, 1990). Slow diffusion rate can accelerate spoilage. In addition, heavy metal ions contained in salt often increase the oxidation rate of fatty acids in fish oil, resulting in low quality fish sauce. Lowering water activity (Aw) reduces water for all metabolic activities causing a longer lag phase. Sodium (Na^+) and chloride (Cl^-) interrupt transferring acyl group in some bacteria. In a very high ionic environment, enzymes are easily denatured and inactivated. Tungkawachara *et al*. (2003) reported that the high salt concentration and high temperature (35 °C) resulted in denaturation and a decline of enzyme activity. The decrease of enzyme activity during

fermentation was also found by Gildberg (1992). Hight salt concentration (25%) prolonged fish sauce shelf-life but it inhibited peptidase activity and hence retarded protein hydrolysis (Gildberg et al., 1989; Sikorski *et al.,* 1995). However, salt reduction from 25% to 5-15% accelerated autolysis during fish sauce fermentation (Gildberg 1989; Sikorski *et al.,* 1995). In addition, fish guts or proteinases accelerated the autolysis of fish proteins, and reduced the autolysis time at a salt concentration < 20% (Dohmoto *et al.,* 2001). Furthermore, maintaining lower or higher temperatures than room temperature during the autolysis period regulated the growth of microrganism. The organoleptic test of the hydrolytic extracts prepared by addition digestive tract and reduced the autolysis time at a salt concentration < 20% showed that the odor of fish sauce was mild and the color was light, whereas the taste was sweet and somewhat bitter (Dohmoto *et al.,* 2001).

The fish to salt ratio is another factor affecting fish sauce quality. The concentrations of salt affect the function of various endogenous enzymes that plays an important role in protein degradation during fermentation. In different countries, the ratio of fish to salt (w/w) varied greatly depending on the type of fish sauce. In Japanese fish sauce (Shottsuru), the

ratio of fish to salt is about 5:1 (Beddow, 1985). Korean fish sauce (aekjeot) producer use a fish: salt ratio of 3:1 – 4:1 (Kim *et al.,* 1999). Nampla is made using fish/salt ratio of 1:1 to 5:1 ratio. Generally, the fish/salt ratio varies depending on the size of fish used in the production and the desired final product taste. At different salt concentrations, bacterial and enzymatic activities are changed, resulting in different flavors. Gildberg *et al.,* (1984) also observed that acceptable fish sauce flavor could be produced after 2 months from anchovies (*Stolephorus spp*) using a 5% salt concentration (low salt concentation) at pH 4 for the initial fermentation. However, inferior quality and bitter taste were problems in most cases when the process was accelerated (Sikorski *et al.,* 1995).

The chemical composition of salt also affects the type of microbiological flora during fermentation, which in turn affects the quality of fish sauce. The volatile acids in fish sauce with added KCl were higher than the controls. Sensory evaluation showed slight differences in odor between the samples with added KCl and the controls, but the differences did not affect the acceptability of the product (Sanceda *et al*., 2003).

6.3 Effect of time and temperature

In patis production, increasing temperature (45°C) and reducing salt concentration can reduce fermentation time (Lopetcharat *et al*., 2001). In general, the optimum temperature for fish sauce fermentation is between 35 and 45°C (Lopetcharat *et al*., 2001). However, Korean fish sauce is usually fermented at 20-25°C in order to maintain traditional taste and flavor. The effective enzymes in fermentation were heat stable and salt tolerant. Fermentation of fish sauce from Pacific whiting and by–products mixed with 25% salt at 50°C gave higher yields than at 35°C (Lopetcharat and Park, 2002). Total nitrogen content of whole fish fermented at 50°C reached the equivalent level of commercial fish sauce before 15 days, supporting the strong degradation effects of Pacific whiting enzymes at earlier stages.

By supplementing minced capelin with 5-10% enzyme-rich cod pyloric caeca, a good recovery of fish sauce protein (60%) from male Arctic capelin and Atlantic cod intestines was obtained after 6 months of storage (Gildberg, 2001). Although, the proteases present in cod pyloric caeca are cold adapted enzymes, a temperature of 26°C gave a higher fish sauce recovery than 21°C.

7. Formation and degradation of histamine and other biogenic amines in fish and fish sauce

7.1 Histamine and biogenic amine in fish

Scombroid fish belonging to the families Scombridae (e.g., tuna and mackeral) and Scomberesocidae (e.g., saury) are most commonly associated with histamine fish poisoning, but non-scombroid species (e.g., mahi-mahi, sardines, pilchards, anchovies, herring, marlin and blue fish) can also be involved. These species are characterized by having relatively high levels of histidine in their flesh (Taylor, 1986). Histidine levels vary from 1 g/kg in herring to as much as 15 g/kg in tuna (Ijomah *et al.,* 1992). Fresh fish contains negligible quantities of histamine, usually < 0.1 mg/100 mg (Frank *et al.,* 1985).

Histamine can be produced rapidly by bacterial decarboxylases in scombroid and other fish that have relatively high free histidine levels in their muscles when alive (Love, 1980). Post-mortem proteolysis liberates additional histidine from muscle protein, and explains why histamine can reach high concentrations without the formation of organoleptic (sensory) spoilage indicators (Sapin-Jaloustre and Sapin-Jaloutre, 1957). Eitenmiller *et al.* (1982) concluded that the

ready availability of free histidine in the muscle to act as both an inducer and substrate makes it an ideal environment for histamine formation.

The main bacteria responsible for histidine decarboxylation and histamine fish poisoning are members of the family *Enterobacteriaceae* (Taylor and Summer, 1986; Frank *et al*., 1985). Endogenous production of decarboxylase enzymes in fish muscle is insignificant when compared with the exogenous (bacterial) pathway (Rawles *et al.,* 1996). Spoilage, ammonia production and biogenic amine production by these bacteria are enhanced at elevated storage temperatures (Arnold *et al.,* 1980). Once a large bacterial population has been established, residual enzyme activity continues slowly at refrigeration temperatures, although bacterial growth ceases (Stratton and Taylor, 1991; Klausen and Huss, 1987). Histamine is also produced, but to a lesser extent, by bacteria that can grow at refrigeration temperatures (Okuzumi *et al.,* 1981).

Histamine formation is most often induced by high temperature abuse of fish after harvest, and the accumulated level is affected by the combination of time and temperature. Regardless of the food type, high amounts of biogenic amines have been reported during fermentation process and/or ripening (essentially fish, fermented sausage, cheese and fermented

alcohol beverages). The prevailing biogenic amines in fermented and/or ripening foodstuffs are histamine, tyramine, cadaverine, while fresh products only show significant amounts of the polyamines, spermidine and spermine. The production of biogenic amine in foodstuffs is a characteristic of several groups of microorganisms, which are able to decarboxylate amino acid, such as Enterobacteriaceae, *Pseudomonas* spp., Micrococcaceae, Enterococci and lactic acid bacteria (Giraffa, 2002; Silla Santos, 1996)

Histamine production showed different patterns at different temperatures, indicating the strong effect of storage temperature on histamine production. Capillas and Moral (2001) found that histamine, tyrosine, lysine, arginine, and ornithine, which can be decarboxylated and form biogenic amines, were present at different levels throughout the storage of hake in ice. The biogenic amines with the highest concentrations at the beginning of the storage were putrescine and spermidine, with levels of 3.08 and 2.71 mg/kg, respectively. Histamine, cadaverine and agmatine had very low initial levels. Tyramine was not detected at the beginning of storage. The levels of these biogenic amines, expect spermidine, in hake stored in ice increased progressively throughout storage (Capillas and Moral, 2001).

Histamine formation occurred very quickly in yellowfin tuna stored at 20 °C. The concentration of histamine became 10 times higher within 24 h of storage increasing from an initial concentration of 1.18 mg/100 g tuna to 11.14 mg/100 g tuna. The concentration reached a value of 67.53 mg/100 g tuna after 48 h of storage (Guizani *et al.,* 2005). Kim *et al.* (2001) observed only 50 ppm of histamine in the mackerel held at 25 °C for 24 h. Histamine content was found to be between 61 and 93 ppm in tuna held on deck at 15 - 33 °C (Ben–Gigirey *et al.,* 1998). Shakila *et al.* (2003) found 175 ppm of histamine in the mackerel held at 32 °C for 24 h. Veciana-Nogués *et al.* (1997) found that histamine increases were lower in samples kept at low temperatures than in samples kept at ambient temperature. However, there were increases in histamine and some other amines in some samples kept at 8-10 °C. Karacam *et al* (2002) reported that histamine in the salted anchovies depended on storage temperatures. While very low levels < 0.05 mg/100 g of histamine were found in the samples stored at low temperatures, very high levels were observed with the samples stored at ambient temperature depending on the time and salt concentrations. This result indicates that low temperature has a beneficial effect on the product safety. Rodtong *et al* (2005) found that accumulation of histamine in Indian anchovy

(*Stolephorus indicus*) was 1.9 mg/100 g after 15 days at ice storage, but it increased to 19.0 mg/100 g after 32 h at 15 °C. Histamine rapidly increased to 25.4 mg/100 g when stored at 35 °C for 8 h.

7.2 Histamine and biogenic amines in fish sauce

Fish sauce has been reported to contain histamine and biogenic amine differently. Yongsawatdigul *et al.* (2004) reported the formation of histamine and biogenic amines in fish sauce made from fresh and temperature-abused (left at 35 °C for 8 and 16 h) Indian anchovy (*Stolephorus indicus*). Histamine, cadaverine, putrescine, and tyramine were the predominant biogenic amines found in anchovy left at 35 °C for 16 h and its fish sauce. Changes of biogenic amines were subtle during the course of fermentation at room temperature (RT) and at 40 °C, Therefore, the main source of biogenic amines was associated with raw material, rather than with the fermentation process. Fish sauce prepared from temperature-abused anchovy contained less free histidine and arginine. Brillantes *et al* (2002) reported the formation of histamine in fish sauce made from *Stolephorus sp.* Histamine was formed both in the raw material

and during fermentation. It was speculated that histidine decarboxylase formed prior to fermentation produced histamine during fermentation.

7.3 Degradation of histamine and biogenic amine

Histamine and biogenic amine are degrade by two enzymes: diamine oxidase (histaminease; E C 1.4.3.6) and histamine *N* -methyltransferase (E C 2.1.1.8) (Schayer, 1959). Diamine oxidase catalyzes the oxidative deamination of histamine to imidazoleacetaldehyde. Histamine *N*-methyltransferase catalyzes the *N*-methylation of histamine to *N*-methyl histamine (Schayer, 1960).

Biogenic amines can be degraded by amine oxidases. Diamines, such as histamine and putrescine, are degraded by diamine oxidase (DAO). The activity of those enzymes is maximum under neutral to alkaline conditions, and oxygen is necessary for their action (Dapkevicius *et al.,* 2000; Roscoe and Kupfer, 1972). DAO can provide a means of controlling histamine accumulation during the first day of ensilage, while the pH is high enough and some

oxygen is still available within the product. Mono and diamine oxidases are present in higher organisms and were also described for bacteria (Isuhizuka *et al.,* 1993; Yamashita *et al.,* 1993).

The amount of histamine accumulated in the sample depended both on the bacteria production and decomposition of histamine (Fujii *et al.,* 1994). Hayashi (1970) found that once accumulated, histamine content may decrease and/or disappear possibly due to bacteria decomposition of histamine. Dapkevicius *et al.* (2000) found that lactic acid bacteria isolated from naturally fermented mackerel- sucrose (88:12, w/w) pastes could degrade histamine and biogenic amine. DAO is able to degrade histamine during the early stage of fish silage fermentation, when the pH is still above 4.5 and there is some oxygen left within the fish mass (Depkevicius *et al.,* 2000).

Furthermore, Leuschner *et al.* (1998) reported that 27 strains of lactic acid bacteria were able to degrade histamine and one strain is able to degrade tyramine. Martuscelli *et al*. (2000) reported that some strains of *Staphylococcus sylosus* isolated from fermented sausage were able to degrade histamine and biogenic amines.

Objectives

1. To characterize the proteinases in tuna internal organs and the changes during fermentation.
2. To study the influence of the storage conditions of tuna internal organs on the fermentation and characteristics of resulting fish sauce.
3. To investigate the possible acceleration process for fish sauce production from tuna internal organs and the effect of those processes on aging.