ABSTRACT OF THE DISSERTATION OF

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Title: Characterization of Biochemical, Functional Properties, and Market Potential of Pacific Whiting Fish Sauce

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Jae W. Park

Biochemical properties, functional properties, and market potential of Pacific whiting (PW) fish sauce were investigated. Biochemical properties of fish sauce made from whole fish (W) and a mixture (1:1) of whole fish and surimi byproducts (WB) were compared. Market potential was evaluated through phone interviews and consumer panelists. Proteolysis was primarily affected by cathepsin B-like and L-like enzymes. Acidic pH (4-5) with low salt concentration (15-20%) provided a greater degree of hydrolysis (DH), total nitrogen, and amino nitrogen content in PW fish sauce compared to the traditional process. The greatest Angiotensin Converting Enzyme (ACE) inhibition (96.8%) was found in samples fermented with 15% salt at pH 5.0 for 30 days. Anti-oxidative activity (AT) increased when fermentation continued and depended on fermentation pH. Peptides with MW <590 Da possibly played an important role in ACE inhibition. Consumer tests disclosed no significant difference in flavor liking and overall liking among fish sauce samples (W, WB, and commercial anchovy fish sauce).

Characterization of Biochemical, Functional Properties, and Market Potential of Pacific Whiting Fish Sauce

By

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Somjintana Tungkawachara, Author

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CONTRIBUTION OF AUTHORS

Prof. Jae W. Park assisted with data collection and writing of all chapters.

Prof. Yeung J. Choi was involved with some data collection of chapter 2 and 4.

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Chapter I

LITERATURE REVIEW

INTRODUCTION

Fish sauce has long been a traditional fermented fish product and is an important source of protein in Southeast Asia. It is also consumed in Europe and North America. Fish sauce is a liquid product with amber color and a salty, sweet taste. Typical examples of fish sauce are the nuoc-mam produced in Vietnam and Cambodia, nam-pla in Thailand, patis in the Philippines, uwoshoyu in Japan, and ngapi in Burma (Sikorski and Ruiter 1994).

Traditional fish sauce production is conducted using combined reactions of salting, enzyme hydrolysis, and bacterial fermentation (Saisithi 1994). Traditional fish sauce in Thailand is produced by storing a mixture of salt and minced anchovies (1:3) at tropical temperatures (30-35 °C) for 9 to 18 mo. As for raw materials, fresh anchovies (*Stolephourus spp.*) can produce high quality nam-pla, which consumers prefer for its typical flavor much better than that made from other fish, such as sardines, lizard fish, mackerel, etc. At present, mussels are also used as a raw material for nam-pla. Nam-pla can also be made from freshwater fish such as pla-soi

(*Cirrhina spp.*) either in the home or small industries, but only in limited quantities (Sikorski and others 1995).

The fermentation process is conducted through the proteolysis of enzymes from the viscera along with the function of salt-tolerant microorganisms (Sikorski and others 1995). Endogenous fish enzymes are primarily responsible for the degradation of muscle proteins during the fish sauce process, while bacteria play only a minor role because of the high salt content (Orejana and Liston 1982). Lopetcharat and others (2001) reported that there were five major factors influencing fish sauce quality: fish species, type of salt, the ratio of fish and salt, minor ingredients, and fermentation conditions.

To speed up protein solubilization during the fish sauce process, several methods have been proposed to reduce the production time. The first is to raise the fermentation temperature. The total production time can be reduced from 1 year to 2 months when the temperature is raised to 45 °C (Gildberg 1993) or 50 °C (Lopetcharat 1999). The second is addition of acid combined with reduced salt content. Some endogenous fish enzymes, such as pepsin (Gildberg and others 1984) and serine proteases (Cho and others 2000), are inhibited by salt. Thus, an initial phase of rapid autolysis is carried out at acidic pH (obtained by addition of hydrochloric acid) and low salt concentration. After the initial phase (5 days), salt may be added to the normal level (25% w/w) to obtain the traditional salty fish sauce taste. The third is initial alkalization at low salt concentration (obtained by addition of sodium hydroxide solution). The forth is the addition of enzyme-rich components from plants,

such as unripe papaya (papain), pineapple stems (bromelain), and fig (ficin) (Beddows and Ardeshir 1979), or the addition of enzyme-rich components from animals, such as squid hepatopancreas tissue (Haard and Simpson 1994), cod intestines, and squid pancreas (Gildberg 2000) or the addition of exogenous commercial enzymes, such as Neutrase and fungal protease (Aquerreta and others 2001).

When fish sauce was made from cod viscera, mixed with salt (25% w/w) and stored at 22-27 °C, only a 25 day fermentation period was needed (Gildberg and Xian-Quan 1994). The downside of the accelerated process, however, is the possiblity of inferior flavor, either too weak taste or too bitter. Bitterness is caused by the formation of peptides containing a bulky hydrophobic group toward their C-terminal (Raksakulthai and others 1986). The intensity of bitterness depends on the degree of hydrolysis and the specificity of the protease. The addition of enzyme-rich components from animals appeared to have positive results including sensory acceptability (Raksakulthai and others 1986).

BIOCHEMICAL PROPERTIES

The major change during the fish sauce fermentation period is the conversion of proteins to small peptides and free amino acids. Total nitrogen (total N) content is used as an indicator to determine the grade and price of fish sauce in Thailand (Lopetcharat 1999). Fish sauce contains a high concentration of NaCl salt (25-30%)

and nitrogen based compounds, such as volatile base nitrogen and trimethylamine. It has a pH between 5.1-6.7, and low acidity (0.1-2.7%) (Itoh and others 1993). Lopetcharat and others (2001) compared the chemical compositions of fish sauce and soy sauce (Table 1.1).

Many vitamins and minerals are also found in fish sauce. Fish sauce is a good source of vitamin B_1, B_2, and minerals such as sodium (Na), calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), and phosphorus (P) (Wilaipun 1990).

Table 1.1: Chemical composition of fish and soy sauce

Composition	Fish sauce	Soy sauce
pH	5.3-6.7	4.7-4.9
NaCl (g/dL)	22.5-29.9	16-18
Total amino acids (g/dL)	2.9-7.7	5.5-7.8
Glutamic acid (g/dL)	0.38-1.32	0.9-1.3
Total organic acids (g/dL)	0.21-2.33	1.4-2.1
Acetic acid (g/dL)	0.0-2.0	0.1-0.3
Lactic acid (g/dL)	0.06-0.48	1.2-1.6
Succinic acid (g/dL)	0.02-0.18	0.04-0.05
Reducing sugar (g/dL)	trace	1.0-3.0
Alcohol (g/dL)	trace	0.5-2.0

Adapted from Lopetcharat and others (2001)

Ammonia and trimethyl amine are also found in fish sauce. Lactic acid, acetic acid, and pyroglutamic acid are generally found as well, including citric and succinic acid. They are generally liberated from substrates and then controlled by bacteria. Fish sauce with high concentrations of isovaleic and butyric acids delivers a pungent odor (Itoh and others 1993). Low molecular weight volatile fatty acids (VFA), in particular acetic acid, ethanolic, propionic, n-butyric, and isovaleric acids have been identified as contributing to the cheesy note of fish sauce (Sanceda and others 1996). These VFA were produced from the autoxidation of polyunsaturated acids and by bacterial action on amino acids, which are used as a carbon source. For example, ethanoic and n-butanoic acids can be produced by the oxidation of glutamate and ethanoic acids from tryptophan (Lopetcharat and others 2001), isobutyric acid can be produced by the oxidation of valine, and isovaleric acid from leucine (Sanceda and others 2001). Different amino acids often contribute to different flavors. For example, glutamic acid produces meaty flavor and aroma. Lopetcharat and others (2001) reports the amino acid composition of fish sauces from various countries (Table 1.2).

Amino acids and peptides control the flavor of fish sauce. Glutamic acid in particular contributes a mild flavor and meaty aroma to the product (Itoh and others 1993). The characteristic aroma and taste are primarily derived from protein and lipid degradation by autolytic and bacterial enzymes during fermentation (Itoh and others 1993). Cha and Cadwallader (1995) reported that the major volatile compounds in anchovy products are composed of aldehydes, ketones, alcohols, esters,

Table 1.2: Amino acid compositions (mg/100 mL) of fish sauce

Amino acid	China	Korea	Philippines	Thailand	Vietnam
Taurine	124.5	207.2	211.6	102.1	169.0
Aspartic acid	362.9	28.0	415.7	609.7	430.3
Threonine	222.2	90.7	298.7	379.4	534.6
Serine	138.9	ND	274.3	260.4	393.3
Glutamic acid	823.1	1803.0	944.1	1205.1	3031.9
Proline	86.4	321.7	143.8	178.7	193.0
Glycine	186.5	591.9	323.0	268.3	232.6
Alanine	437.8	1234.0	506.9	670.8	328.9
Cysteine	115.2	287.0	ND	ND	38.1
Valine	338.0	681.1	358.7	476.1	350.1
Methionine	159.5	133.7	217.3	167.0	294.6
Isoleucine	282.5	720.2	355.7	298.4	511.4
Leucine	375.4	1217.7	466.1	343.6	895.1
Tyrosine	38.4	25.0	58.4	37.2	44.9
Phenylalanine	176.2	65.5	201.5	226.7	129.5
Histidine	99.8	341.3	222.8	269.7	307.3
Lysine	667.7	1058.8	696.4	956.5	634.0
Arginine	19.0	57.8	29.9	6.8	14.9
Total	4654.0	8864.6	5724.9	6456.5	8533.5

Adapted from Lopetcharat and others (2001)

aromatics, nitrogen, and sulfur-containing compounds, including lipid-derived components such as aldehydes, alcohols, and esters.

In nature, fish contain highly unsaturated fatty acids so that it easily induces lipid oxidation. However, anaerobic fermentation conditions in the presence of NaCl prevent fat oxidation. The brine also constitutes good protection against excessive lipid oxidation. Since fish enzymes and microorganisms compete with lipids for oxygen, at low oxygen concentrations, lipid oxidation would be reduced but not eliminated (Wheaton and Lawson 1985).

Lipoxygenase enzymes are important in initiating lipid peroxidation in anchovy but other enzyme systems, such as myoloperoxidase of blood leucocytes may also be active, especially after salting. After evisceration, blood and other organic matter are spread over the surface, which may activate the peroxidase of blood leucocytes in the presence of NaCl and O_2 (Kanner and Kinsella 1983).

Histamine content in fish sauce vary widely from 100-1000 ppm (Brillantes and Samosorn 2001). Histamine content in fish sauce was due to the combination of histamine in the raw material and the presence of histidine decarboxylase that can continue to convert histidine to histamine even after the fish is mixed with salt. Histidine decarboxylase enzymes were produced from histamine-forming bacteria prior to the fermentation stage (Brillantes and others 2002).

Cho and others (2000) found that protein hydrolysis in salted anchovy was inhibited at salt concentrations higher than 25% at 5 °C. Reducing salt content,

therefore, was one of the recommended methods in order to accelerate the fish sauce process (Sikorski and others 1995). In addition, Beddows and Ardeshir (1979) suggested acid hydrolysis preparation to increase the rate of fish sauce production. They reported that the adjustment of pH and salt to 2.0 and 10% or 3.0 and 15%, respectively, before fermenting for 6 days at 30 °C gave a high degree of protein hydrolysis, resulting in nitrogen compounds similar to traditional fish sauce. However, Beddows and Ardeshir (1979) observed the precipitation of calcium phosphate when the pH of fish sauce pretreated with acid hydrolysis was adjusted to 5.65, which is the typical pH of naturally fermented fish sauce. Gildberg and others (1984) reported that the pH adjustment to 4.0 for anchovy fish sauce with 5-10% salt concentration resulted in acceptable flavor after fermenting at 40 °C for 2 mo. However, low salt concentration possibly controls the product's shelf life. A minimum of 15% salt concentration is required to prevent the growth of putrefactive bacteria (Putro 1993).

Chymotrypsin and Trypsin

Chymotrypsin and trypsin are serine proteinases and they have been described as a group of endoproteinases with a serine residue in their catalytic site (Simpson 2000). Their catalytic sites contain a serine residue, together with an imidazole group and an aspartyl carboxyl group. Trypsin has a very narrow specificity for the peptide bonds on the carboxyl side of arginine and lysine. Chymotrypsin 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 (Simpson 2000). Trypsin and chymotrypsin from marine digestive organs are found in the pancreatic tissues, pyloric ceca, and intestines of animals. They are active at neutral to slightly alkaline pH and unstable and/or inactive under acid conditions (Simpson 2000).

Chymotrypsins have been isolated and characterized from marine animals such as anchovy (*Engraulis japonica*) (Heu and others 1995), Atlantic cod (*Gadus morhua*) (Heu and others 1995; Asgiersson and Bjarnasson 1991), capelin (*Mallotus villosus*) (Kalac 1978), herring (*Clupeas harengus*) (Kalac 1978), rainbow trout (*Oncorhynchus mykiss*)(Kristjansson and Nielson 1992), and spiny dogfish (*Squalus acanthias*)(Racicot 1984). In general, chymotrysins have molecular weights ranging between 25 to 28 kDa. They are most active within the pH range of 7.5 to 8.5, and are most stable at around pH 9.0 (Kristjansson and Nielson 1992). Chymotrypsins from marine animals have a higher catalytic activity and hydrolyzed more peptide bonds in various protein substrates (casein, collagen, and bovine serum albumin) at subdenaturation temperatures than mammalian chymotrypsins. In addition, they were more heat-labile than mammalian chymotrypsins (Asgiersson and Bjarnasson 1991). Dogfish chymotrypsin was more active toward soy protein isolate from 5 to 35 °C than bovine chymotrypsin (Ramakrishna and others 1987). Chymotrypsins from herring and capelin exhibited greater hydrolytic activities toward the synthetic substrate BTEE than the bovine enzyme (Kalac 1978).

Trypsin is a major member of the serine proteases that consists of a single peptide chain with a typical molecular weight of 24 kDa (Torrissen and Male 2000).

It is synthesized in the pancreas and secreted as an inactive precursor, trypsinogen. Trypsin is a key enzyme for activating all other pancreatic zymogens. Several isoforms of trypsin have been reported in fishes (Torrissen and Male 2000). These isozymes possess differences in their catalytic efficiency (k_{cat}/K_m) and the distribution of charged amino acids appears to be responsible for different substrate binding preferences (Torrissen and Male 2000). Trypsins have been isolated from the pancreatic tissues, pyloric ceca, and intestines of several marine animals: anchovy (Engraulis encrasicholus) (Martinez and others 1988), Greenland cod (Gadus ogac) (Simpson and Haard 1984a; Simpson and Haard 1984b), Atlantic cod (Gadus morhua) (Simpson and others 1989; Gildberg 1988), capelin (Mallotus villosus) (Hjelmeland and Raa 1982), mullet (Mugil cephalus)(Guizani and others 1991), sardine (Sardinos melanostica)(Noda and Murakami 1981), catfish (Parasilurus asotus)(Yoshinaka and others 1984), starfish (Chen and others 1978), and cunner (Tautogolabrus adspersus)(Simpson and others 1989; Simpson and Haard 1987).

Trypsins from marine animals resemble mammalian trypsins with respect to their molecular size (22.5-24 kDa), amino acid composition, and sensitivity to inhibitors such as aprotinin, soybean trypsin inhibitor (SBTI), phenylmethylsulfonyl fluoride (PMSF), and N-tosyl-L-lysine chloromethyl ketone (TLCK). Their pH optima for the hydrolysis of various substrates have been reported to range from 7.5-10.0, while their temperature optima for hydrolysis of those substrates ranged from 35 to 45 °C (Vecchi and Coppes 1996). Trypsins from marine animals tend to be more

stable at alkaline pH, but are unstable at acid pH, unlike mammalian trypsins that are most stable at acid pH (Simpson 2000).

FUNCTIONAL PROPERTIES

Angiotensin-I Converting Enzyme (ACE) Inhibition

The angiotensin I-converting enzyme (EC 3.4.15.1 ACE) catalyzes the hydrolysis of decapeptide (angiotensin I) to the octapeptide (angiotensin II) by hydrolytic removal of the C-terminal dipeptide, histidylleucine (Cushman and Cheung 1971). The ACE is primarily responsible for the conversion *in vivo* of circulating inactive angiotensin I to a potent vasoconstrictor, angiotensin II and inactivates the vasodilator bradykinin (Matsui and others 1992). This enzyme plays an important role in the rennin-angiotensin system to regulate both the arterial blood pressure and salt/water balance (Laragh and others 1972). Thus, the inhibition of ACE could be of enormous value for the prevention of hypertension (Matsui and others 1992). In fact some commercial ACE inhibitors such as captopril (D-3-mercapto-2-methylpropyl-L-proline) have been used for hypertension therapy (Okamoto and others 1995a).

Recently, inhibitory action of food components on ACE has drawn much attention in tempting to prevent hypertension. Okamoto and others (1995a) surveyed the ACE inhibitory effect of various fermented foods such as fish sauce, mirin, sake,

soy sauce, natto, cheese, and etc. They reported that amongst the liquid fermented products tested, fish sauce and soy sauce showed the strongest ACE inhibitory action.

Short chain peptides and organic compounds contained in fermented products exhibited inhibitory action on ACE (Kinoshita and others 1993; Matsui and others 1993; Okamoto and others 1995b). Kinoshita and others (1993) found that the major part of the ACE inhibitory action in soy sauce was nicotianamine. Okamoto and others (1995b) fractionated fish sauce with ethyl alcohol and the supernatant fraction and orally administered at a dose of 166 mg/kg body weight (BW) to spontaneously hypertensive rats. They found that it significantly reduced the blood pressure in vivo.

Similar positive results were also found when they tested the isolated peptides from fish sauce (at dose of 200 mg/kg BW). They concluded that the strong ACE inhibitory activity of fish sauce was caused, not by a single compound like that in soy sauce, but by the combined action of many substances. Okamoto and others (1995b) found that sardine fish sauce processed by the traditional method showed 2 times stronger inhibitory action than that processed by the enzymatic method. They also found that fish sauce made from various raw materials (salmon, sardine, and anchovy) showed different ACE inhibitory action.

Anti-Oxidative Property

Oxidation leads to deterioration of lipids, and secondary reactions between lipid oxidation products and proteins may cause browning, loss of protein quality, and

impaired organoleptic quality (Karel and others 1966). Antioxidants scavenge free radicals and reactive oxygen species and can be extremely important in inhibiting oxidative mechanisms that lead to chronic diseases (Martinez and others 2002). The most widely used antioxidants, tert-butyl-4-hydroxyanisol (BHA), and tert-butyl-4-hydroxytoluene (BHT), however, are suspected of causing liver damage (Osawa and Namiki 1981).

Peptides and amino acids also exhibit the anti-oxidative activity in a liquid system. They could also be important for the removal of toxic products of lipid oxidation in vivo (Decker and others 2000). Food components, though, also exhibit anti-oxidative properties and thus, inhibit cancer cells in vitro. Some examples include, mustard leaf kimchi (Lim and others 2000) and common beans (*Phaseolus vulgaris*) (Martinez and others 2002). In addition, peptides and amino acids exhibit anti-oxidative activity in a liquid system. Foods containing peptides and amino acids include, soy protein hydrolysate, kimchi, and gelatin hydrolysate (Chen and others 1995; Choi and others 2001; Kim and others 2001) The proposed anti-oxidative mechanisms of peptides include interference with metal ions, which catalyze breakdown of hydroperoxides, a simple pH effect due to the buffering capacity, scavenging of singlet oxygen, scavenging of hydroxyl radicals and peroxide radicals, and quenching of secondary lipid oxidation products (Yamashoji and Kajimoto 1980; Aruoma and others 1989; Kansri and others 1997; Zhou and Decker 1999; Carlsen and others 2002).

The anti-oxidative activity of the hydrolysates was likely dependent on the characteristic amino acid sequences of the peptides derived from protein (Chen and others 1995). The anti-oxidative effect of hydrolysate from sardine myofibril protein depended mainly on the amino acid composition and molecular size, but not on the kind of terminal amino acid residues (Hatate and others 1990). In contrast Chen and others (1996) found that the synthetic peptides (Leu-Leu-Pro-His-His) deleted the C-terminal His and therefore, decreased the anti-oxidative activity, whereas the deletion of the N-terminal Leu had no effect. The peptide structures that have potential anti-oxidative activity of pepsin digestion from prawn muscle were identified as Ile-Lys-Lys, Phe-Lys-Lys, and Phe-Ile-Lys-Lys (Suetsuna 2000). The purified peptide, containing 16 amino acid residues from gelatin hydrolysate of Alaska Pollock skin, was a natural antioxidant that also has potentential anti-oxidative activity (Kim and others 2001).

Yamaguchi and others (1975) studied the anti-oxidative activities of various dipeptides on linoleic acid and found that anti-oxidative activity of the dipeptides varied with the position of amino acid in the peptide. They found that dipeptides consisting of L-tryptophan and L-tyrosine at the N-terminal amino acid showed more inhibitive activities on the oxidation of linoleic acid than those of dipeptides consisting of the respective amino acids at the C-terminal (Yamaguchi and others 1975). In addition, dipeptides showed greater anti-oxidative activities than the constituent amino acid mixtures in an aqueous system (Chan and Decker 1994).

Several amino acids, such as metionine, histidine, alanine, and lysine, are generally accepted as antioxidants, in some cases, in spite of their pro-oxidative effects. They reduced oxygen absorption of linoleate by as much as 50-80%. Each amino acid had an optimum concentration for the anti-oxidative activity and at high concentrations showed an activity inversion, becoming pro-oxidative rather than anti-oxidative in its action (Marcuse 1961; Karel and others 1966). Histidine, lysine, b-amino-n-butyric acid, cysteine, and e-amino-n-caproic acid showed the greatest anti-oxidative activity in the early stages of autoxidation. These amino acids could prolong the induction period and affect the initial rate of oxidation (Karel and others 1966). Wade and Tucker (1998) reported that free histidine, histidine containing peptides such as carnosine, and histidine residues in the proteins, scavenged both the hydroxyl radical and singlet oxygen, a toxic oxygen species. The anti-oxidative activity of amino acids depends on pH, the presence of other antioxidants or synergists, and the state of oxidation of the linoleic acid (Marcuse 1961).

MICROBIOLOGICAL PROPERTIES

Fish sauce is made without starter cultures and relies on salt and enzymes to achieve the desired decomposition and preservative action. In addition to the chemical composition of fish, microorganisms in fish are also important to the quality of fish sauce. Microorganisms vary depending upon season, place, transportation,

species, storage, and catching methods. Microorganisms found in fish and seafood are shown in Table 1.3 (Lopetcharat and others 2001).

Table 1.3: Genera of bacteria most frequently associated in fish and seafood

Genus	Gram reaction	Frequency
Acinetobacter	-	x[a]
Aeromonas	-	x
Alcaligenes	-	x
Bacillus	+	x
Corynebacterium	+	x
Enterobacter	-	x
Enterococcus	+	x
Escherichia	-	x
Flavobacterium	-	x
Lactobacillus	+	x
Listeria	+	x
Microbacterium	+	x
Moraxella	+	x
Psychrobacter	-	x
Shewanella	-	xx[b]
Vibrio	-	x
Pseudomonas	-	xx

Adapted from Lopetcharat and others (2001)

[a]X indicates known to occur
[b]XX indicates most frequently reported

The microbial flora in fish sauce is composed of gram-positive mesophilic, *Micrococcus, Bacillus, Sarcina, Leuconostoc,* and *Brevibacterium* genera with some gram negative *Pseudomonas* spp. and *Flavobacterium* spp (Sikorski and others 1995). The microbial flora of these products is derived from the fish by the selective action of salt, or from the salt or brine (Hobbs 1987).

Salt is the second main ingredient in fish sauce production. It controls the type of microorganisms and retards or kills some pathogenic microbes during fermentation (Lopetcharat and others 2001). The gram-negative flora of freshly caught fish are not halotolerant and are, for the most part, replaced by halophilic and halotolerant gram-positive Micrococci, Coryneforms and lactic acid bacteria and to a lesser degree by yeasts and molds (Hobbs 1987).

Fish sauce is a kind of fermentative fishery product. From a microbiological point of view the preservative action results from a reduction of water activity, a reduction of pH and the combination of these factors (Sikorski and others 1995). Wheaton and Lawson (1985) concluded that as the fish salt content increased above 1%, bacteria associated with spoilage of fresh fish were increasingly stressed. At salt concentrations of 6 to 8%, most of these bacteria would die or at least stop growing. A second large group of bacteria and molds are adapted to grow well at salt contents of 6 to 8% up to 10 to 13%. Above 12 to 13% this second group would tend to die or at least stop growing and a third group, called the extreme halophiles begin to grow

(Wheaton and Lawson 1985). Fish sauce, however, contains very high concentration of salt (25-30% w/w), therefore, all pathogens are inhibited.

Fish sauce, though, can be spoiled by halophillic bacteria and halophillic molds. Nevertheless, some bacterial growth is essential for production of the characteristic odors and flavors that are generally produced by microbial action on the free amino acids (Hobbs 1987). The precise composition of these odors and flavors though has not been determined. However, amongst the compounds responsible are low molecular weight fatty acids and amines (Hobbs 1987). These are produced from amino acids by bacterial action which, if allowed to continue, results in spoilage (Hobbs 1987).

Itoh and others (1993) reported a list of bacteria involved in the fish sauce process. During fermentation, there are *Bacillus coagulans*, *B. megaterium* and *B. subtilis*, *B. licheniformis*, *M. colpogenes, S. epidermidis*, *M. varians* and *S. saprophyticus*. The heterofermentative lactic acid producing bacteria were identified as *Peptococcus anaeobius, Staphylococcus sprophyticus* and *Micrococcus varians*. The homofer-mentative lactic acid bacteria were identified as *Pediococcus halophilus*. The pink colored halophilic bacteria were isolated as *Paracoccus halodenitrificans* (Itoh and others 1993). There were no heat resistant bacteria and gram-negative bacteria in fish sauce but there were lactobacillus, osmophilic yeasts, and osmophilic molds (Itoh and others 1993).

Herrero and others (1999) found that during ripening of anchovy fish sauce in 19% salt concentration for 2 weeks, the psychrotrophic bacteria, moderately

halophilic bacteria, Enterobacteria, and Enterococci counts decreased. However, the extremely halophilic counts showed an increased trend during ripening. They suggested that due to high salt concentration, the water activity (a_w) was, therefore, more suitable for growth of most halophilic bacteria (Herrero and others 1999). Sanceda (1996), on the other hand, found some fish sauce containing low content of histamine and explained that the very high concentration of salt might inhibit the growth of microorganisms that could decarboxylate free histidine to histamine during fermentation. Unless the salt content of the raw material is raised rapidly during the initial stages, changes can occur as a result of growth of the normal gram-negative spoilage bacteria before the water activity reaches an inhibitory level (Sanceda 1996).

Yeast can cause a fruity, musty taint and the obligate anaerobic, halophilic gram-negative rods produce off-flavors in products (Knochel, 1993). When the process has been carried out properly, except the products are stored at too high temperature, two kinds of spoilage by halophilic organisms often occur: a condition known as "Pink" caused by halophilic bacteria of the genera Halococcus and Halobacterium; and a condition known as "dun" or "mite", caused by halophilic molds of the groups Sporendonema and Oospora (Hobbs 1987). The pink condition first appears as a delicate pink slime on the surface of the product. This gradually spreads and the fish are degraded by the active proteolytic enzymes produced by the bacteria. The bacteria are strictly aerobic, obligate halophiles; they will grow in saturated brine, with an optimum growth temperature of 37 °C or higher, a minimum

growth temperature of 5 °C and grow between pH 6.0 and 10.0 (Hobbs 1987). These bacteria originate primarily from solar salts. Control is therefore readily achieved by using mined or manufactured salt, and frequent cleaning of the processing area with fresh water (Hobbs 1987).

CONSUMER LIKING

Sensory attributes of quality guide the consumer in his selection of foods. Such attributes are measured by the processor to determine consumer preference in order to manufacture an acceptable product with maximum production economy (Pigott and others 1988; Zeuthen and others 1990). The selection, acceptance, and digestibility of a food are largely determined by its sensory properties. Evaluation of sensory properties, is, however, affected by personal preference, which is influenced by factors ranging from the caprices of fashion to the prevalence of dentures; social, cultural, and religious patterns; psychological factors; variations in climate and in the general physical status of the individual; availability; and nutritional education (Amerine and others 1965; Zeuthen and others 1990). To minimize the effects of such factors, different procedures for sensory evaluation have been devised and the results are evaluated by statistical methods. Large consumer groups are generally used to determine consumer reaction. Highly trained experts are employed for evaluating small differences in high-quality foods (Pomeranz and Meloan 1994).

Meilgaard and others (1999) stated that a consumer test involves 100 to 500 target consumers divided over three or four cities. Potential respondents are screened

by phone or in a shopping mall. Results are calculated in the form of preference scores overall and for various subgroups. Study designs need to be carefully tailored to the expected consumer group. The most effective tests for preference or acceptance are based on carefully designed test protocols run among selected subjects with representative products. The choice of test protocol and subjects is based on the project objective. The reasons for conducting consumer tests usually fall into one of the following categories: product maintenance, product improvement/ optimization, new product development, assessment of market potential, product category review, or support for advertising claims (Meilgaard and others 1999).

Chapter 2

BIOCHEMICAL PROPERTIES AND CONSUMER LIKING OF PACIFIC WHITING FISH SAUCE

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ABSTRACT

Biochemical characteristics of fish sauce made from Pacific whiting whole fish and a mixture (1:1) of its byproducts were investigated at 0, 1, 3, and 9 mo. As fermentation time extended, the degree of hydrolysis, total nitrogen content, amino nitrogen content, and hypoxanthine content increased, while inosine content, moisture content, and pH decreased. Degree of hydrolysis was significantly different at 9 mo of fermentation. All cathepsin activities were negligible after 3 mo. Consumer test showed a nonsignificant difference in overall liking between our samples and commercial anchovy fish sauce ($P>0.05$). This study demonstrated that surimi byproducts can be utilized as raw material for fish sauce.

Key words: Fish sauce, Pacific whiting, Byproducts, Consumer test, Cathepsin activity

INTRODUCTION

Fish sauce is a clear liquid condiment, with an amber color and mild cheesy/salty flavor commonly used as a flavor enhancer or salt replacement in various food preparations. Fish sauce from anchovies is produced by storing a mixture of salt and minced fish (1:3) at ambient temperatures. Fermentation is conducted through the proteolysis of enzymes from the viscera along with the function of salt-tolerant microorganisms (Uyenco and others 1953; Sikorski and others 1995). Endogenous fish enzymes are primarily responsible for the degradation of muscle proteins during the fish sauce manufacturing process (Orejana and Liston 1982). During storage, the enzymes slowly hydrolyze the fish proteins (Sikorski and others 1995). Gildberg (2001) reported the utilization of minced capelin with 5-10 % enzyme-rich (trypsin and chymotrypsin) cod intestines and a fish sauce product was obtained after 6 mo of fermentation. In Thailand, which produces the majority of commercial fish sauce, fish sauce is manufactured through fermentation at ambient temperatures (30-35 °C) for 12 to 18 mo.

Pacific whiting (*Merluccius productus*), which is abundant off the Pacific Northwest coast, was not commercially utilized until 1991 due to proteolytic enzymes that cause texture softening. An and others (1994) reported that cathepsin B was the most active cysteine protease in Pacific whiting fish fillets. During fish sauce fermentation, cysteine proteases were mainly responsible for the degradation of proteins in Pacific whiting byproducts, while serine proteases and trypsin-like enzymes had a minor role in hydrolyzing byproducts (Lopetcharat 1999). Lopetcharat

and Park (2002) studied the feasibility of fish sauce production from Pacific whiting for the first time. However, a consumer liking test for fish sauce from Pacific whiting was not done.

Our objective was to evaluate the biochemical properties and consumer liking of fish sauce made from Pacific whiting and a mixture of surimi byproducts from Pacific whiting. Free amino acid contents and the changes of the nucleotides (inosine and hypoxanthine contents) at different fermentation times were also investigated.

MATERIALS AND METHODS

Chemicals

All chemicals, except salt, used in the biochemical experiments were reagent grade. Sodium phosphate dibasic (Na_2HPO_4) was from Matheson Coleman & Bell (Norwood, OH). Sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$) was from Mallinckrodt Chemical Inc. (St. Louis, MO). Trinitrobenzenesulfonic acid (TNBS), sodium sulfite, L-leucine, uric acid, ammonium sulfate $(NH_4)_2SO_4$, iodoacetate, aminomethylcoumarine, potassium sulfate (K_2SO_4), mercuric oxide (HgO), and nucleoside phosphorylase (EC 2.4.2.1, N-8264) were purchased from Sigma Chemical Co. (St. Louis, MO). Sulfuric acid (H_2SO_4), sodium hydroxide (NaOH), trichloroacetic acid (TCA), perchloric acid (PCA), potassium hydroxide (KOH), and hydrochloric acid (HCl) were purchased from Fisher Scientific (Pittsburgh, PA). Sodium thiosulfate ($Na_2S_2O_3 \cdot 5\ H_2O$) was purchased from EM Science (Gibbstown,

NJ). Adenosine deaminase and xanthine oxidase were purchased from Roche Diagnostics (Mannheim, Germany).

Raw materials

Pacific whiting (*Merluccius productus*) was harvested off the Oregon coast in August 2000, kept in a storage tank with champagne ice before off-loading at a local seafood processing plant. Fresh fish (more than 100 fish) and surimi byproducts including heads, guts, skins, and bones were randomly selected, stored on ice, and transported to the Oregon State University Seafood Laboratory.

Preparation of fish sauce

Two sets of samples were ground: W for whole fish and WB for a mixture (1:1) of whole fish and surimi byproducts. Ground samples (W and WB) were homogeneously mixed with table salt at 3:1, placed in polyethylene bottles closed loosely with a lid, and kept at 35 °C.

Fermented fish mince was filtered using 8-fold cheesecloth followed by filter papers (Whatman No. 41 and No.1) with vacuum at 0, 1, 3, and 9 mo. The filtrate (liquid portion) was subjected to biochemical analysis.

Degree of hydrolysis

An aliquot of 0.75 mL of 20% trichloroacetic acid (TCA) was added to 0.75 mL fish sauce, for a final concentration of 10% TCA. After centrifuging at 10,000 x g using an Eppendorf centrifuge (Model No. 5415C, Brinkmann Instruments, Inc., Westbury, NY), the supernatant was analyzed for TCA-soluble nitrogen by the micro-Kjeldahl method (AOAC 1995). Degree of hydrolysis was calculated according to the method previously described by Hoyle and Merritt (1994): DH = [(10% TCA-soluble N in sample)/ total N in filtered fish sauce] x 100.

Activity of cathepsin L-like, B-like, and H-like enzymes

Fish sauce was fractionated by ammonium sulfate 30-70 % and dialyzed with 5 X 500 mL of deionized water at 4 °C for 12-15 h. Dialysis membrane sack had molecular weight cut off of 12-14 kDa (Spectra/POR, regenerated cellulose, Fisher Scientific, Pittsburg, PA, USA). Cathepsin activities were determined according to the method of Barrett and Kirschke (1981). Substrates for cathepsin L, B, and H were 20 μM of N-CBZ-Phe-Arg-7-amido-4-methylcoumarin, N-CBZ-Arg-Arg-7-amido-4-methylcoumarine, and L-Arg-7-amido-4-methylcoumarine, respectively. Optimum pH and temperature were used for cathepsin L-like (at 55 °C, pH 5.5), B-like (30 °C, pH 6.0), and H-like (20 °C, pH 6.8) activity. An and others (1994) determined the cathepsin activities in crude extract of Pacific whiting using the method of Barrett and Kirschke (1981). They found that the optimum temperature of cathepsin L-like, B-like, and H-like were 55 °C, 20-37 °C, and 20 °C, respectively. The optimum pH of cathepsin L, B, and H, were 5.5-6.0, 6.0, and 6.5-6.8, respectively (Kirschke and

others 1998). Concentration of aminomethylcoumarine was observed using a luminescence spectrophotometer (Model No. LS 50B: Perkin Elmer, Norwalk, CT) at an excitation wavelength of 370 nm and emission wavelength of 460 nm. For the blank, 5 mM iodoacetate was added before crude extract.

One unit of enzyme activity was defined as 1 μmole of aminomethyl-coumarine released per min for cathepsin L-like at 55°C/pH 5.5, cathepsin B-like at 30°C/pH 6.0, and cathepsin H-like at 20°C/pH 6.8.

Determination of pH, moisture, total nitrogen, amino nitrogen, and salt content

Fish sauce (approximately 3g) was dried for moisture analysis using a hot-air oven (VWR Scientific, Inc., So. Plainfield, NJ) at 105 °C until a constant weight was achieved (AOAC 1995). Total nitrogen in the fish sauce was determined according to the micro-Kjeldahl method (AOAC 1995). Protein concentration (%) was calculated based on the total nitrogen content (%) multiplied by 6.25. Total amino nitrogen (mg %) was determined according to the method of Nissen (1979). Reaction of amino nitrogen and 0.01% trinitrobenzenesulfonic acid (TNBS) was spectrophotometrically measured at 420 nm (Beckman Instruments, DU 640, Inc., Redmond, WA). Distilled water and L-leucine were used as a blank and standard reagent, respectively. Total amino nitrogen (mg %) was calculated by concentration x (14/131.2) x 100. Based on the composition of the fish sauce preparation (75% fish and 25% salt), fat content in fish sauce was assumed to be 0.75% (Park and Morrissey 2000). Salt content was calculated by subtracting % protein, % moisture, and 0.75% fat from 100. The pH

was measured directly by submerging a probe connected to the pH meter (Model A-15; Fisher Scientific, Pittsburg, PA).

ATP-related compounds: Inosine and hypoxanthine

One mL of fish sauce and 0.5 mL of 3 enzyme solutions prepared as described by Cho and others (1999). Adenosine deaminase (4U), xanthine oxidase (0.03U), and nucleoside phosphorylase (0.02U) were added into the mixture of deionized water (1.5 mL) and 1/15 M sodium phosphate buffer (pH 7.6, 2 mL). The blank was prepared using 1 mL deionized water instead of sample. All tubes were incubated at 37 °C for 40 min and absorbance was measured at 290 nm. (Model No. DU 640; spectrophotometer, Beckman Instruments, Inc., Redmond, WA). The uric acid concentration was determined using a standard curve. Inosine concentration (mM) and hypoxanthine concentration (mM) was calculated from the difference of uric acid content in samples according to the method of Cho and others (1999).

Amino acid composition

A mixture of 1.0 mL fish sauce and 50 mg 5'-sulfosalicylic acid was allowed to stand for 30 min at room temperature before centrifuging at 3,000 x g for 10 min. The supernatant was diluted with lithium citrate buffer (pH 2.2) to a final volume of 10 mL. The diluted solution was subjected to the auto amino acid analyzer (LKB-Biochrom 20, Pharmacia-Biotech, Buckinghamshire, UK). Standard amino acids

were used to identify the amino acid profile in Pacific whiting fish sauce samples. Type and concentration of amino acids in Pacific whiting fish sauce samples were obtained by comparing the retention time as well as the peak area between the standard amino acids and the unknown compounds.

Consumer acceptability

Volunteer consumers were randomly selected from the Uwajimaya grocery store (Beaverton, Oregon). A brief interview was used to screen for panelists that were familiar with fish sauce and usually used fish sauce at home. People who are allergic to seafood were not included in this study. Randomized complete block design (RCBD) was used to arrange the serving order of fish sauce for each panelist. Panelists were asked to give liking scores for three attributes: color, flavor, and overall preference using the 9-point hedonic scale. Three fish sauce samples (W, WB, and commercial fish sauce from anchovies) were presented to each panelist.

Unlike samples used for biochemical experiments, fish sauce used for consumer test was fermented for 12 mo before filtration. Since the commercial fish sauce (anchovy fish sauce, Squid brand, Thai Fish Sauce Factory Co., Ltd., Bangkok, Thailand) contains 2% sucrose, the same amount of sucrose was added into the filtered fish sauce in order to equilibrate the sweetness. The fish sauce (5 mL) was poured into polypropylene containers with lids. Cooked squid in uniform strips (1 cm width) was used for dipping with fish sauce samples. Fish sauce samples were served at ambient temperature. Fish sauce samples were served to each panelist in a random

order. Panelists were asked to use drinking water and sliced white bread for rinsing their mouths before tasting the next sample.

Statistical analysis

The biochemical analysis was conducted in triplicate for every treatment. Two-sample t-test for the means was performed for biochemical analysis (Ramsey and Schafer 1997). Randomized complete block design (RCBD) was used in consumer test. Seventy-five panelists were treated as blocks in order to obtain the degree of freedom for error among panelists. Mean comparisons among each liking scores of three fish sauce samples (commercial, W, and WB fish sauce) were compared using General Linear Model (GLM) and Bonferroni comparison method at a 95% confidence interval (SAS version 8, SAS Institute, Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Degree of hydrolysis

The degree of hydrolysis in the WB fish sauce increased as fermentation period increased, while degree of hydrolysis in W fish sauce increased up to 3 mo, and then decreased (Fig. 2.1). However, a significant difference in the degree of hydrolysis in W and WB fish sauces was only found at 9 mo ($P<0.05$). Degree of hydrolysis represents the extent of the hydrolytic degradation of the protein (Adler-

Nissen 1986). Orejana and Liston (1982) concluded that endogenous enzymes are the major agents responsible for protein digestion during the fish sauce process.

Cathepsin-like enzymes activities

The activities of cathepsin H-like enzymes were extremely low during fish sauce fermentation, while the cathepsin B-like enzyme was most active at the incubation temperature (Fig. 2.2). Cathepsin L, B, and H are lysosomal cysteine proteases that are also acid proteases (Kang and Lanier 2000). They are responsible for the intracellular protein degradation of fish muscle during fermentation. An and others (1994) reported that cathepsin L has highest activity at 55 °C, while cathepsin B has highest activity at 20-37 °C, and cathepsin H has highest activity at 20 °C. Cathepsin L is a true endopeptidase and cathepsin H is an endoaminopeptidase enzyme. While cathepsin B is both an endo- and exopeptidase enzyme (Kang and Lanier 2000). Consequently, cathepsin B might be one of the important enzymes for flavor development of fish sauce during the fermentation period. In addition, cathepsin B is reported to be the most active cysteine protease in Pacific whiting muscle (Kang and Lanier 2000)

The activities of cathepsin B-like, cathepsin L-like, and cathepsin H-like enzymes decreased rapidly during the first month of fermentation (Fig. 2.2). The decline was thought to be due mainly to inhibition by end products, such as amino acid and short chain peptides (Orejana and Liston 1982). There was no significant difference of cathepsin H-like activity in the 2 fish sauce samples ($P>0.05$). Cathepsin B-like

showed the highest enzyme activity in both fish sauce samples when compared to each other at the same fermentation time. Cathepsin B-like and L-like activities in WB fish sauce were greater than those in W fish sauce during the first month of fermentation ($P<0.05$). However, there was no significant difference between the 2 samples for the cathepsin B-like and L-like activities ($P>0.05$) after 3 mo. Cathepsin B-like, L-like, and H-like activities remained negligibly at 9 mo. According to Kirschke and others (1998), cathepsin B, L, and H are cysteine peptidases. They work well at pH lower than 7.0. Cathepsin B and H is catalytically active and stable in the pH range 5-6.5, but it is irreversibly inactivated at above pH 7.0. While cathepsin L is catalytically active at pH between 3.5 and 7.0. They also stated that their stability was also affected by ionic strength. According to our results, cathepsin-like activities in fish sauce decreased even though pHs of fish sauce decreased during fermentation period. Therefore, it possibly due to high ionic strength of fish sauce affecting on stability of enzymes.

The high salt concentration increased the ionic strength combining with long incubation time at high temperature (35 °C), which resulted in denaturation and a decline of enzyme activity (Stauffer 1989). The decrease of enzyme activity during fermentation was also found by Gildberg (1992). The activity of trypsin-like enzyme in protein hydrolysate made from 75% fish viscera and 25% salt at 27 °C was only 10% after 50 days (Gildberg 1992). High salt concentration (25%) prolonged fish sauce shelf life but it inhibited peptidase activity and hence retarded protein hydrolysis (Gildberg 1989; Sikorski and others 1995). However, salt reduction from

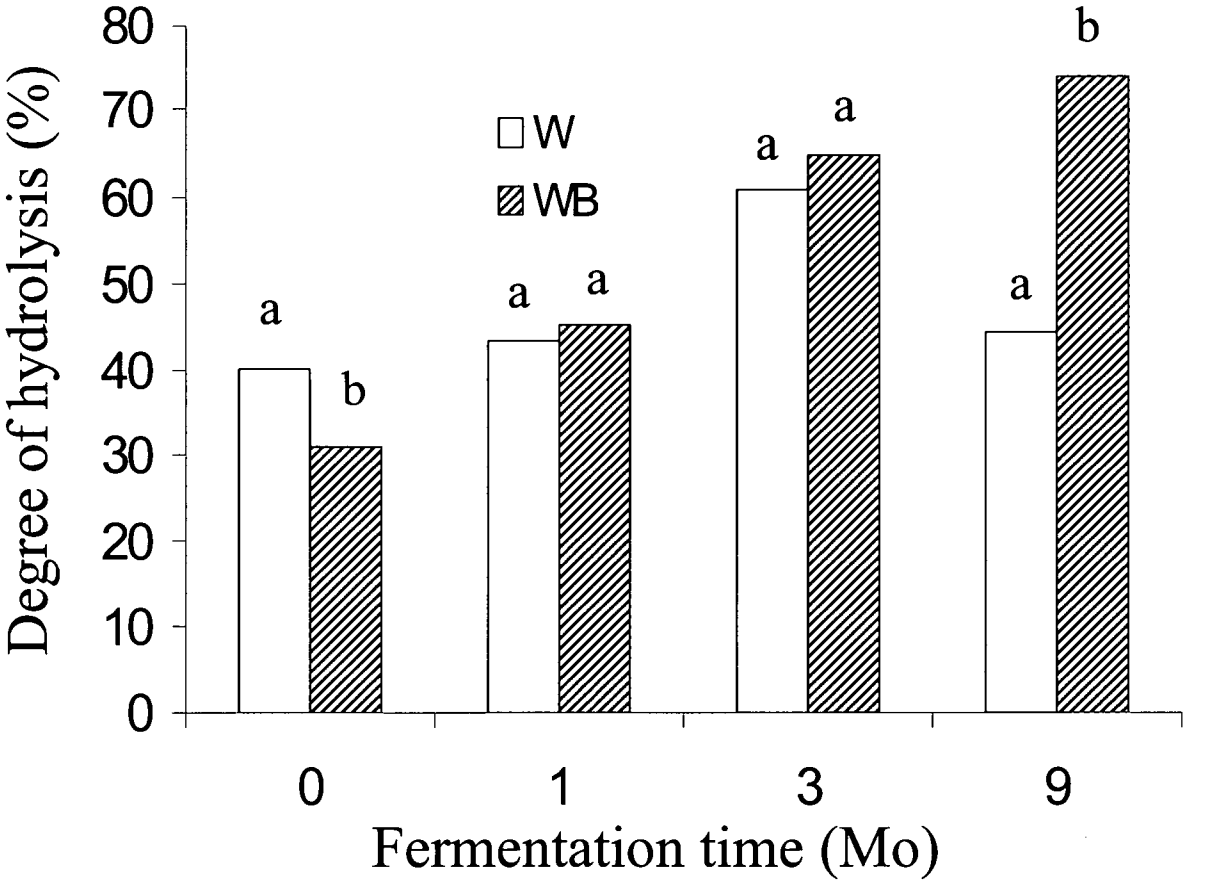

Figure 2.1: Degree of hydrolysis of fish sauce during fermentation

W = Fish sauce made from whole Pacific whiting
WB = Fish sauce made from the mixture (1:1) of whole fish and surimi byproducts
Different letters at the same fermentation time indicate a statistical difference ($P<0.05$)

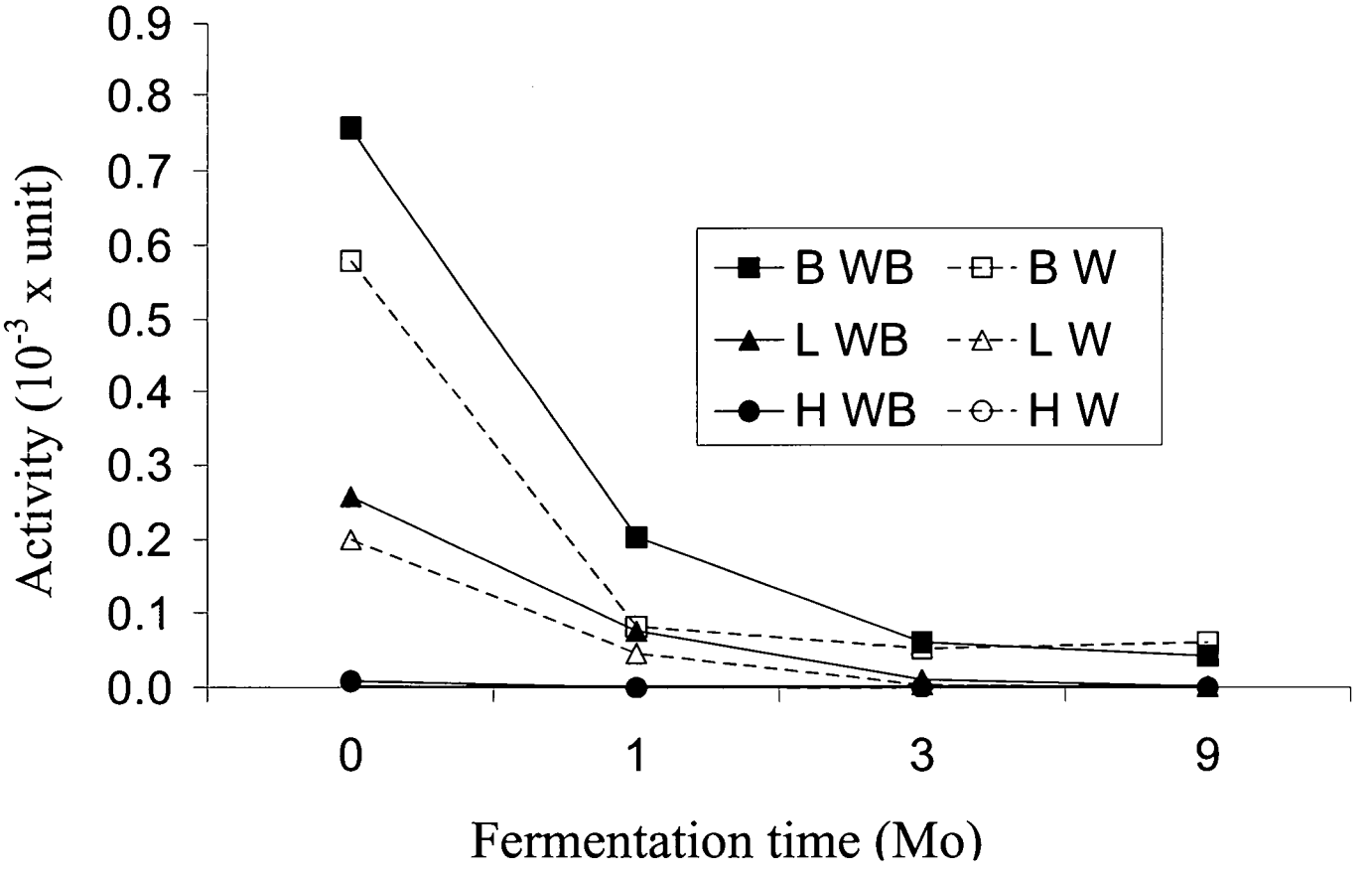

Figure 2.2: Cathepsin activity during fish sauce fermentation

B WB, L WB, H WB = Cathepsin B-like, L-like, and H-like enzyme activity in WB fish sauce
B W, L W, H W = Cathepsin B-like, L-like, and H-like enzyme activity in W fish sauce

25% to 5-15% accelerated autolysis during fish sauce fermentation (Gildberg and others 1984; Sikorski and others 1995).

Moisture, total protein, salt contents, and pH

Moisture content decreased and soluble protein content increased as fermentation continued, most probably due to protein hydrolysis and/or the dehydration effect (Table 2.1). At an early stage of fermentation, fish sauce made from the mixture (1:1) of whole fish and surimi byproducts (WB) had higher soluble protein content than fish sauce from whole fish (W)($P<0.05$). But there was no significant difference at 9 mo ($P>0.05$). At 0 mo of fermentation, moisture content of 2 fish sauce samples was not significantly different ($P>0.05$). After the first month of fermentation, WB fish sauce had a lower moisture content than W fish sauce ($P<0.05$). The lower moisture content and higher soluble protein content in WB fish sauce were thought to be due to the greater protein hydrolysis.

Total nitrogen in fish sauce increased during fermentation (Table 2.1). At early stage of fermentation (1 and 3 mo), WB fish sauce had higher total nitrogen than W fish sauce ($P<0.05$), possibly due to the greater degree of hydrolysis. Total nitrogen content in fish sauce depends on the fish species and chemical composition of the fish. About 80 percent of total nitrogen in fish sauce remains in the form of amino acids (Dougan and Howard 1975). W and WB fish sauces had a total nitrogen content of 1.28 and 1.36 %, respectively. Japanese fish sauce made from mackerel, sardine, and squid at 12 mo fermentation had a total nitrogen content of 1.89, 1.52,

and 1.48 %, respectively (Funatsu and others 2000). Total nitrogen in fish sauce is composed of protein nitrogen and nonprotein nitrogen (NPN) compounds such as free amino acids, nucleotides, peptides, ammonia, urea, TMAO, and so forth. These compounds contribute to the specific aroma and flavor (Finne 1992; Shahidi 1994). Salinity was calculated by subtracting %protein, %moisture, and 0.75% fat from 100. Moisture content decreased and soluble protein content increased as fermentation time continued, most probably due to protein hydrolysis and/or the partial dehydration effect. WB at 9 mo had lower moisture than that at other fermentation times. Consequently, salinity in WB increased at 9 mo.

The fresh Pacific whiting flesh had a neutral pH (6.93). The pH decreased gradually during fermentation reaching 5.6 after 9 mo (Table 2.1). The pH of WB fish sauce was lower than the pH of W fish sauce ($P<0.05$). This pH difference probably resulted from fermentation products containing organic acids, such as lactic acid, acetic acid, etc (Itoh and others 1993; Funatsu and others 2000; Michihata and others 2000). Ijong and Ohta (1996) reported the pH of bakasang (Malaysian fish sauce) ranged from 5.95 to 6.5. Lopetcharat (1999) reported the pH of Pacific whiting fish sauce, fermented for 40 days ranged between 6.1 and 6.3.

Amino nitrogen

Amino nitrogen content increased as fermentation continued (Fig. 2.3). The amino nitrogen concentration represents the amount of primary amino groups in fish sauce. An increase in amino nitrogen concentration is related to the degradation of the

polypeptide. Up to 1 mo of fermentation, WB fish sauce had greater amino nitrogen content than W fish sauce ($P<0.05$). Amino nitrogen contents were about 50-52 % of the total nitrogen in both fish sauce samples at 3 mo of fermentation. According to the Thai Industrial Standard, amino nitrogen content must be 40-60% of total nitrogen (Thai Industrial Standard 1983). Our results indicated the potential of Pacific whiting as a resource for acceptable fish sauce.

Table 2.1 Moisture, total nitrogen, salinity and pH of fish sauce from Pacific whiting (W) and a mixture (1:1) of whole fish and surimi by-product (WB)

Fermentation time (Mo)	Sample	Moisture (%)	Total nitrogen (mg/ 100 g)	Salinity[a] (%)	pH
0	W	73.4±0.5	375±49	23.6	7.05±0.01
	WB	73.0±0.1	553±45	22.9	6.66±0.01
1	W	71.7±0.6	743±72	23.0	6.64±0.01
	WB	70.8±0.1	853±43	23.2	5.75±0.01
3	W	68.0±0.1	1128±30	24.3	5.72±0.01
	WB	66.8±0.1	1322±18	24.2	5.64±0.01
9	W	68.0±0.0	1282±2	23.3	5.42±0.01
	WB	64.4±0.1	1357±75	26.4	5.34±0.01

The mean and standard deviation were derived based on data obtained from triplicate runs.
[a]Salinity was calculated by subtracting % protein, % moisture, and 0.75% fat from 100.

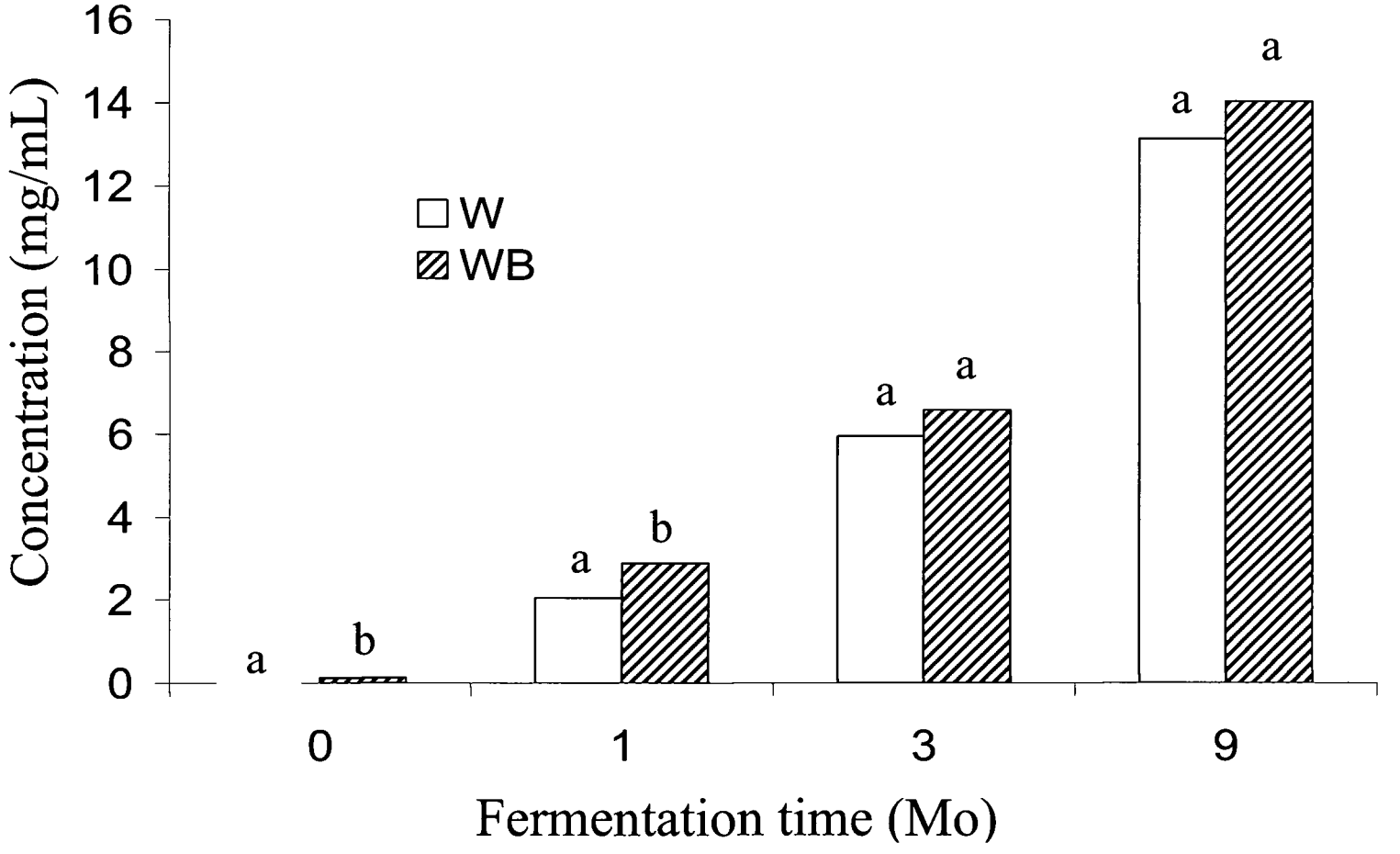

Figure 2.3: Amino-nitrogen content in fish sauce during fermentation

See Figure 2.1 for legend

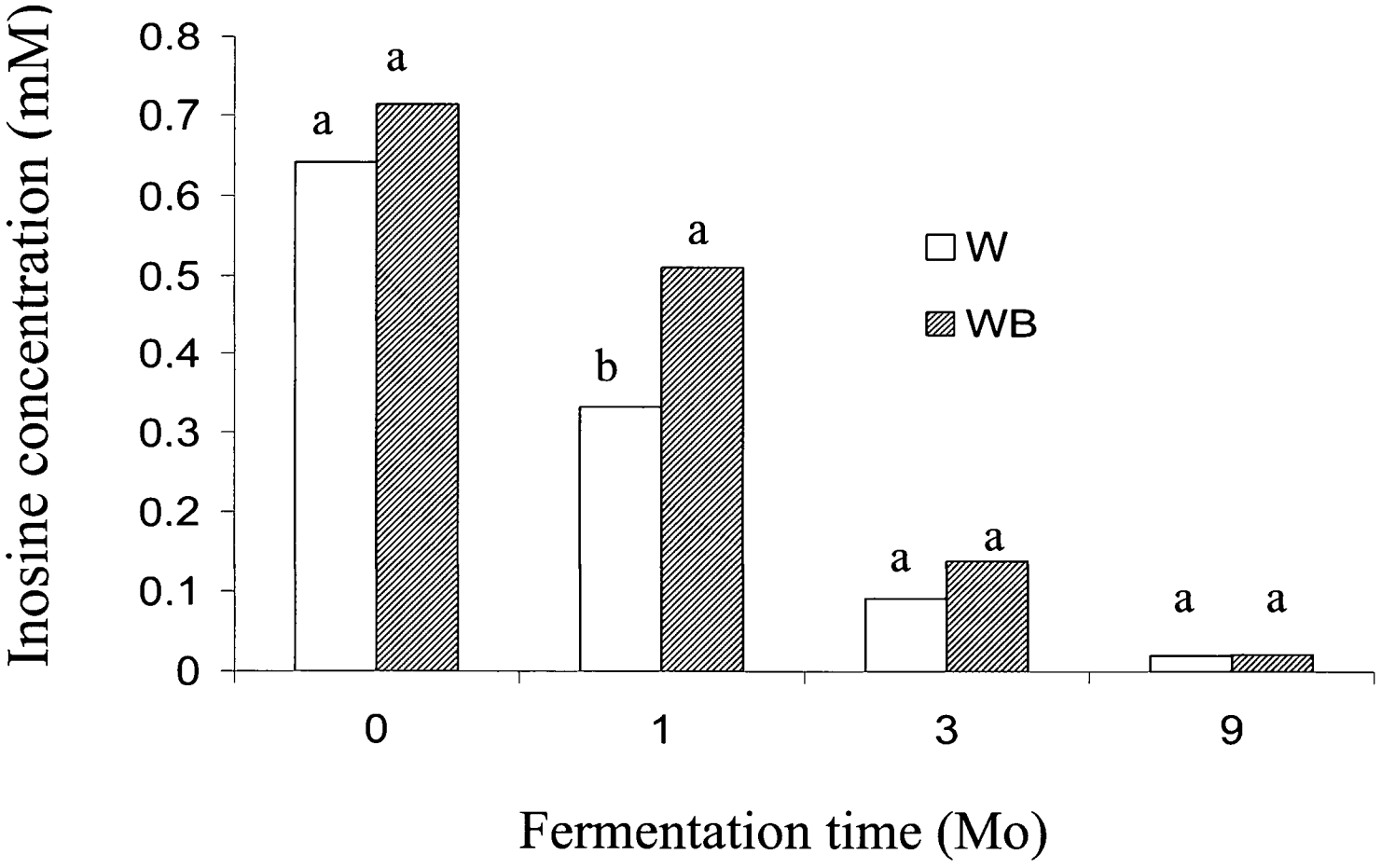

Figure 2.4: Inosine content in fish sauce during fermentation

See Figure 2.1 for legend

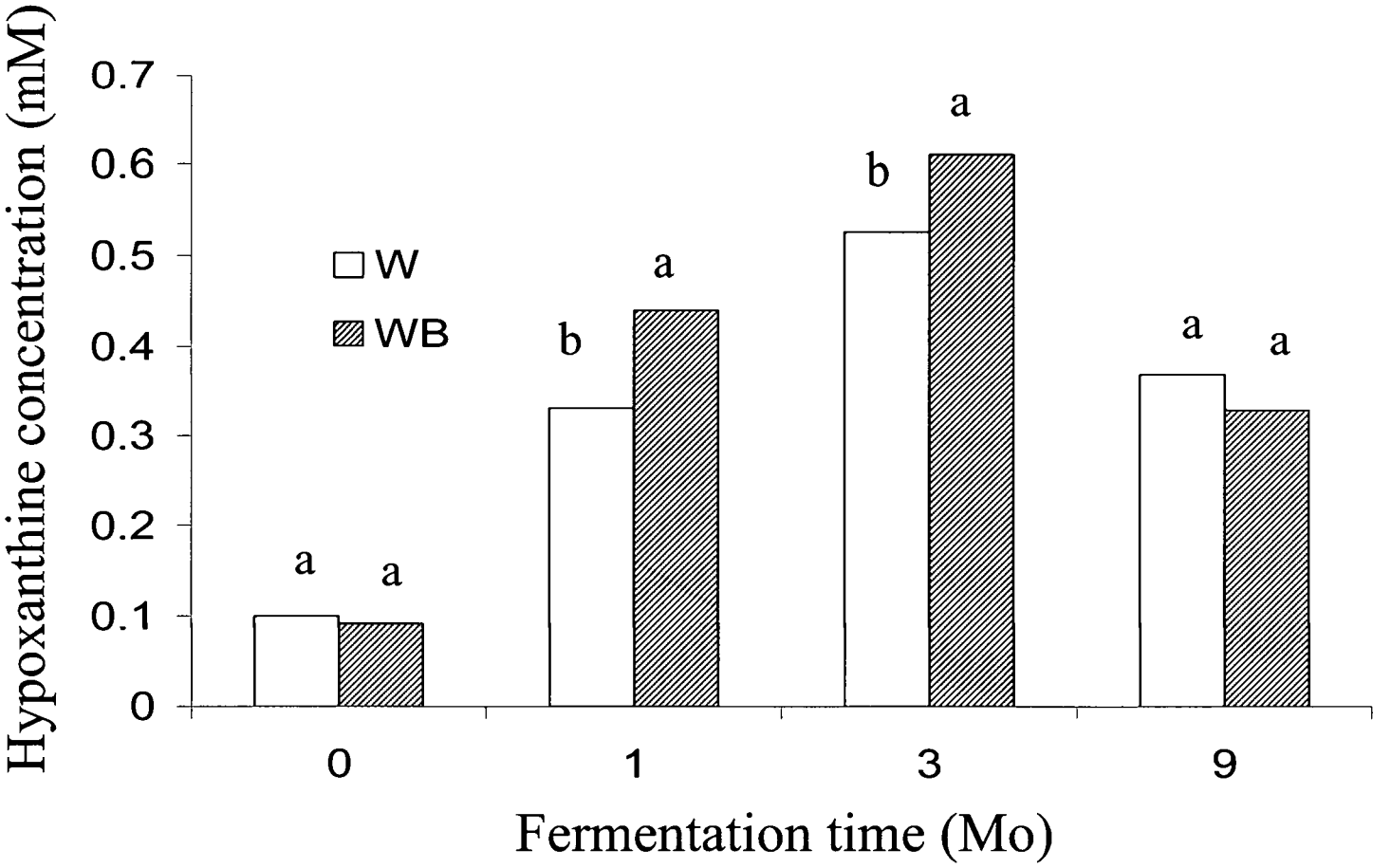

Figure 2.5: Hypoxanthine content in fish sauce during fermentation

See Figure 2.1 for legend

ATP-related compounds: inosine and hypoxanthine

Inosine decreased while hypoxanthine increased up to 3 mo fermen-tation (Fig. 2.4 and Fig. 2.5). Hypoxanthine decreased at 9 mo of fermentation. W and WB fish sauces contained a similar amount of the ATP-related compounds at 9 mo of fermentation ($P>0.05$). In ATP catabolism, adenosinetri-phosphate (ATP) was hydrolyzed to inosine (HxR), hypoxanthine (Hx), xanthine (X), and then further hydrolyzed to uric acid. This trend of hydrolysis was thought to be due to both endogenous enzymes in fish and the bacterial inosine nucleosidase (Surette and others 1980; Shahidi 1994; Cho and others 1999).

In general, the change of purine derivatives was used as an index of the freshness of fish. For example, the level of hypoxanthine of 1-4 μg/g tissue is the quality index (Hughes and Jones 1966; Botta 1995). Shahidi (1994) reported that the free amino acids and nucleotides are the most important components contributing to the desirable flavor of seafoods. The contribution of 5'nucleotides (5'-IMP and 5'-GMP) to the umami taste of seafood is well recognized (Komata 1990; Shahidi 1994). However, hypoxanthine gives a bitter taste (Hughes and Jones 1966). The disappearance of the nucleotide is related to hypoxanthine formation, and it is to be expected that the concentration of purine would be indirectly related to flavor. However, since the fish sauce contained a significant amount of free amino acid and short-chain peptides that contribute to the favorable flavor, the bitter taste of hypoxanthine could be easily masked.

Amino acid composition

The amino acid compositions of Pacific whiting fish sauce fermented for 9 mo are shown in Table 2.2. The amino acid compositions of W fish sauce WB and fish sauce were quite similar. Total free amino acids increased as fermentation continued. However, after 9 mo of fermentation, total free amino acids in W fish sauce (38.37 mg/mL) were twice as high as the amount in WB fish sauce (16.56 mg/mL). Glutamic acid, alanine, leucine, lysine, and arginine were rich in the 9-mo samples and accounted for 43.27% and 54.08 % of total amino acids in W and WB fish sauce, respectively. According to Benjakul and Morrissey (1997), Pacific whiting muscle is composed of 6 major amino acids (glutamic acid, aspartic acid, lysine, leucine, arginine, and alanine) that account for 56.54 % of total protein.

Similar free amino acid profiles were found in skipjack tuna sauce (Cha and Cadwallader 1998), as well as in herring protein hydrolysate (Liceaga-Gesualdo and Li-Chan 1999). Ijong and Ohta (1996) found that alanine, isoleucine, glutamic acid, and lysine were high in a traditional Indonesian fish sauce. Some differences in amino acid profile between W and WB fish sauces were possibly due to the differences in protein composition. WB fish sauce contained a significant amount of fish skins, bones, heads, and other connective tissues, unlike W fish sauce manufactured from whole fish.

Amino acids dictate the taste of seafood (Sikorski 1994). Taste values (Table 2.2) were calculated based on the amino acids from concentration and taste threshold

Table 2.2: Amino acid composition of Pacific whiting fish sauce after 9 mo fermentation

Amino acid	Concentration (mg/mL)		Taste threshold[a]	Taste value[b]	
	W	WB	(g/dL)	W	WB
Arginine	3.074	1.516	0.050	61.5	30.3
Aspartic acid	1.698	0.891	0.003	566.0	297.0
Threonine	1.716	1.145	0.260	6.6	4.4
Serine	1.684	0.935	0.150	11.2	6.2
Glutamic acid	3.435	1.623	0.005	687.0	324.6
Proline	2.660	0.000	0.300	8.9	0.0
Glycine	1.036	0.482	0.130	8.0	3.7
Alanine	2.743	1.664	0.060	45.7	27.7
Valine	2.404	1.083	0.040	60.1	27.1
Methionine	1.366	0.217	0.030	45.5	7.2
Isoleucine	1.709	0.814	0.090	19.0	9.0
Leucine	3.528	1.873	0.190	18.6	9.9
Phenylalanine	1.310	0.625	0.090	14.6	6.9
Lysine	3.825	2.277	0.050	76.5	45.5
Histidine	0.199	0.065	0.020	10.0	3.3
Ammonia	0.200	0.286	NA	-	-
DL-allohydroxylysine	0.136	0.055	NA	-	-
Ornithine	0.031	0.000	NA	-	-
1-methylhistidine	0.270	0.064	NA	-	-
Taurine	0.529	0.335	NA	-	-
Urea	0.000	0.189	NA	-	-
Phosphoserine	0.104	0.037	NA	-	-
α-aminoisobutyric acid	0.026	0.000	NA	-	-
Hydroxyproline	3.656	0.000	NA	-	-
Tyrosine	0.525	0.263	NA	-	-
Cystathionine	0.201	0.115	NA	-	-
Total	38.374	16.555	-	-	-

W = Fish sauce made from whole Pacific whiting
WB = Fish sauce made from the mixture (1:1) of whole fish and surimi byproducts
Taste threshold[a] (Kato and others 1989)
Taste value[b] calculated using Cha and Cadwallader (1998)
NA = Not available for taste threshold data.

data according to the method described by Cha and Cadwallader (1998). Taste values of amino acids from W fish sauce were 2 times higher than those from WB fish sauce. Glutamic acid and aspartic acid showed the highest taste values and the lowest thresholds in both samples. Various amino acids carry their own taste. Lysine,

alanine, glycine, serine, and threonine provide a sweet taste, while arginine, leucine, valine, phenylalanine, histidine, and isoleucine give a bitter taste, and aspartic acids give a sour taste (Kato and others 1989). High content of glutamic acid in fish sauce might make an important contribution to the development of the umami taste (Komata 1990; Sanceda and others 1990). Cha and Cadwallader (1998) stated that the specific free amino acids having sweet, sour, and bitter tastes may play a prominent role in the overall taste of fish sauce. Some volatile amino acids contribute to the flavor (taste and aroma) of fish sauce. Glutamic acid gives meaty taste. Isoleucine and leucine give sweet taste while methionine gives methyl sulfide-like aroma and taste, and phenylalanine gives strong rose-like flavor (Saisithi and others 1966).

Consumer acceptability

A majority of panelists (93.3%) were fish sauce users at home at least once a month. As for ethnic groups, Asians were 53.3% and Caucasians 46.7% of the panelists. As for gender, 68.0% of the panelists were female. A majority (89.3%) were 20-59 years old. There was no significant difference in overall sensory liking and flavor liking for all fish sauce samples ($P>0.05$) (Table 2.3). However, WB fish sauce had lower color liking scores than commercial anchovy fish sauce ($P<0.05$). No significant difference in color liking was detected between W fish sauce and commercial anchovy fish sauce ($P>0.05$).

Regarding consumer liking by the ethnic group, Asians (n=40) showed no significant difference of liking among W fish sauce, WB fish sauce, and commercial

anchovy fish sauce for color, flavor, and overall liking ($P>0.05$) (Table 2.3). Likewise, Caucasians (n=35) showed no significant difference of liking among W fish sauce, WB fish sauce, and commercial anchovy fish sauce for flavor and overall liking ($P>0.05$), except for color liking ($P<0.05$) (Table 2.3). In terms of color liking, they showed no significant difference of liking between W fish sauce and commercial anchovy fish sauce ($P>0.05$). However, they rated greater color liking scores for commercial anchovy fish sauce than for WB fish sauce ($P<0.05$). Regarding consumer liking by gender, there was no difference. Both genders rated the same liking levels for all fish sauce samples with regard to color, flavor, and overall liking ($P>0.05$) (Table 2.3).

CONCLUSION

Two fish sauce samples developed from Pacific whiting showed similar biochemical properties during 9 mo of fermentation. Based on the biochemical aspects of fish sauce (total nitrogen, amino nitrogen, and amino acid composition), the quality fish sauce could be made from Pacific whiting and its surimi byproducts. Consumer panelists demonstrated that fish sauce from Pacific whiting could successfully replace imported anchovy fish sauce. Furthermore, solid byproducts from surimi processing can be utilized as a raw material for fish sauce.

Table 2.3: Mean score for color, flavor, and overall liking of fish sauce

Attributes	Consumer group	Fish sauce samples		
		W	WB	C
Color liking	All consumers (n=75)	6.40^{ab}±1.77	6.12^{bc}±1.94	6.92^{a}±1.50
	Asian (n=40)	6.58^{a}±1.66	6.28^{a}±2.01	6.80^{a}±1.59
	Caucasian (n=35)	6.20^{ab}±1.89	5.94^{b}±1.88	7.06^{a}±1.39
	Female (n=51)	6.32^{a}±2.01	6.22^{a}±2.05	7.00^{a}±1.64
	Male (n=24)	6.33^{a}±1.24	5.92^{a}±1.72	6.75^{a}±1.15
Flavor liking	All consumers (n=75)	6.07^{a}±2.02	5.76^{a}±2.07	6.31^{a}±2.22
	Asian (n=40)	5.93^{a}±1.95	5.35^{a}±2.29	6.30^{a}±2.33
	Caucasian (n=35)	6.22^{a}±2.12	6.23^{a}±1.70	6.31^{a}±2.13
	Female (n=51)	5.77^{a}±2.19	5.61^{a}±2.20	6.33^{a}±2.29
	Male (n=24)	6.33^{a}±1.61	6.08^{a}±1.77	6.25^{a}±2.13
Overall liking	All consumers (n=75)	6.09^{a}±1.90	5.93^{a}±1.91	6.37^{a}±1.98
	Asian (n=40)	5.90^{a}±1.97	5.58^{a}±2.10	6.35^{a}±2.09
	Caucasian (n=35)	6.31^{a}±1.83	6.34^{a}±1.61	6.40^{a}±1.87
	Female (n=51)	5.88^{a}±2.10	5.82^{a}±2.02	6.39^{a}±1.96
	Male (n=24)	6.54^{a}±1.32	6.17^{a}±1.69	6.33^{a}±2.06

Results are expressed as the means ± standard deviation of the liking of panelists (n = number of panelists).
Liking score: 9 = extremely like, 5 = neither like nor dislike, 1 = extremely dislike
Means with the same letter in each row are not significantly different.
W = Fish sauce made from whole Pacific whiting
WB = Fish sauce made from the mixture (1:1) of whole fish and surimi byproducts
C = Commercial anchovy fish sauce

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Chapter 3

INTERACTIVE EFFECT OF SALT CONCENTRATION AND pH ON ENDOGENOUS ENZYME STABILITIES AND BIOCHEMICAL PROPERTIES OF PACIFIC WHITING FISH SAUCE

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ABSTRACT

Pacific whiting (PW) fish sauce containing lower salt concentration had a greater degree of hydrolysis (DH). At early fermentation (Day 3), the greatest activities of cathepsin B (B) and cathepsin L (L) remained in fish sauce processed at pH 5 and 4, respectively, and the greatest activities of chymotrypsin (C) and trypsin (T) remained at pH 7. The greatest DH, total nitrogen, and amino nitrogen were found at processing pH between 4.0-5.0 (15-20% salt), with 1.8-2.4 times greater proteolysis than samples processed at pH 7.0. B and L enzymes played an important role in proteolysis. Adjustment of pH and salt to 4.0-5.0 and 15-20%, respectively, were recommended for accelerating proteolysis.

Key words: fish sauce, degree of hydrolysis, total protein, amino nitrogen, endogenous enzymes

INTRODUCTION

Traditional fish sauce in Thailand is produced by storing a mixture of salt and minced anchovies (1:3) at tropical temperatures (30-35 °C) for 9 to 18 mo. Fermentation is conducted through the proteolysis of enzymes from the viscera along with the function of salt-tolerant microorganisms (Uyenco and others 1952; Sikorski and others 1995). Endogenous fish enzymes are primarily responsible for the degradation of muscle proteins during the fish sauce process, while bacteria play a minor role due to the high salt content (Uyenco and others 1952; Orejana and Liston 1982).

The enzymes slowly hydrolyze the fish proteins during storage (Sikorski and others 1995). An attempt to accelerate the hydrolysis process in fish protein hydrolysate product with the addition of papain and alcalase enzymes results in a bitter taste (Hoyle and Merritt 1994). Cho and others (2000) found that the protein hydrolysis in salted anchovy was inhibited at salt concentrations higher than 25% at 5 °C. Reducing salt content was therefore suggested in order to accelerate the fish sauce process (Hamm and Claque 1950; Sikorski and others 1995). Beddows and Ardeshir (1979) also suggested acid hydrolysis as a means of accelerating fish sauce production. Consequently, both salt reduction and pH adjustment appear to be solutions for accelerating fermentation. However, little information is available regarding the interactive effect of salt concentration and pH on the enzyme activity in fish sauce, particularly made from Pacific whiting.

Beddows and Ardeshir (1979) reported that the adjustment of pH to 2-3 and salt concentration to 10-15%, respectively, gave a higher degree of protein hydrolysis, resulting in a nitrogen content similar to that of Malaysian fish sauce (Budu). However, there was a problem with calcium phosphate precipitating when the pH of the acid fish sauce was re-adjusted to the pH similar of naturally produced fish sauce (5.65) (Beddows and Ardeshir 1979). Gildberg and others (1984) reported that anchovy fish sauce fermented at pH 4 and with 5-10% salt for 2 mo at 40 °C gave acceptable flavor. However, low salt concentration could negatively affect product shelf life. According to Putro (1993), at least 15% salt concentration is required to prevent the growth of putrefactive bacteria.

Pacific whiting (*Merluccius productus*), which is abundant off the Pacific Northwest, was limited from commercial utilization (surimi) until 1991 due to proteolytic enzymes, which cause texture softening. Lopetcharat and others (2002) studied the feasibility of fish sauce production from enzyme-laden Pacific whiting for the first time. In addition, it was thought that fish sauce made from lean fish like Pacific whiting would be more consumer-friendly than anchovy fish sauce since the histamine hazard is reduced. Tungkawachara and others (2003) also characterized the biochemical and sensory properties of fish sauce made from a mixture (1:1) of Pacific whiting and its surimi byproducts at 25% salt and physiological pH (approximately 6.5-7.0).

Our objective was to evaluate the interactive effect of salt concentration and pH on some endogenous enzyme activities during the fermentation of Pacific whiting

fish sauce. Degree of hydrolysis, amino nitrogen, and total nitrogen in fish sauce at different fermentation times were also measured and compared.

MATERIALS AND METHODS

Chemicals

All chemicals, except salt, used in the biochemical experiments were reagent grade. Sodium phosphate dibasic (Na_2HPO_4) was from Matheson Coleman & Bell (Norwood, OH). Sodium phosphate monobasic ($NaH_2PO_4.H_2O$) was from Mallinckrodt Chemical Inc (St. Louis, MO). Ammonium sulfate $(NH_4)_2SO_4$, iodoacetate, aminomethylcoumarine, potassium sulfate (K_2SO_4), sulfuric acid (H_2SO_4), sodium hydroxide (NaOH), trichloroacetic acid (TCA), perchloric acid (PCA), potassium hydroxide (KOH), and hydrochloric acid (HCl) were purchased from Fisher Scientific (Pittsburgh, PA). N-benzoyl-L-tyrosine ethyl ester (BTEE), p-toluenesulfonyl L-arginine methyl ester (TAME), Tris (Hydroxymethyl aminomethane), and calcium chloride ($CaCl_2$) were purchased from Sigma chemical Co (St. Louis, MO).

Preparation of fish sauce

Frozen Pacific whiting (Merluccius productus) was ground and mixed homogeneously with salt (Morton International, Inc., Chicago, IL) at 15 %, 20 %, and 25% (W/W). The pH was adjusted to 4.0, 5.0, 6.0, 7.0, 8.0, or 9.0 using 5 N HCl and

5 N NaOH. After pH adjustment, de-ionized water was added to the fish paste to obtain a total volume of 60 mL The pH was not changed significantly by the addition of de-ionized water. The paste, after mixing, was then placed in glass bottles closed tightly with a lid and kept at 35 °C for fermentation. At each fermentation time (at 3, 30, and 60 days), fermented fish paste samples were diluted (1:1) with cold 20 mM phosphate buffer (pH 7.0) and homogenized for 2 min before centrifuging at 10,000 x g for 30 min at 4 °C. The supernatant was then filtered through filter paper (Qualitative P8, Fisher Scientific, Pittsburgh, PA) and the filtrate was subjected to various biochemical analyses.

Enzyme activity measurement

Sample preparation – Proteins and peptides in fish sauce were fractionated using two-step approaches with 25 and 85% ammonium sulfate, respectively. The precipitate fraction was collected by centrifugation at 15,000 x g for 30 min. Four milliliters of de-ionized water were added to the precipitate fraction before it was subjected to dialysis against de-ionized water (5 X 500 mL) for 12-15 h at 4 °C. The dialysis membrane sack had a molecular weight limit of 12-14 kDa (Spectra/POR, regenerated cellulose, Fisher Scientific, Pittsburg, PA, USA). The changed volume of dialyzed samples was used to calculate the dilution factor and enzyme activity unit.

Cathepsin L-like and cathepsin B-like enzyme activities

Cathepsin-like enzyme activities were determined according to the method of Barrett and Kirscke (1981). Substrates for cathepsin L-like and B-like were 20 μM of N-CBZ-Phe-Arg-7-amido-4-methylcoumarin and N-CBZ-Arg-Arg-7-amido-4-methylcoumarine, respectively. An and others (1994) determined the cathepsin activities in crude extract of Pacific whiting using the method of Barrett and Kirschke (1981). They found that the optimum temperature of cathepsin L-like, B-like, and H-like enzymes were 55 °C, 20-37 °C, and 20 °C, respectively. The optimum pH of cathepsin L, B, and H, were 5.5-6.0, 6.0, and 6.5-6.8, respectively (Kirschke and others 1998). Optimum pH and temperature were used for cathepsin L-like (at pH 5.5 and 55 °C,) and B-like (30 °C, pH 6.0) enzyme activities.

Enzyme activity was measured from the production rate of aminomethyl-coumarine produced from the enzyme reaction. A luminescence spectrophotometer (Model No LS 50B: Perkin Elmer, Norwalk, CT) was used to measure the fluorescence intensity at an excitation wavelength of 370 nm and emission wavelength of 460 nm. For the blank, 200 μL of 5 mM iodoacetate was added before the crude extract. One unit of enzyme activity was defined as enzyme concentration involving in the production rate of 1 μmole of aminomethylcoumarine released per min for cathepsin L-like enzyme at 55°C and pH 5.5, cathepsin B-like at 30°C and pH 6.0, and cathepsin H-like at 20°C and pH 6.8.

In order to report the activity unit as an international unit (U), our preliminary test was conducted to assure that there was a linear relationship between the

aminomethyl-coumarine concentration released and the incubation time and also that the test conditions obey the steady state assumption.

Chymotrypsin-like and trypsin-like enzyme activities

Chymotrypsin-like and trypsin-like enzyme activities were determined according to the method of Walsh and Wilcox (1970). Substrate for chymotrypsin-like activity was 1mM N-benzoyl-L-tyrosine ethyl ester (BTEE) in 50% w/w aqueous methanol and substrate for trypsin-like activity was 1.04 mM p-toluenesulfonyl L-arginine methyl ester (TAME) in 0.04 M Tris pH 8.1 containing 0.01 M $CaCl_2.2H_2O$. Buffer for chymotrypsin-like activity was 0.1 M Tris-HCl pH 7.8, containing $CaCl_2$ 0.1 M, while the buffer used for trypsin-like activity was 0.04 M Tris-HCl, pH 8.1, containing 0.01 M $CaCl_2.2H_2O$.

Enzyme reactions were measured from the absorbance change per minute at 256 nm for chymotrypsin-like activity and at 247 nm for trypsin-like activity. The change of absorbance during the first 5 minutes of the reaction at 30 °C was recorded and the activity was calculated from the slope of the linear portion of the reaction curve. One unit of chymotrypsin-like activity was defined as enzyme concentration involving in the hydrolysis rate of BTEE 1 μmole/min and it was calculated as: 1 Unit = $[(\Delta A_{256}nm/min) \times 1000x\ 3] / 960$, where 960 is the extinction coefficient (ε), and 3 is total volume of reaction mixture (Walsh and Wilcox 1970). In addition, one unit of trypsin-like activity was defined as the enzyme concentration involving in the hydrolysis rate of TAME 1 μmole/min and it was calculated as: 1 Unit =

[(ΔA_{247}nm/min) x 1000 x 3] / 410, where 410 is the extinction coefficient (ε), and 3 is total volume of reaction mixture (Walsh 1970).

Degree of hydrolysis

An aliquot of 10 mL of 20% trichloroacetic acid (TCA) was added to an equal volume of fish sauce, to obtain a concentration of 10% TCA. After centrifuging at 10,000 x g, TCA-soluble nitrogen in the supernatant and total nitrogen in the whole sample (mixture of fish paste and liquid) were analyzed for nitrogen content by the Kjeldahl method (AOAC 1995). Degree of hydrolysis was calculated according to the method described by Hoyle and Merritt (1994):

DH (%) = [(TCA-soluble Nitrogen in sample)/ Total Nitrogen in sample] x 100.

Total nitrogen

Total nitrogen in fish sauce was determined according to the Kjeldahl method (AOAC 1995) with slight modification. Regarding environmental and health concerns, copper sulfate ($CuSO_4$) was used as a catalyst instead of mercuric oxide (HgO). Copper catalyst was prepared by mixing potassium sulfate with copper sulfate at a ratio of 3.5:1 (by weight) (NFTA, 2001).

Amino nitrogen

Amino nitrogen content (mg/mL) in fish sauce was determined according to the method of Nissen (1979). Reaction of amino nitrogen and 0.01% trinitrobenzenesulfonic acid (TNBS) was spectrophotometrically measured at 420 nm (Beckman Instruments, DU 640, Inc., Redmond, WA). Distilled water and L-leucine were used as a blank and standard reagent, respectively. Total amino nitrogen (mgN/mL) was calculated by leucine equivalent concentration (mg/mL) x (14/131.2).

RESULTS AND DISCUSSION

Cathepsin L-like and B-like enzyme activities

The highest cathepsin L-like and cathepsin B-like enzyme activities were found in samples processed at pH 4.0 (15% salt) and pH 5.0 (25% salt), respectively (Fig. 3.1 and Fig. 3.2). Except at pH 4.0, cathepsin L-like and B-like activities at 25 % were greater than those in samples containing 20 % and 15 % salt. This was probably due to the chloride ions activating the cathepsin L-like and B-like enzymes during the first stage of fermentation.

Simpson (2000) stated that cathepsins are activated by chloride ions. However, there was no information regarding the concentration of chloride ions required for activation. Our results showed that cathepsin L-like and B-like activities in samples at acidic pH were greater than samples at basic pH. At 15% salt, cathepsin L-like activity remaining in samples at 3-day incubation had the highest activity at pH 4

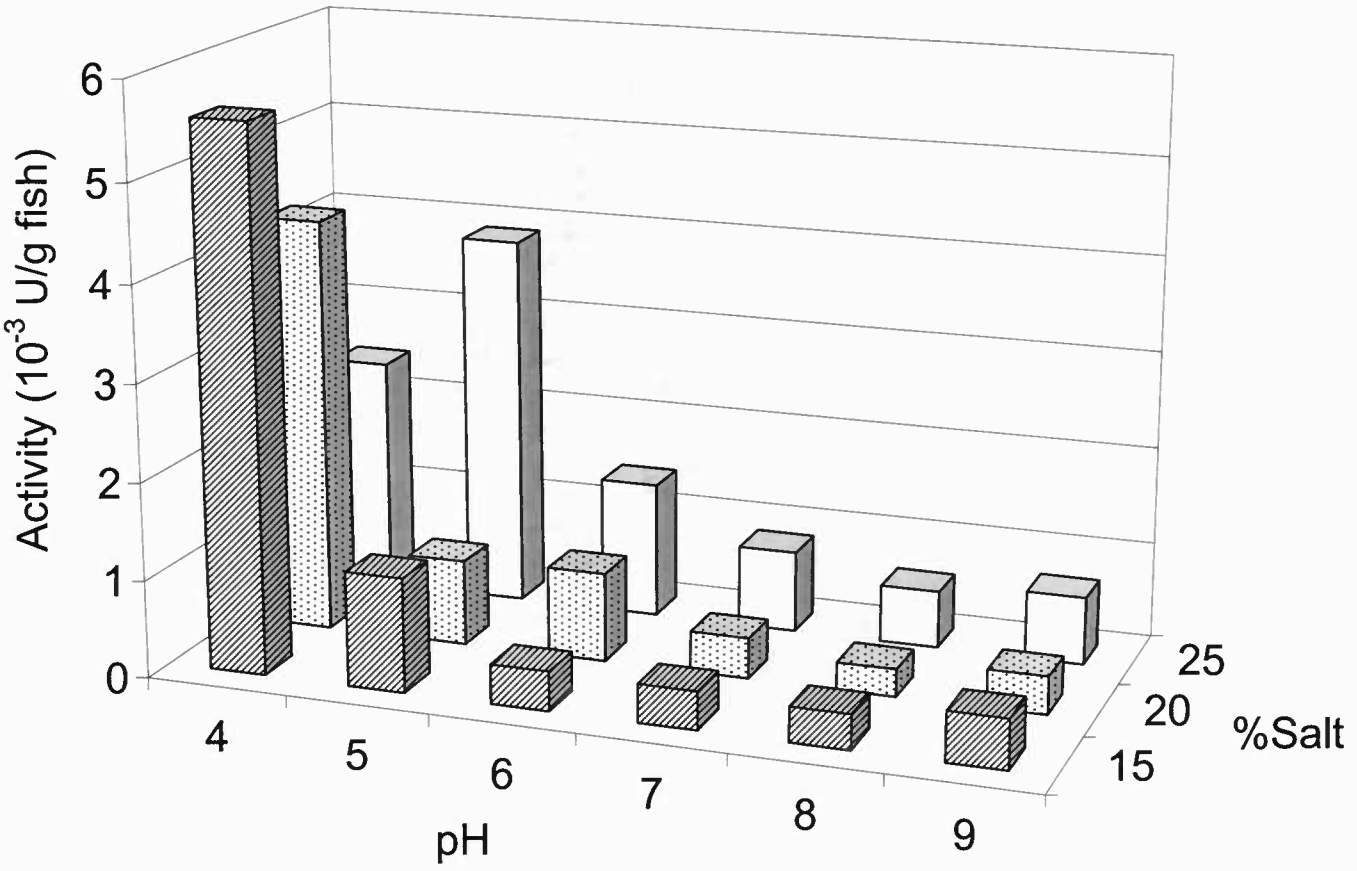

Figure 3.1: Active cathepsin L-like activity remained in fish sauce fermented for 3 days

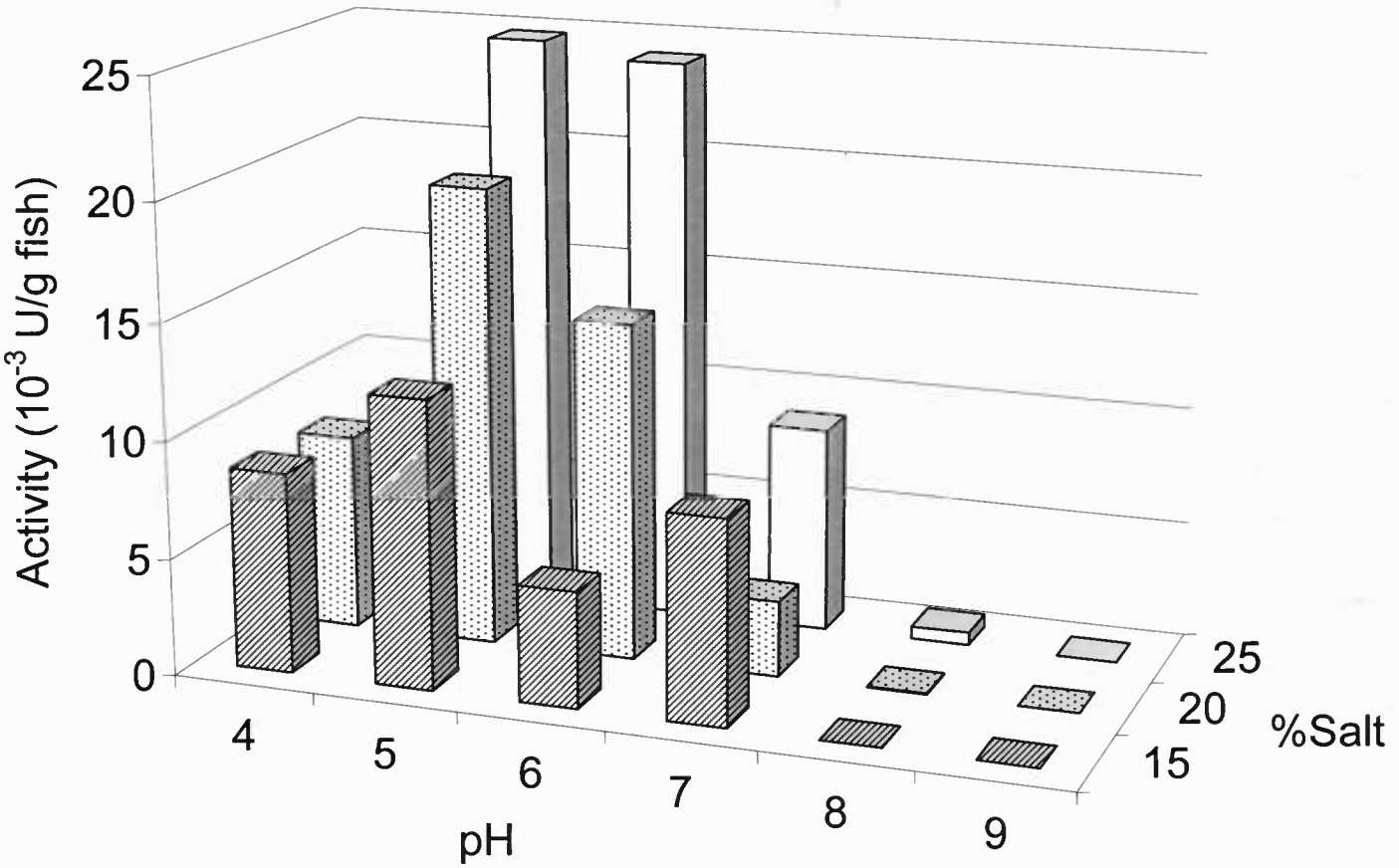

Figure 3.2: Active cathepsin B-like activity remained in fish sauce fermented for 3 days

(Table 3.1) and then reduced dramatically at pH 7.0, 8.0, and 9.0. This was probably because cathepsin L and cathepsin B are acidic proteases (Kang and Lanier 2000). The optimum pH of cathepsin L on Z-phe-arg-NHmec was reported to be 5.6 and the optimum pH of cathepsin B on Z-arg-arg-NHmec was reported to be 6.0 (Yamashita and Konagaya 1990). Eisen and Jeffrey (1969) also reported that cathepsin B activity toward collagenase from mussel showed an optimum pH between 3.0 and 4.5. Our results showed the interactive effect of salt and pH on cathepsin L-like and B-like activities remaining for the duration of incubation. At pH above 4.0, cathepsin L-like and B-like activities remaining in samples containing 25% salt were greater than samples containing 15% salt. At various pH, the activity of enzyme is dependent on ionic strength (Mort 1998). Salt addition increases ionic strength and

Table 3.1: Enzyme activity summary

Enzymes	Optimum pH	pH at highest remained activity @ 3 days (15% Salt)	Highest Stability @ 60 days (Salt/pH)
Cathepsin L	5.5 [1]	4.0	20/5.0
Cathepsin B	6.0 [1]	5.0	15/5.0
Chymotrypsin	7.0-8.0 [2]	7.0	20/5.0
Trypsin	7.5 [3]	7.0	20/4.0

[1] Yamashita and Konagaya (1990)
[2] Kristjansson and Nielson (1992)
[3] Simpson and Haard (1984)

causes a change in the ionization of amino acid side chains on the enzyme catalytic site and thus, affects enzyme activity and stability (Palmer 1995). The interactive effect of salt and pH on the activities of enzymes may be due to the changes in the ionic charge of the active site. The interactive effect may also alter the susceptibility of the two molecules to form an enzyme-substrate complex (ES). In addition, the protonated status of the amino acid side chains in the active site of the ES may be changed, resulting in a change in the ability of ES to break down into product (Palmer 1995).

Chymotrypsin-like and trypsin-like enzyme activities

It was found that chymotrypsin-like activity at 3-day fermentation was highest in samples at pH 7.0 with 15 % salt followed by samples containing 20% and 25% salt (Fig. 3.3). Trypsin-like activity at 3-day fermentation was highest at pH 7.0 in sample containing 15 % salt followed by samples containing 20% and 25% salt (Fig. 3.4).

Chymotrypsin from rainbow trout (Oncorhynchus mykiss) showed maximum activity at pH 7.0-8.0 (Kristjansson and Nielson 1992). Trypsin from Greenland cod (Gudus orac) showed maximum activity at pH 7.5 (Simpson and Haard 1984). Our results also showed that chymotrypsin-like and trypsin-like activities were higher in samples at neutral pH than at acidic pH. This is probably because serine proteases have a catalytic triad (serine, histidine, aspartic acid) at their catalytic sites and their activities depend on the ionization of imidazole moiety of the histidine residue (pK_a of

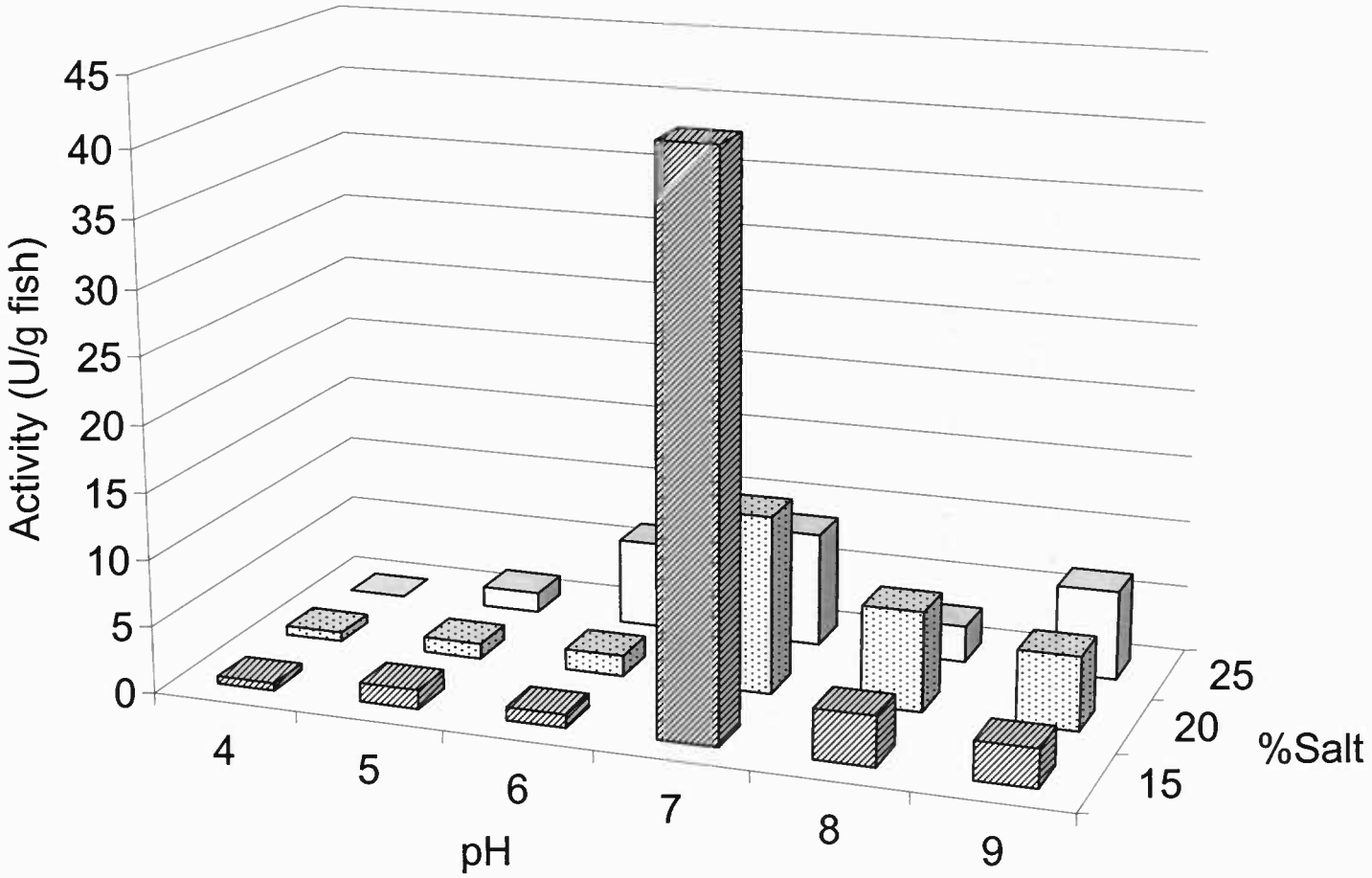

Figure 3.3: Active chymotrypsin-like activity remained in fish sauce fermented for 3 days

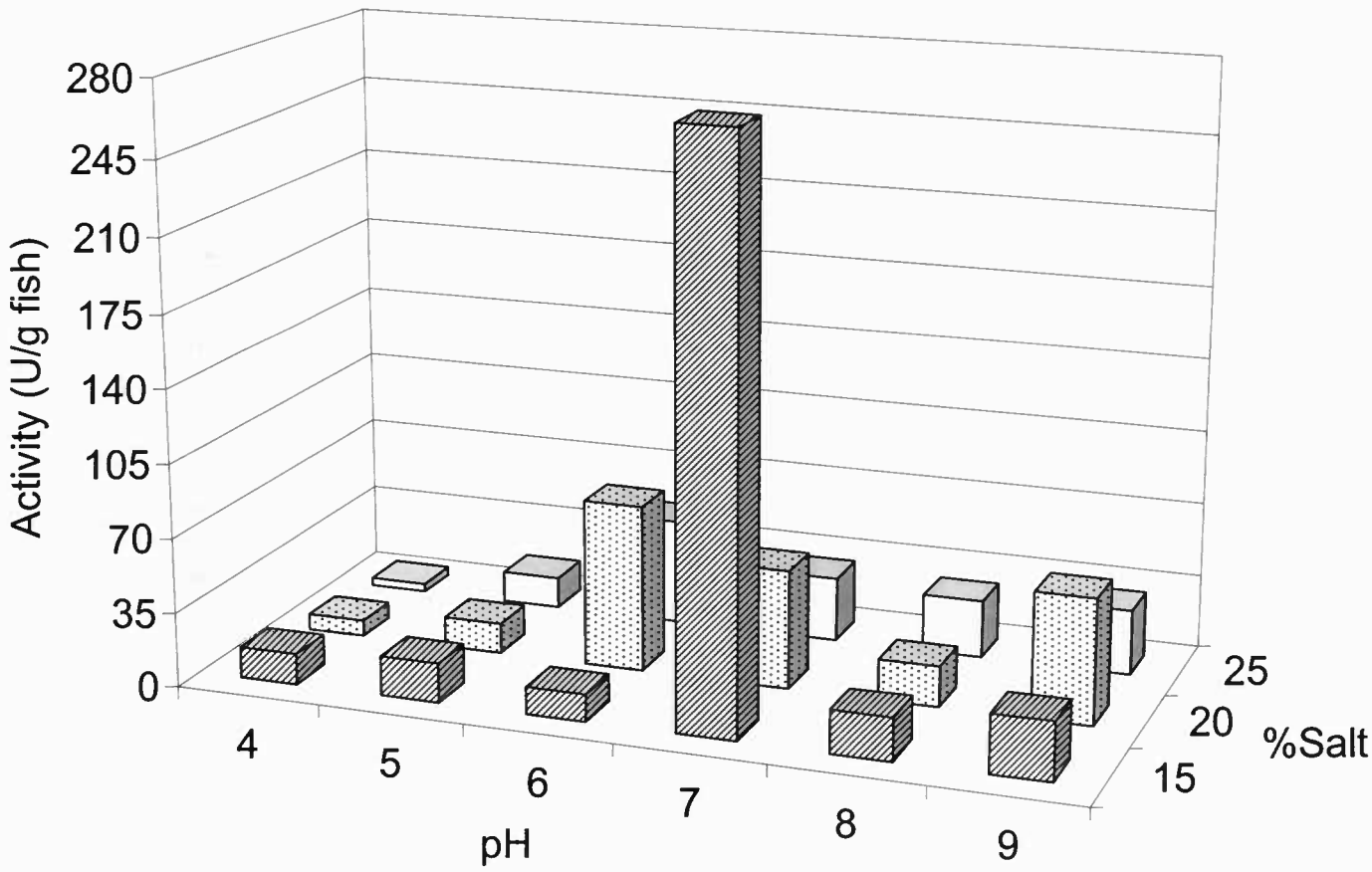

Figure 3.4: Active trypsin-like activity remained in fish sauce fermented for 3 days

7.0). The nonprotonated form of the histidine residue on the catalytic site is the active form of the serine proteases (Polgar 2000). The remaining activity of chymotrypsin and trypsin were higher in samples containing lower salt concentration. These results were similar to the work of Gilberg (1989) where at high salt concentration (26%), trypsin-like activity was reduced during fish sauce fermentation.

Enzyme stability after 60-days fermentation was calculated from the percentage of enzyme activity remaining at 60-day compared to activity at 3-day fermentation. Regarding Table 3.1, cathepsin B-like enzymes had the highest stability at pH 5.0 (salt 15%), cathepsin L-like had the highest stability at pH 5.0 (salt 20%), chymotrypsin-like had the highest stability at pH 5.0 (20% salt) and the stability of trypsin-like enzymes was highest at pH 4.0 (20% salt).

Stability of cathepsin B-like enzymes at pH 5.0 was 30.8% at low salt concentration (15%) and decreased as salt concentration increased: 17.9% at 20% salt and 9.3% at 25% salt. Trypsin-like stability was highest (45.6%) at pH 4.0 in samples containing 20% salt followed by samples containing 25% (43.4 %) and 15% salt (19.9%). Gildberg (1992) found that there was only 10% trypsin-like enzyme activity remaining in protein hydrolysate prepared by fermentation of salted fish viscera (25% salt without pH adjustment) at 27 °C for 50 days.

Stability of enzymes is a function not only of temperature but also of pH, ionic strength and nature of buffer, presence or absence of substrate, concentration of enzyme as well as other proteins in the system, time of incubation, and the presence or absence of activators and inhibitors (Whitaker 1994). In general, an enzyme is

more stable to temperature in an intact tissue or in a homogenate, where its structure is protected by the presence of other colloidal material such as proteins, carbohydrates, than it is in a purified form (Whitaker 1994).

Salt addition (15-25%) increased the ionic strength in samples. Combined with long incubation time at high temperature (35 °C), enzyme activity decreased. Our results showed that the highest stability of cathepsin L-like, B-like, and chymotrypsin-like enzymes were at pH 5.0 and that of trypsin-like enzyme was at pH 4.0. The pH that showed the highest stability of catheptic enzyme was close to the isoelectric point of protein from Pacific whiting (pH 5.0) (Choi and Park 2002).

Whitaker (1994) reported that the maximum stability of trypsin was found at pH 5.0 and the stability was enhanced at higher protein concentrations. In addition, they irreversibly lost all activities above 70 °C. In contrast, solutions of crystallized trypsin are most stable at pH 2 to 3 and can be briefly heated to boiling at this pH without permanent loss in activity (Whitaker 1994). The characteristics of purified enzymes, however, may be different from those existing in fish sauce.

El-Shemy and Levin (1997) found that purified tilapia trypsin showed no detectable loss of enzyme activity at a holding temperature of 0 °C to 40 °C for 30 min. In general trypsins from aquatic species are unstable at acid pH but stable at alkaline pH (Chen and others 1978; Yoshinaka and others 1984). In contrast, Kristjansson (1991) reported that purified trypsin from the pyloric ceca of rainbow trout was stable at pH 5-11 when incubated at 30 °C for 30 min.

Cathepsin L-like and chymotrypsin-like activities in fish sauce after 60-days incubation increased when compared to 3-day fermentation. Cathepsin L-like stability at pH 5.0 was highest (435.1%) in samples containing 20 % salt followed by samples containing 15% salt (218.0%) and 25% salt (42.5%). Chymotrypsin-like activity was highest in samples containing 20% salt at pH 5.0 (109.9%) followed by samples containing 15% salt (37.7%) and 25% salt (0%).

Several possible reasons can be speculated to explain the increase of cathepsin L-like and chymotrypsin-like activities in samples. These include: the activation of procathepsin L (inactive form) to cathepsin L (active form) at pH 5.0-6.0 (Barrette and Rawlings 1998), hydrolysis of chymotrypsinogen (inactive form) to chymotrypsin (active form), and/or by hydrolytic action of trypsin (Torrisen and Male 2000). Furthermore, the increase of enzyme activity was possibly due to the reversible form of the enzyme-inhibitor complex that occurs during autolysis at acidic pH values (Barrette and Rawlings 1998). The reversible inhibitors naturally found in fish sauce fermentation might be peptide aldehydes that were normally found in fermented products. Moreover, the increase of cathepsin L-like and chymotrypsin-like activity at 60-day fermentation may have resulted from the activity of other enzymes, such as those from halophilic bacteria.

Since intracellular fluid of halophilic bacteria contains a considerable concentration of NaCl and KCl, making it possible for their enzymes to work under high salt concentration, their enzymes are thus active at salt concentrations that inhibit or denature many enzymes of non-halophilic organisms (Norberg and Hofsten 1968).

The larger the enzyme, the more complex the structure, and the more susceptible it is to high temperatures (Whitaker 1994). Ingram (1947) has suggested that the halophilic enzymes are smaller than most other enzymes, which would make them more resistant to salting out.

Degree of hydrolysis, total nitrogen, and amino nitrogen

Fish sauce samples containing 15 % salt had the highest degree of hydrolysis (DH) value at pH 4.0 (Fig. 3.5). Samples containing 20 % salt had the highest DH value at pH 5.0, and samples containing 25 % salt had the highest DH value at pH 5.0 (Fig. 3.5). As indicated by the great stability of endogenous enzymes (i.e., cathepsin L-like, B-like, and chymotrypsin-like enzymes) at pH 5.0, the extent of proteolysis of Pacific whiting fish sauce was more affected by endogenous enzyme activities than acid hydrolysis.

It was interesting to see that pH adjustment to 4.0 and 5.0 provided 1.8-2.4 times greater DH compared to pH 7.0 (Fig. 3.5). Fish sauce containing 15% salt showed 2.2 times greater DH at pH 4.0 than at pH 7.0 (Fig. 3.5). DH of fish sauce containing 20% salt at pH 5.0 was 2.4 times greater than that at pH 7.0, while DH of fish sauce containing 25% salt at pH 5.0 was 1.8 times greater than that at pH 7.0 (Fig. 3.5). An interactive effect of pH and salt concentration on the extent of protein hydrolysis was possibly due to the effect of endogenous enzyme activities in Pacific whiting. Beddows and Ardeshir (1979) reported similar results that the presence of high salt concentration generally decreased the extent of proteolysis. Orejana and

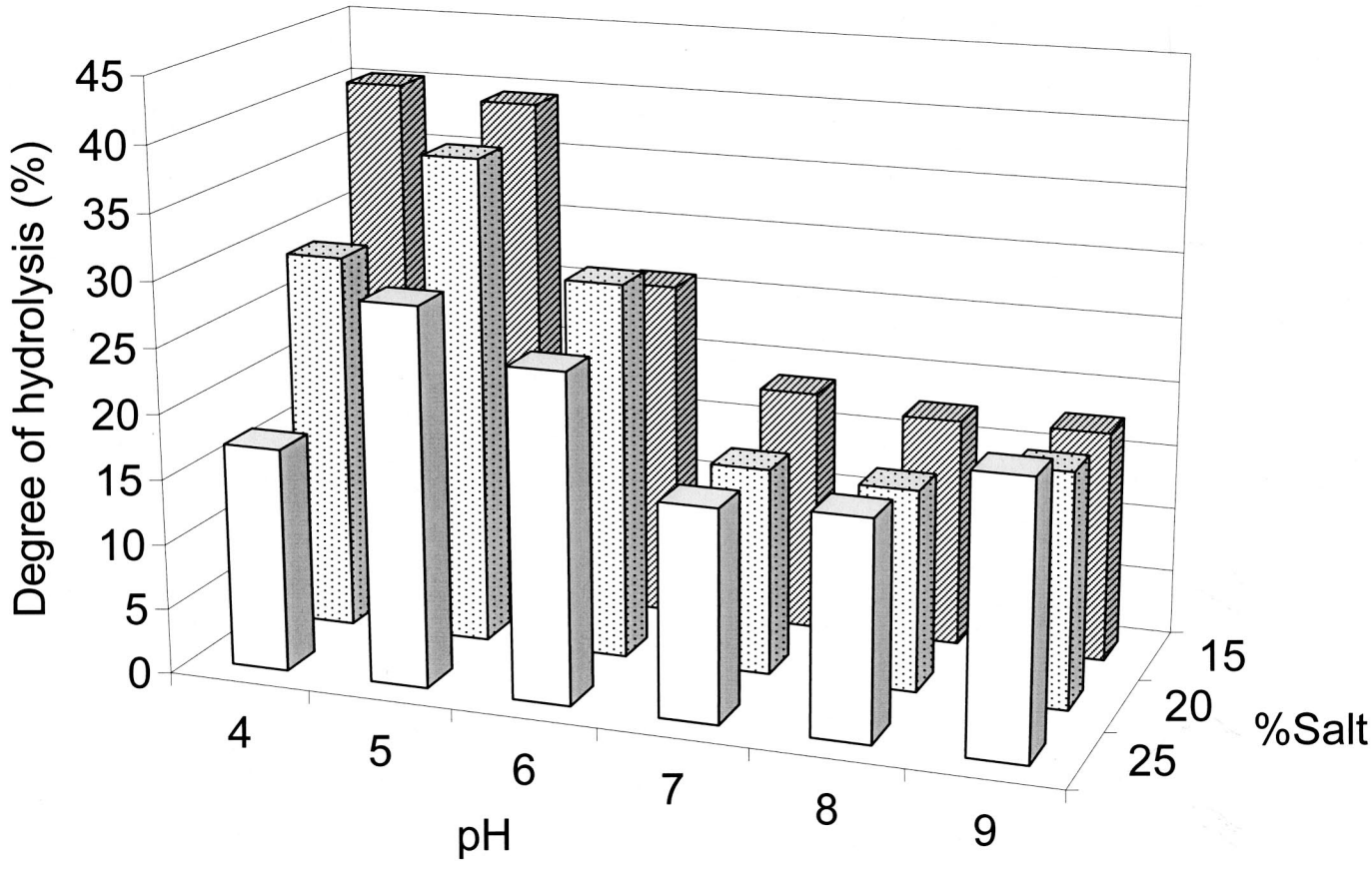

Figure 3.5: Degree of hydrolysis in fish sauce fermented for 60 days

Liston (1982) reported that endogenous fish enzymes are primarily responsible for the hydrolysis of intracellular muscle proteins during the fish sauce fermentation process.

In this experiment, the extent of protein hydrolysis of Pacific whiting fish sauce was greatest at a processing pH between 4 and 6 (Fig. 3.5). Endogenous enzymes had different optimum pH for their respective activities and various salt concentrations affected the ionic strength. Cathepsin L and cathepsin B are predominant in Pacific whiting muscle and have an optimum pH between 5.0 and 6.0 (An and others 1994). Cathepsin B was the most active cysteine protease in Pacific whiting fish fillets (An and others 1994; Kang and Lanier 2000). Cysteine proteases were primarily responsible for the degradation of proteins in Pacific whiting byproducts, while serine proteases and trypsin-like enzymes had a minor role in hydrolyzing byproducts during fermentation (Lopetcharat 1999).

Amino nitrogen content (Fig. 3.6) and total nitrogen content (Fig. 3.7) showed similar trends compared to DH values. Lower salt concentration resulted in greater amino nitrogen content (Fig. 3.6). Adjusting the processing pH lower than neutral pH resulted in a greater amino nitrogen content (Fig. 3.6). The greatest amino nitrogen value was found in samples at pH 4.0 with 15% salt (Fig. 3.6). Cho and others (2000) found similar results that amino nitrogen content in salted anchovy, containing lower salt concentrations (8% and 15%), were greater than samples containing higher salt concentrations (25% and 35%) at both 3-day and 10-day fermentation.

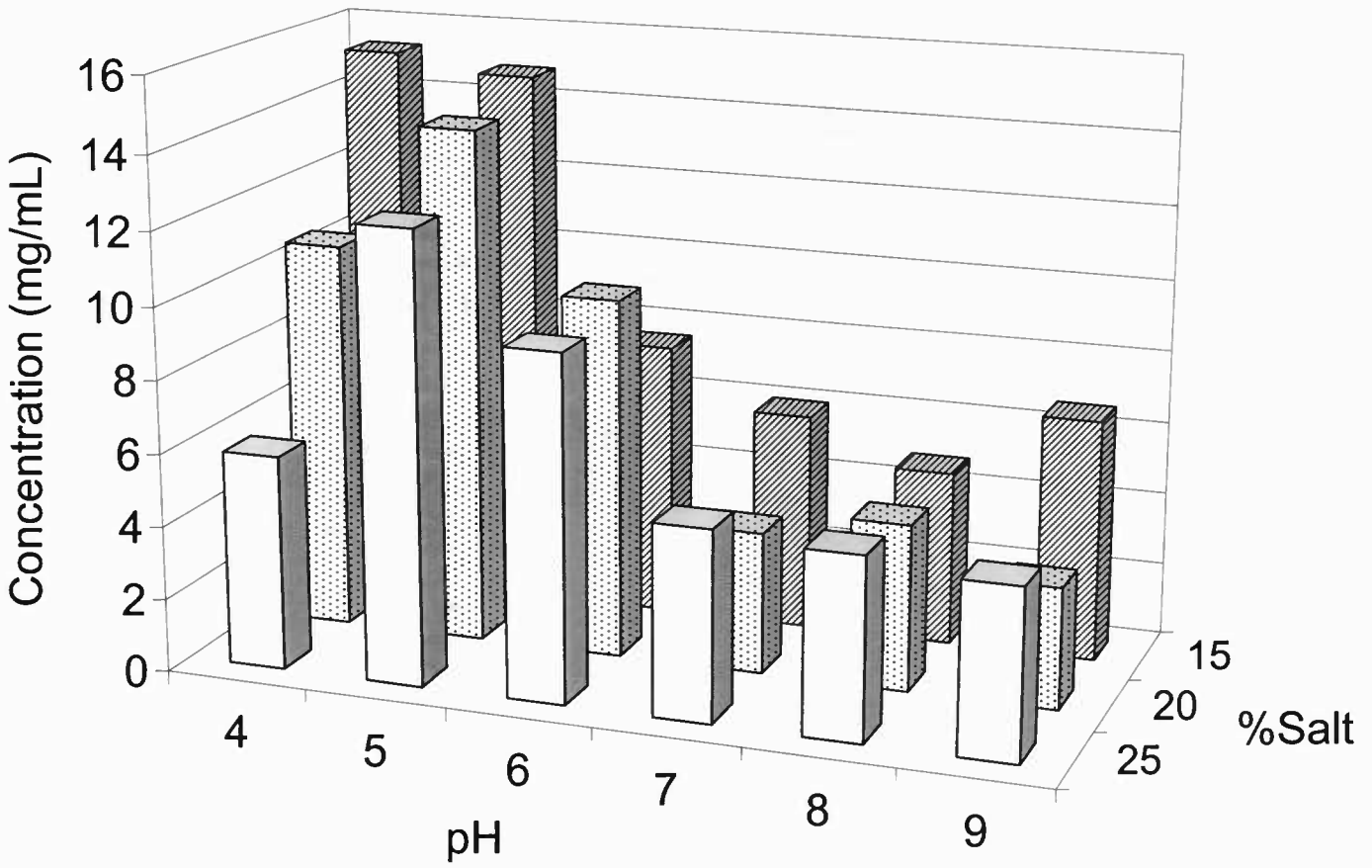

Figure 3.6: Amino nitrogen in fish sauce fermented for 60 days

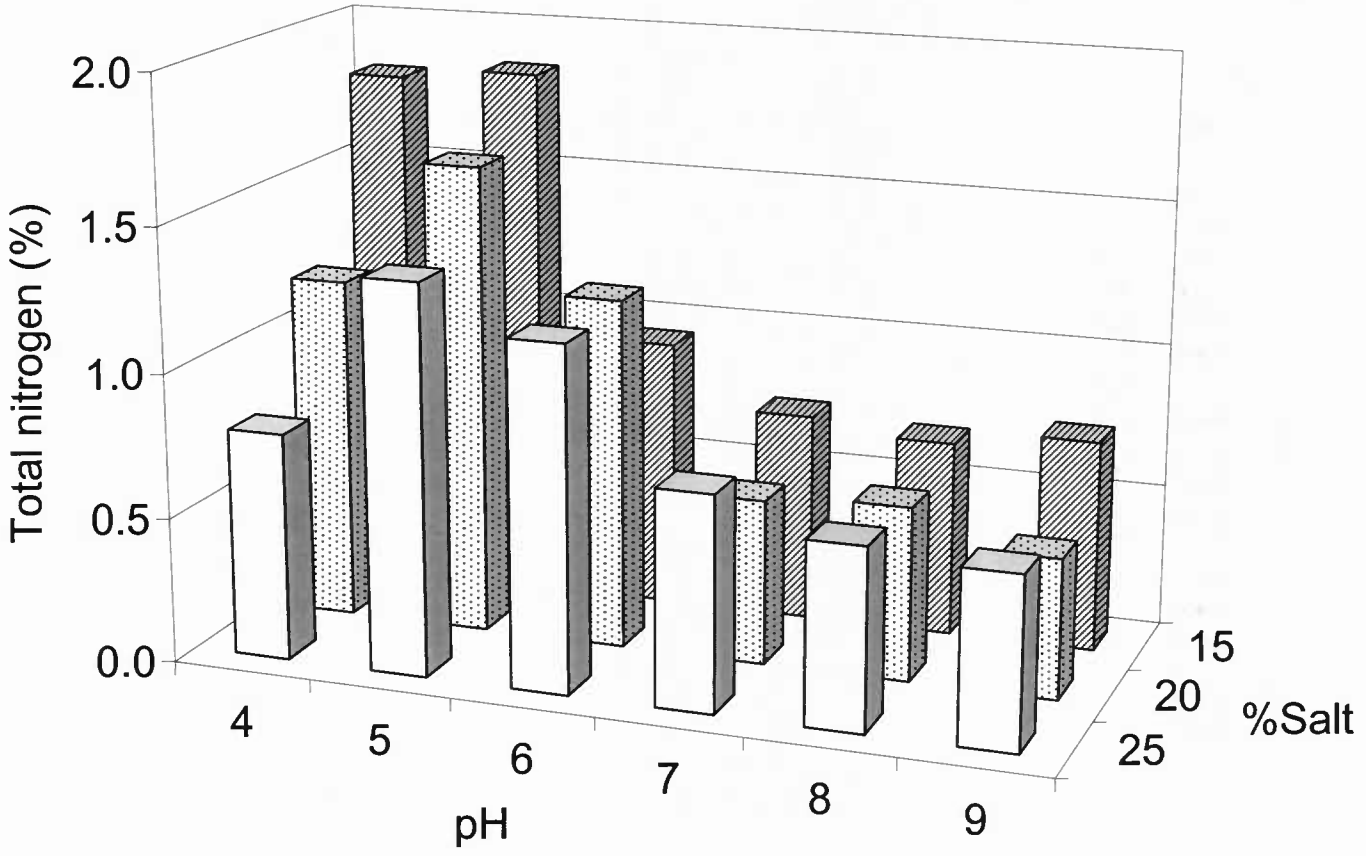

Figure 3.7: Total nitrogen in fish sauce fermented for 60 days

An increase in amino nitrogen concentration is related to the degradation of polypeptides. It was found that lower salt concentration provided a greater amino nitrogen concentration (Fig. 3.6) and thus a greater degree of protein degradation. Similar trends were found in total nitrogen content in fish sauce fermented for 60 days (Fig. 3.7). Our results showed that the greatest total nitrogen content at 60-day fermentation was found at pH 5.0 and lower salt concentration produced greater total nitrogen content (15%>20%>25%) (Fig. 3.6). Similar trends were reported that the extractive nitrogen content in salted anchovy, after fermenting for 10 days at 20 °C, were higher in samples containing lower salt (8% and 15%) than those containing higher salt concentrations (25% and 35%) (Cho and others 2000).

CONCLUSION

Due to the specificity of enzymes, an interactive effect of pH and salt concentration showed various effects on the endogenous enzyme activities in Pacific whiting fish sauce. Proteolysis in Pacific whiting fish sauce was primarily affected by endogenous enzyme actions. Adjustment of pH and salt concentration accelerated the fermentation process. Acidic pH with low salt concentration provided a greater degree of hydrolysis, total nitrogen, and amino nitrogen content in fish sauce compared to fish sauce obtained from the traditional process (neutral pH with 25% salt).

ACKNOWLEDGEMENT

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Chapter 4

DEVELOPMENT OF PACIFIC WHITING FISH SAUCE: MARKET POTENTIAL AND MANUFACTURING IN THE UNITED STATES

S. Tungkawachara and J.W. Park

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Alaska SeaGrant

ABSTRACT

Manufacturing fish sauce from whole Pacific whiting (W) and a mixture (50/50) (WB) of surimi byproducts and whole fish was investigated to develop histamine-free fish sauce and to better utilize solid byproducts. This study also discusses the market potential of the respective fish sauces.

Market potential for Pacific whiting fish sauce in the United States was evaluated through phone interview and consumer panelists. Fish sauce import reached $16.6 million in the United States in 2000. Anchovy was the most popular raw material used (47%) in the fish sauce process and brand name was the most important factor in the purchase decision (52%). Based on market info, good quality, and low cost, fish sauce from Pacific whiting had great potential in replacing imported anchovy fish sauce, which contains histamine.

INTRODUCTION

Fish sauce is a clear liquid condiment with an amber color and salty/mild cheesy flavors that is commonly used as a flavor enhancer or salt replacement for various food preparations in Southeast Asia. Fish sauce from anchovies is produced through natural fermentation by storing a mixture (1:3) of salt and minced fish at subtropical temperatures. In Thailand, where the majority of commercial fish sauce in

the world is manufactured, fish sauce is processed through fermentation at ambient temperatures (30-35 °C) for 12 to 18 mo.

Fermentation is conducted by the combined function of proteolytic enzymes and halophilic microorganisms from the viscera (Sikorski and others 1995; Uyenco and others 1953). Endogenous fish enzymes are primarily responsible for degradation of muscle proteins during fermentation (Orejana and Liston 1982). The enzymes slowly hydrolyze the fish proteins during storage (Sikorski and others 1995).

Pacific whiting (*Merluccius productus*), which is abundant off the Pacific Northwest, was excluded from commercial utilization until 1991 due to endogenous proteolytic enzymes that caused texture softening. An and others (1994) reported that cathepsin B was the most active cysteine protease in Pacific whiting fish fillets. Serine proteases, cathepsin B-like enzymes, trypsin-like enzymes, and metalloproteases were almost equally responsible for protein hydrolysis at 35 °C during fermentation of whole Pacific whiting. In contrast, cysteine proteases were primarily responsible for the degradation of proteins from byproducts during fermentation at 35 °C (Lopetcharat 1999).

Lopetcharat and Park (2002) were the first to study the feasibility of fish sauce production from Pacific whiting. However, a consumer liking profile for fish sauce from Pacific whiting was neither evaluated nor compared with the biochemical properties. Our objective, therefore, was to evaluate the market potential, in the United States, of fish sauce made from whole Pacific whiting or a mixture of surimi byproducts and whole fish based on phone interview.

METHODS

Market potential

Information regarding imported fish sauce in the United States, including market size and the country of origin, was obtained from the USITC Trade Database (USITC 2000). A list of importers and dealers of fishery products was obtained from the U.S. Department of Commerce (NMFS 1999). A market survey was performed using phone interview to obtain various product information including the country of origin, package size, raw material, and other factors affecting the purchase decision. Consumer liking test was performed with 75 volunteer consumers. The examples of ballots for phone interview of importers, dealers, and retailers as well as for consumer liking test were shown in the appendix A, B, C, and D.

Biochemical properties

Biochemical properties (pH, moisture content, total nitrogen, salinity) of Pacific whiting fish sauce were determined according to AOAC (1995). Free amino acid content was determined auto amino acid analyzer (LKB-Biochrom 20, Pharmacia-Biotech, Buckinghamshire, UK). Standard amino acids were used to identify the amino acid profile in Pacific whiting fish sauce samples. Type and concentration of amino acids in Pacific whiting fish sauce samples were obtained by

comparing the retention time as well as the peak area between the standard amino acids and the unknown compounds.

RESULTS AND DISCUSSIONS

Market survey

Market size and country of origin

In dollar value, fish sauce imported to the USA was $16.6 million in the year 2000 (USITC 2000). It increased by 3.7% when compared to imports from two years ago. The majority of import (72% of total value) was from Thailand (Fig. 4.1). More than 60 % of the total import was brought into the United States through the West Coast ports and distributed to dealers and/or directly to retailers. The three major custom districts of entry for fish sauce import to the United States in 2000 were Los Angeles (47%), San Francisco (17%), and New York (16.3 %)(Fig. 4.2).

Dealers/importers interview

According to one of the largest fish sauce importers, the import price of fish sauce products was about $0.75/Kg. According to phone interview of two importers and a dealer, it was concluded that brand name, customer's need, quality, and price were the factors affecting the purchasing decision for importers. The retail bottle (710

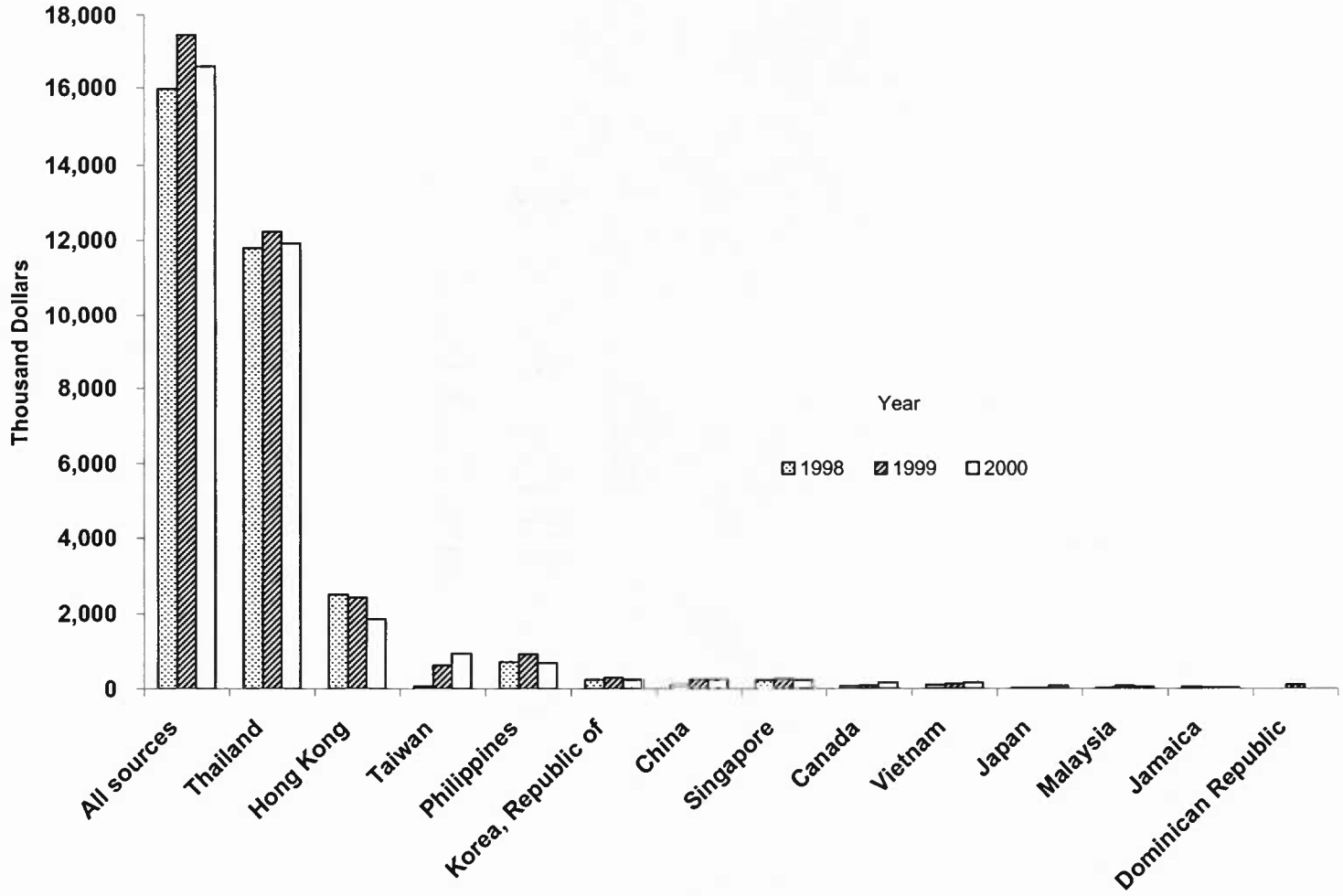

Figure 4.1: Country of exported fish sauce
Source of data: U.S. International Trade Commission-2000 Tariff Database

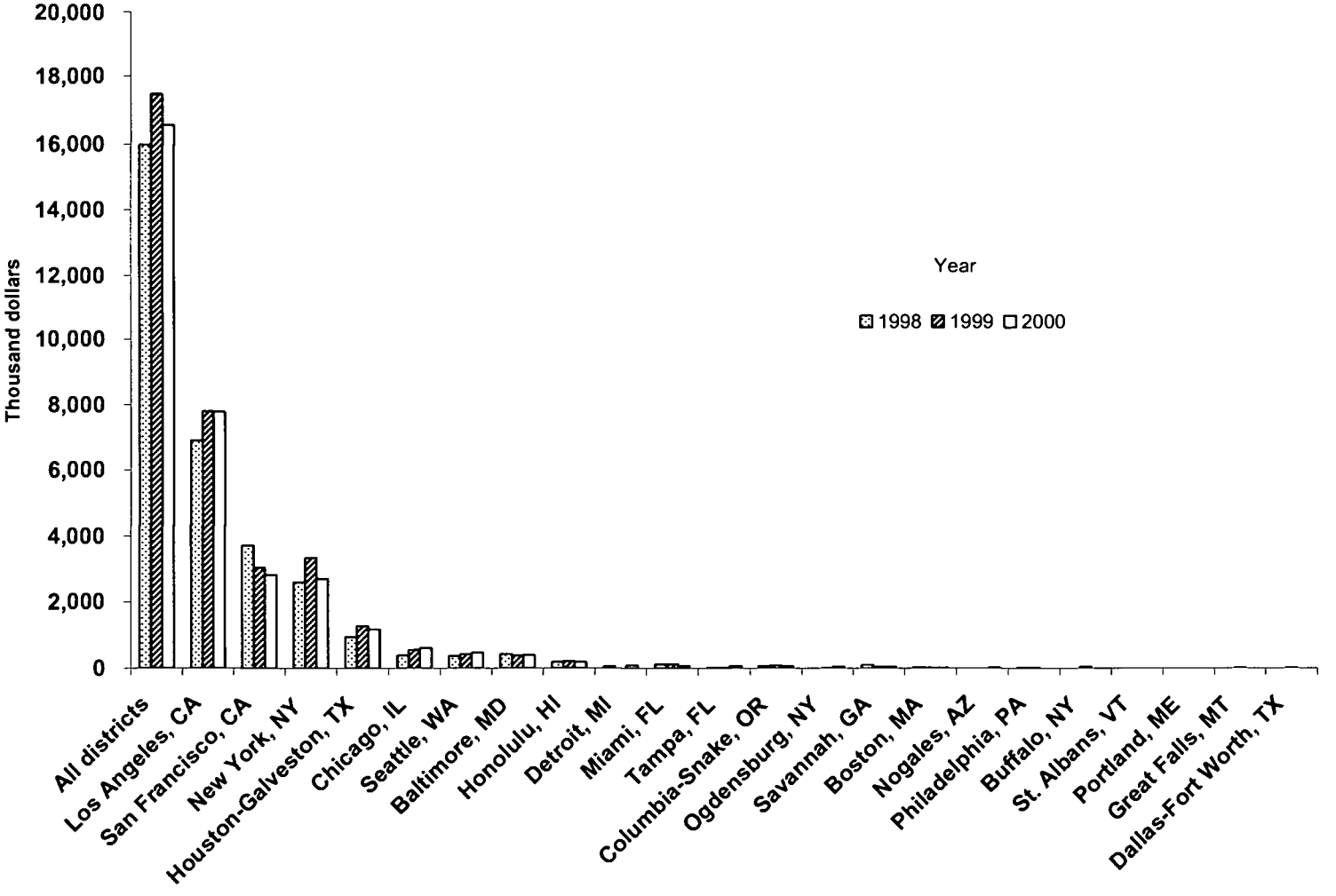

Figure 4.2: Customs district of entry for fish sauce imported in USA
Source of data: U.S. International Trade Commission-2000 Tariff Database

mL) made from either glass or plastic was the most frequently used container. Products were distributed with the original package without repackaging.

Importer A in Seattle (WA) imports fish sauce from Thailand, Malaysia, the Philippines, Vietnam, Japan, China, and Korea. This company distributes the products to the Northwest region through wholesale brokers in Seattle as well as through direct sales to some Asian groceries in the Northwest. This importer carries both fish sauce and soy sauce products in equal proportions based on dollar value. Its customers make their purchasing decision based primarily on brand name and sweet flavor.

Dealer B, in Portland, OR, buys fish sauce from an importer in Seattle. Its products were primarily from Thailand and the Philippines. It distributes products to grocery stores and carries many kinds of Asian food products. Fish sauce covers about 10 to 20 % of total products based on dollar values. Customer's need and brand name were considered as the most important purchasing factors.

Importer C in San Francisco, CA, imports fish sauce only from Thailand. It works as an import agent for the biggest fish sauce company in Thailand. The three most famous brand names of fish sauce are directly distributed to groceries in the Northwest US region. The yearly import quantity of fish sauce was about 5,000 M/T, which is about 30 % of the total fish sauce imported to the United States each year. All of the imported fish sauce was made from anchovies. The grade of fish sauce was considered the most important attribute in making a purchase decision followed by brand name and price. Color and sweet flavor also affected its decision-making.

FDA has listed the tolerance level for histamine in fish products at 50 ppm in its HACCP guideline (Gingerich 1999). Ironically, the histamine content in fish sauce was not a concern for any of the fish sauce dealers/importers. Furthermore, they do not believe there is a potential problem of high histamine content in anchovy fish sauce. However, Putro (1993) reported that sardine fish sauce in Indonesia had histamine content in the range of 140 to 230 ppm. In addition, Sanceda and others (1996) found histamine in fish sauce from many countries, for example, 40 ppm in the Philippines fish sauce and 430 ppm in Thai fish sauce.

Retailer interviews

According to the retailer survey via phone interview, twenty-one grocers out of 89 grocers throughout the USA participated in this study. Information surveyed included country of origin, proportion of fish sauce products to entire food products sold (in dollars), package size, raw material used in fish sauce, and factors affecting the purchase decision. We found that most fish sauce products in groceries were made in Thailand (36 %), followed by the Philippines (10%), Vietnam (10%), and Japan (10%) (Fig. 4.3). In more than one third of groceries the proportion of fish sauce was less than 10% of the entire food products based on dollar value. Most fish sauce products (55 %) were distributed in medium-size packages (401-750 mL) and 21% of fish sauce products were distributed in large-size packages (751-1000 mL) (Fig. 4.4). Anchovy was the most popular (47 %) source of raw material in fish sauce products followed by sardine (10%) and krill/shrimp (10%) (Fig. 4.5). Brand

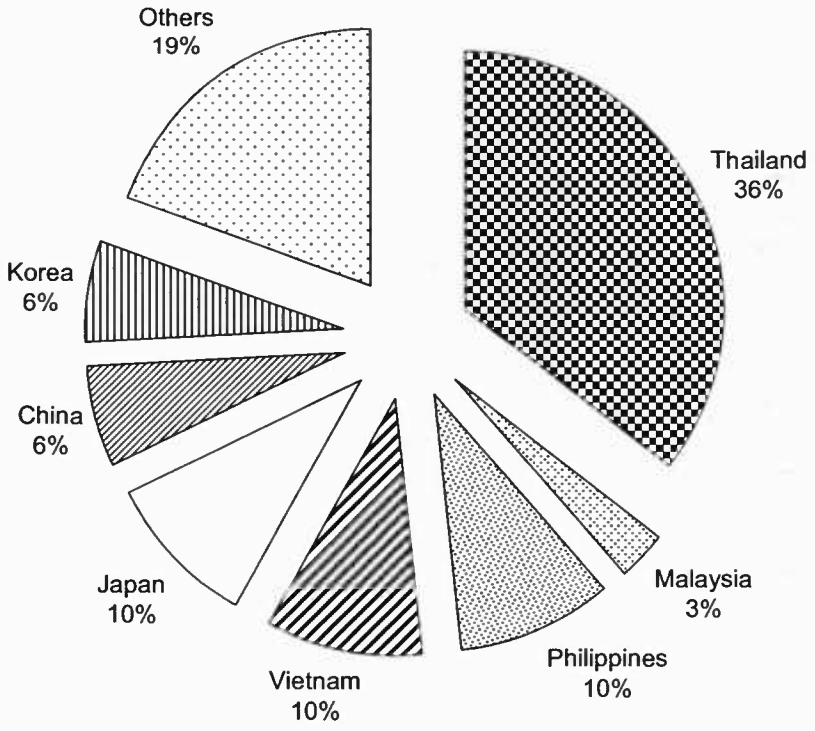

Figure 4.3: Country of origin of fish sauce
Source of data: Retailer interviews

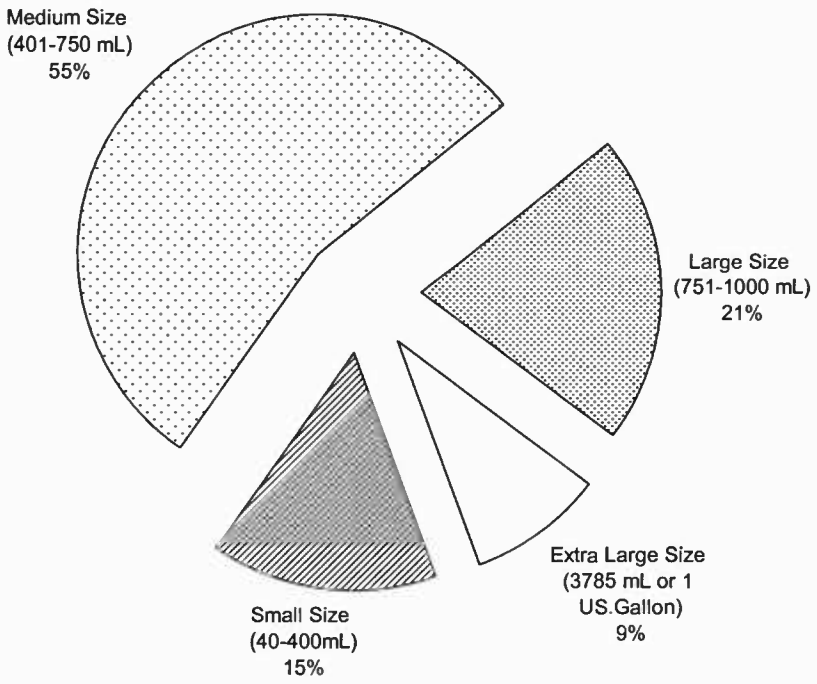

Figure 4.4: Package size of fish sauce products
Source of data: Retailer interviews

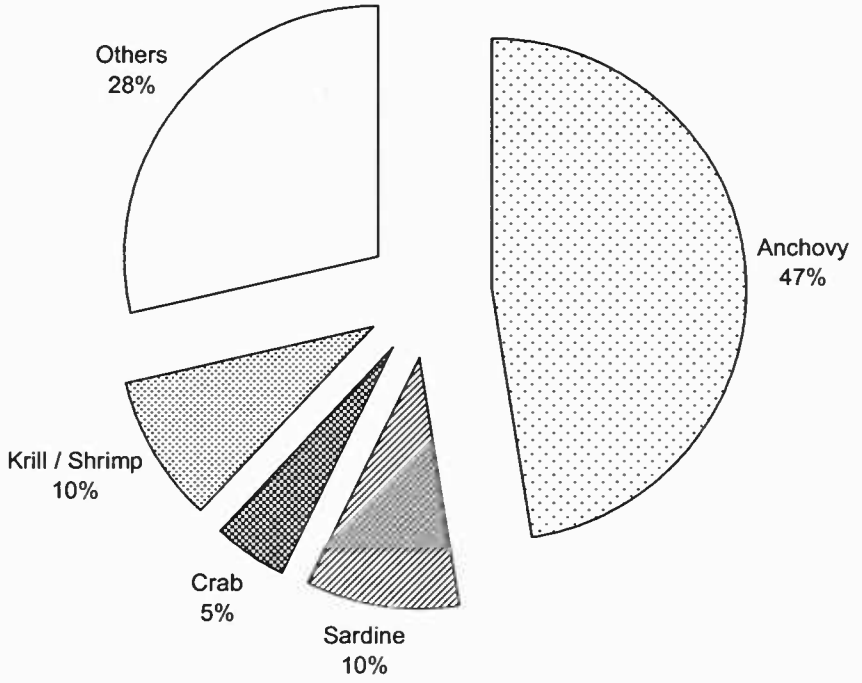

Figure 4.5: Raw materials commonly used for fish sauce

Source of data: Retailer interviews

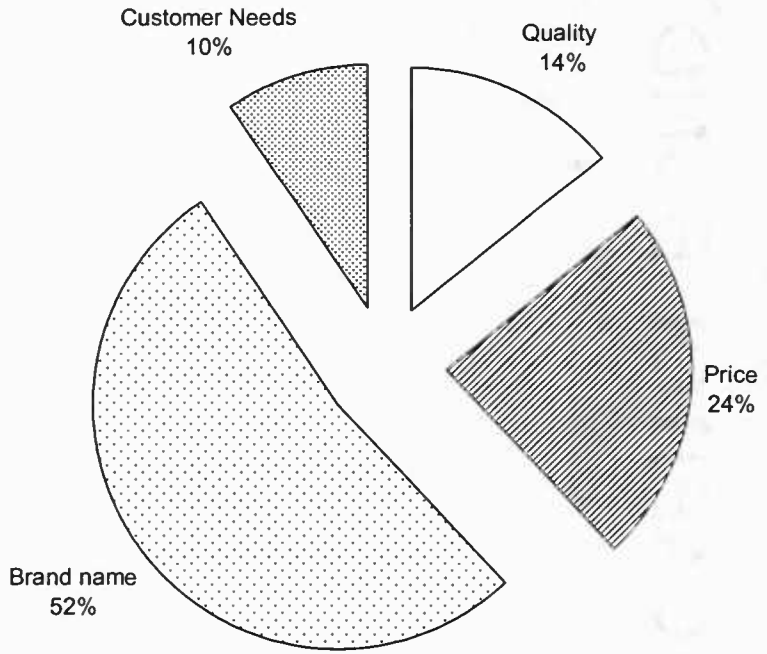

Figure 4.6: Factors affecting a purchase decision for fish sauce

Source of data: Retailer interviews

Table 4.1: Consumer liking of fish sauce fermented for 12 months

Attributes	Consumer group	Fish sauce samples		
		W	WB	C
Color liking	All consumers (n=75)	6.40^{ab}±1.77	6.12^{bc}±1.94	6.92^{a}±1.50
	Asian (n=40)	6.58^{a}±1.66	6.28^{a}±2.01	6.80^{a}±1.59
	Caucasian (n=35)	6.20^{ab}±1.89	5.94^{b}±1.88	7.06^{a}±1.39
	Female (n=51)	6.32^{a}±2.01	6.22^{a}±2.05	7.00^{a}±1.64
	Male (n=24)	6.33^{a}±1.24	5.92^{a}±1.72	6.75^{a}±1.15
Flavor liking	All consumers (n=75)	6.07^{a}±2.02	5.76^{a}±2.07	6.31^{a}±2.22
	Asian (n=40)	5.93^{a}±1.95	5.35^{a}±2.29	6.30^{a}±2.33
	Caucasian (n=35)	6.22^{a}±2.12	6.23^{a}±1.70	6.31^{a}±2.13
	Female (n=51)	5.77^{a}±2.19	5.61^{a}±2.20	6.33^{a}±2.29
	Male (n=24)	6.33^{a}±1.61	6.08^{a}±1.77	6.25^{a}±2.13
Overall liking	All consumers (n=75)	6.09^{a}±1.90	5.93^{a}±1.91	6.37^{a}±1.98
	Asian (n=40)	5.90^{a}±1.97	5.58^{a}±2.10	6.35^{a}±2.09
	Caucasian (n=35)	6.31^{a}±1.83	6.34^{a}±1.61	6.40^{a}±1.87
	Female (n=51)	5.88^{a}±2.10	5.82^{a}±2.02	6.39^{a}±1.96
	Male (n=24)	6.54^{a}±1.32	6.17^{a}±1.69	6.33^{a}±2.06

Results are expressed as the means ± standard deviation of the liking of panelists (n = number of panelists).
Liking score: 9 = extremely like, 5 = neither like nor dislike, 1 = extremely dislike
Means with the same letter in each row are not significantly different.
W = Fish sauce made from whole Pacific whiting
WB = Fish sauce made from the mixture (1:1) of whole fish and surimi byproducts
C = Commercial anchovy fish sauce

Adapted from Tungkawachara and others (2003)

name was the primary reason (52%) for grocery stores to make a purchasing decision, followed by price (24%), quality attributes (sweetness, saltiness, and color) (14 %), and customer's needs (10%) (Fig. 4.6).

Consumer acceptability

Tungkawachara and others. (2003) conducted a consumer acceptability test for Pacific whiting fish sauce. The majority of panelists (93.3%) were fish sauce users at home, consuming fish sauce at least once a month. As for ethnic groups, Asians composed 53.3% and Caucasians composed 46.7%. The majority (89.3%) was between 20-59 years old and 68.0% of the panelists were female. There was no significant difference in overall sensory liking and flavor liking for all fish sauce samples ($P>0.05$) (Table 4.1). Fish sauce WB, however, had lower color liking scores than commercial anchovy fish sauce ($P<0.05$), whereas, no significant difference in color liking was detected between fish sauce W and commercial anchovy fish sauce ($P>0.05$). Regarding consumer liking by ethnic group, Asians (n=40) preferred fish sauce W and WB as much as commercial anchovy fish sauce for color, flavor, and overall liking ($P>0.05$) (Table 4.1). Caucasians (n=35), on the other hand, preferred fish sauce W and WB as much as commercial anchovy fish sauce with regard to flavor and overall liking ($P>0.05$), but not for color liking ($P<0.05$) (Table 4.1). In terms of color, they rated the same preference levels for commercial fish sauce and fish sauce W ($P>0.05$), but rated commercial anchovy fish sauce greater than fish sauce WB ($P<0.05$). Regarding consumer liking by gender, there was no difference in terms of color, flavor, and overall liking ($P>0.05$) (Table 4.1).

Biochemical properties

Fresh Pacific whiting flesh had neutral pH (6.93). The pH decreased gradually during fermentation reaching 5.6 after 9 mo (Tungkawachara and others 2003) (Table

4.2). The pH of fish sauce WB was lower than that of fish sauce W ($P<0.05$). This was probably due to increased protein hydrolysis by the proteolytic activities of intestines included in fish sauce production, which results in more free hydrogen ions. Fermentation products containing organic acids such as lactic acid and acetic acid also tend to lower the pH of fish sauce (Itoh and others 1993; Funatsu and others 2000; Michihata and others 2000). The degree of hydrolysis in fish sauce WB increased as fermentation time was extended. The degree of hydrolysis of fish sauce W, though, increased up to 3 mo, and then decreased (Tungkawachara and others 2003). Comparing fish sauce W and WB, a significant difference in the degree of hydrolysis was found only at 9 mo ($P<0.05$). Degree of hydrolysis represents the extent of the hydrolytic degradation of protein (Adler-Nissen 1986). Orejana and Liston (1982) concluded that endogenous enzymes are the major agents responsible for protein digestion in the fish sauce process.

Moisture content decreased, while protein content increased as fermentation continued (Table 4.2). This is most likely due to protein hydrolysis and/or the possible moisture evaporation through the loosely closed lid. At the early stage of fermentation, fish sauce WB had higher protein content than fish sauce W ($P<0.05$). However, there was no significant difference at 9 mo ($P>0.05$). At 0 mo of fermentation (referring to 1 day after fermentation), the moisture content of the two fish sauce samples was not significantly different ($P>0.05$). After the first month of fermentation, however, fish sauce WB had a lower moisture content than fish sauce

W ($P<0.05$). The lower moisture content and higher protein content in fish sauce WB was thought to be due to the greater protein hydrolysis by intestines included in the fish sauce production.

Table 4.2: Changes of pH, moisture content, and total nitrogen content of fish sauce during fermentation

Fermentation time (Mo)	Sample	Moisture (%)	Total nitrogen (mg/ 100 g)	Salinity[a] (%)	pH
0	W	73.4±0.5	375±49	23.6	7.05±0.01
	WB	73.0±0.1	553±45	22.9	6.66±0.01
1	W	71.7±0.6	743±72	23.0	6.64±0.01
	WB	70.8±0.1	853±43	23.2	5.75±0.01
3	W	68.0±0.1	1128±30	24.3	5.72±0.01
	WB	66.8±0.1	1322±18	24.2	5.64±0.01
9	W	68.0±0.0	1282±2	23.3	5.42±0.01
	WB	64.4±0.1	1357±75	26.4	5.34±0.01

The mean and standard deviation were derived based on data obtained from triplicate runs.
[a]Salinity was calculated by subtracting % protein, % moisture, and 0.75% fat from 100.
W = Fish sauce made from whole Pacific whiting
WB = Fish sauce made from the mixture (1:1) of whole fish and surimi byproducts

Adapted from Tungkawachara and others (2003)

Total nitrogen in fish sauce is composed of protein nitrogen and non-protein nitrogen (NPN) compounds such as free amino acids, nucleotides, peptides, ammonia,

urea, TMAO, and so forth. These compounds contribute to the specific aroma and flavor (Finne 1992; Shahidi 1994). Total nitrogen content in fish sauce depends on fish species and the chemical composition of fish. About 80 percent of total nitrogen in fish sauce remains in the form of amino acids (Dougan and Howard 1975).

Total nitrogen in fish sauce increased during fermentation (Table 4.2). At the early stages of fermentation (1 and 3 mo), fish sauce WB had higher total nitrogen than fish sauce W ($P<0.05$), possibly due to the greater degree of hydrolysis. After 9 mo fermentation, fish sauce W and WB had a total nitrogen content of 1.28 and 1.36 percent, respectively In comparison, Japanese fish sauce made from mackerel, sardine, and squid after 12 mo fermentation had a total nitrogen content of 1.89, 1.52, and 1.48 percent, respectively (Funatsu and others 2000).

The amino acid composition of Pacific whiting fish sauce fermented for 9 mo is shown in Table 4.3 (Tungkawachara and others 2003). The amino acid compositions of fish sauce WB and W were quite similar. Total free amino acids increased as fermentation continued. However, after 9 mo of fermentation, total free amino acids in fish sauce W (38.37 mg/mL) were twice as much as fish sauce WB (16.56 mg/mL).

Glutamic acid, alanine, leucine, lysine, and arginine were rich in the 9-mo samples and accounted for 43.27 and 54.08 % of total amino acids in fish sauce W and WB, respectively. According to Benjakul and Morrissey (1997), Pacific whiting muscle is composed of 6 major amino acids (glutamic acid, aspartic acid, lysine, leucine, arginine, and alanine), which accounts for 56.54 % of the total protein.

Table 4.3: Amino acid composition in fish sauce fermented for 9 months

Amino acid	Concentration (mg/mL)		Taste threshold[a]	Taste value[b]	
	W	WB	(g/dL)	W	WB
Arginine	3.074	1.516	0.050	61.5	30.3
Aspartic acid	1.698	0.891	0.003	566.0	297.0
Threonine	1.716	1.145	0.260	6.6	4.4
Serine	1.684	0.935	0.150	11.2	6.2
Glutamic acid	3.435	1.623	0.005	687.0	324.6
Proline	2.660	0.000	0.300	8.9	0.0
Glycine	1.036	0.482	0.130	8.0	3.7
Alanine	2.743	1.664	0.060	45.7	27.7
Valine	2.404	1.083	0.040	60.1	27.1
Methionine	1.366	0.217	0.030	45.5	7.2
Isoleucine	1.709	0.814	0.090	19.0	9.0
Leucine	3.528	1.873	0.190	18.6	9.9
Phenylalanine	1.310	0.625	0.090	14.6	6.9
Lysine	3.825	2.277	0.050	76.5	45.5
Histidine	0.199	0.065	0.020	10.0	3.3
Ammonia	0.200	0.286	NA	-	-
DL-allohydroxylysine	0.136	0.055	NA	-	-
Ornithine	0.031	0.000	NA	-	-
1-methylhistidine	0.270	0.064	NA	-	-
Taurine	0.529	0.335	NA	-	-
Urea	0.000	0.189	NA	-	-
Phosphoserine	0.104	0.037	NA	-	-
α-aminoisobutyric acid	0.026	0.000	NA	-	-
Hydroxyproline	3.656	0.000	NA	-	-
Tyrosine	0.525	0.263	NA	-	-
Cystathionine	0.201	0.115	NA	-	-
Total	38.374	16.555	-	-	-

W = Fish sauce made from whole Pacific whiting
WB = Fish sauce made from the mixture (1:1) of whole fish and surimi byproducts
Taste threshold[a] (Kato and others 1989)
Taste value[b] calculated using Cha and Cadwallader (1998)
NA = Not available for taste threshold data.

Adapted from Tungkawachara and others (2003)

Similar free amino acid profiles were found in skipjack tuna sauce (Cha and Cadwallader 1998) as well as in herring protein hydrolysate (Liceaga-Gesualdo and Li-chan 1999). Ijong and Ohta (1996) found that alanine, isoleucine, glutamic acid, and lysine were high in a traditional Indonesian fish sauce.

Some differences in amino acid profile between fish sauce W and WB were possibly due to the differences in protein composition. Fish sauce WB was made from raw materials containing a significant amount of fish skins, bones, heads, and other connective tissues, unlike fish sauce W, which was manufactured from whole fish.

Amino acids dictate the taste of seafood (Sikorski 1994). Taste values (Table 4.3) were calculated based on the amino acid concentration and taste threshold data according to the method described by Cha and Cadwallader (1998). Taste values of amino acids from fish sauce W were 2 times greater than those from fish sauce WB. Glutamic acids and aspartic acids showed the highest taste values and the lowest threshold in both samples. Various amino acids carry their own taste. Lysine, alanine, glycine, serine, and threonine give a sweet taste, while arginine, leucine, valine, phenylalanine, histidine, and isoleucine give a bitter taste, and aspartic acids give a sour taste (Kato and others 1989). High content of glutamic acid in fish sauce might make an important contribution to the good taste due to the development of the umami taste (Komata 1990; Sanceda and others 1990). Cha and Cadwallader (1998) stated that the specific free amino acids having sweet, sour, and bitter tastes may play a prominent role in the overall taste of fish sauce. Some volatile amino acids contribute to the aroma of fish sauce, as well. Glutamic acid gives a meaty aroma, whereas isoleucine and leucine give a sweet aroma. In addition, methionine gives a methyl sulfide-like aroma and phenylalanine gives a strong rose-like aroma (Saisithi and others 1966).

CONCLUSION

Market information of fish sauce in the United States was reviewed along with the biochemical properties of Pacific whiting fish sauce. Brand name was the primary reason for grocers to make a purchasing decision, followed by price, and quality attributes (sweetness, saltiness, and color). The two fish sauce samples developed from Pacific whiting showed similar biochemical properties during 9 mo of fermentation. The consumer test and biochemical properties of fish sauce demonstrated that high quality Pacific whiting fish sauce can successfully replace imported anchovy fish sauce. Furthermore, solid byproducts from surimi processing can be utilized as a raw material for fish sauce.

ACKNOWLEDGEMENT

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Chapter 5

ACE INHIBITION AND ANTI-OXIDATIVE ACTIVITY OF PACIFIC WHITING FISH SAUCE

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To be submitted to Journal of Food Science
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ABSTRACT

The greatest ACE inhibition (96.8%) was found in samples fermented at pH 5.0 for 30 days and 15% salt followed by 20% and 25% salt, respectively (91.2% and 82.1% ACE inhibition). As for the pH effect, ACE inhibition at pH 5.0 was greater than at pH 7.0 and 9.0. Molecular Weight (MW) profiles of fish sauce were in agreement with degree of hydrolysis (DH). Anti-oxidative activity (AT) increased when fermentation continued and depended on fermentation pH. However, a relationship between AT and DH was not established. At low salt (15%), fermentation pH had more influence on AT than at high salt (20% and 25%). Fish sauce fermented with low salt (15%) at pH 5.0 for 60 days exhibited greater AT (98.4%) than at pH 7.0 (96.5%) and 9.0 (93.9%). Pacific whiting fish sauce exhibited a significant level of ACE inhibition and AT. ACE inhibition was highly dependent on DH. Peptides with MW <590 Da possibly played an important role in ACE inhibition. The optimum condition for the greatest ACE inhibition was at pH 5 with 15% salt concentration after 30 days fermentation and the greatest AT was obtained at pH 7.0 or 9.0 and 25% salt after 60 days fermentation.

Keywords: ACE inhibition, anti-oxidative activity, fish sauce, fermentation condition, various pH and salt concentration

INTRODUCTION

Fish sauce is a clear, liquid condiment with amber color and mild cheesy/salty flavor that is commonly used as a flavor enhancer or salt replacement in various food preparations. As a result of enzymatic and microbial fermentation, nitrogenous compounds in fish sauce are short chain peptides, amines, or amino acids. Pacific whiting (*Merluccius productus*), which is abundant off the Pacific Northwest coast, was not commercially utilized until 1991 due to proteolytic enzymes that cause textural softening. Following the study of Lopetcharat and Park (2002) on fish sauce from Pacific whiting, Tungkawachara and Park (2003) reported that fish sauce made from Pacific whiting was successfully accepted by American consumers.

Many food proteins possess specific biological activities, such as anti-hypertensive and anti-oxidative activity (Janitha and others 2002). ACE (Angiotensin-I Converting Enzyme) is primarily responsible for the conversion, *in vivo,* of circulating inactive angiotensin I to a potent vasoconstrictor, angiotensin II and inactivates the vasodilator bradykinin (Matsui and others 1992). ACE catalyzes the hydrolysis of decapeptide (angiotensin I) to the octapeptide (angiotensin II) by hydrolytic removal of the C-terminal dipeptide, histidylleucine (Cushman and Cheung 1971). This enzyme plays an important role in the rennin-angiotensin system to regulate both the arterial blood pressure and salt/water balance (Laragh and others 1972). ACE inhibitors have proven to be a key therapy for hypertension and congestive heart failure (Matsui and others 1992; Bakris 2001). In fact, some synthetic compounds, such as captopril, lisinopril, benazepril, perindopril, etc., have

been used as commercial ACE inhibitors for hypertension therapy (Okamoto and others 1995a; Texas Department of Health 2000).

Okamoto and others (1995a) surveyed the ACE inhibitory effect of various fermented foods, such as fish sauce, mirin, sake, soy sauce, natto, cheese, etc. They reported that amongst the liquid fermented products tested, fish sauce and soy sauce showed the strongest ACE inhibitory action. Okamoto and others (1995b) found that sardine fish sauce processed by the traditional method showed 2 times stronger inhibition than fish sauce processed by the enzymatic method. They found a significant reduction of blood pressure when isolated peptides from fish sauce or crude fish sauce fractionated with ethyl alcohol was applied to spontaneously hypertensive rats. Dipeptides are small enough to pass through the intestine and are relatively resistant to digestive protease. Accordingly, they are likely to exert strong ACE inhibition in vivo (Okamoto and others 1995c). Fish sauce made from different kinds of raw materials (salmon, sardine, and anchovy) showed different levels of ACE inhibitory action (Okamoto and others 1995b).

Oxidation leads to deterioration of lipids, browning, loss of protein quality, and impaired organoleptic quality (Karel and others 1966). Peptides and amino acids exhibit anti-oxidative activity in a liquid system (Decker and others 2000). Anti-oxidants scavenge free radicals and reactive oxygen and can be extremely important in inhibiting oxidative mechanisms that lead to chronic diseases (Martinez and others 2002). Martinez and others (2002) claimed that the anti-oxidative activity of common beans (*Phaseolus vulgaris*) implied its potent antimutagenic activity. Lim and others

(2000) found that mustard leaf kimchi exhibited anti-oxidative activity and it significantly inhibited growth of cancer cells *in vitro*. Several amino acids, such as metionine, histidine, alanine, and lysine, are generally recognized as anti-oxidative in spite of their pro-oxidative effects in some cases. They reduced oxygen absorption of linoleate by as much as 50-80% (Marcuse 1961; Karel and others 1966).

With known information on the functional and biochemical activity of fish sauce or other fermented products, our interest was to investigate the anti-hypertension and anti-oxidative effects of Pacific whiting fish sauce. Our objective was to evaluate the ACE inhibitory action and anti-oxidative activity of Pacific whiting fish sauce with regards to two important processing parameters, pH and salt. Degree of hydrolysis and molecular weight profile of fish sauce were also investigated.

MATERIALS AND METHODS

Chemicals

All chemicals, except salt used in the fish sauce preparation, were reagent grade. Sodium phosphate dibasic (Na_2HPO_4) was purchased from Matheson Coleman & Bell (Norwood, OH). Sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$) and ethyl acetate were purchased from Mallinckrodt Chemical Inc. (St. Louis, MO). Sodium hydroxide (NaOH), trichloroacetic acid (TCA) and hydrochloric acid (HCl) were purchased from Fisher Scientific (Pittsburgh, PA). Ethyl alcohol (≥99.5% absolute,

C_2H_5OH) was purchased from Aldrich Chemical Company, Milwaukee, WI). Ammonium thiocyanate (NH_4SCN), ferrous chloride (≥99%, $FeCl_2.4H_2O$), Sodium chloride (NaCl), Angiotensin converting enzyme (ACE: 2 units) isolated from rabbit lung, linoleic acid (≥99%, cis-9, cis-12-octadecadienoic acid, $C_{18}H_{32}O_2$) and N-Hippuryl-His-Leu tetrahydrate ($C_{21}H_{27}N_5O_5$) were purchased from Sigma Chemical Co. (St. Louis, MO).

Fresh Pacific whiting (*Merluccius productus*) (more than 100 fish), which were harvested off the Oregon coast in August 2001, were randomly selected and frozen at -18 °C for 4 mo before fish sauce preparations.

Preparation of fish sauce

Whole frozen fish were thawed before grinding. The paste was mixed homogeneously with salt (Morton International, Inc., Chicago, IL) at 15 %, 20 %, and 25%, respectively (W/W). The pH was adjusted to 4.0, 5.0, 6.0, 7.0, 8.0, or 9.0 using 5 N HCl and 5 N NaOH. After pH adjustment, de-ionized water was added to the fish paste to obtain a total volume of 60 mL. According to our preliminary experiments, the pH was not significantly changed by the addition of de-ionized water during pH adjustment. The paste, after mixing, was then placed in glass bottles closed tightly with a lid and kept at 35 °C for fermentation. At each fermentation time (3, 30, and 60 days), fermented fish paste samples were diluted (1:1) with cold 20 mM phosphate buffer (pH 7.0) and homogenized for 2 min before centrifuging at 10,000 x g for 30

min at 4 °C. The supernatant was then filtered through filter paper (Qualitative P8, Fisher Scientific, Pittsburgh, PA) and the filtrate was subjected to various biochemical analyses.

ACE inhibition

The *in vitro* ACE inhibition was measured according to the method of Okamoto and others (1995c), which was slightly modified from Cushman and Cheung (1971). In this experiment, concentration of fish sauce in the reaction mixture was 200 μL/mL. ACE solution (50 μL) containing 0.05 U/mL ACE in buffer (pH 8.3) [100 mM potassium phosphate buffer with 300 mM NaCl] was preincubated with each fish sauce (50 μL) for 5 min at 37 °C in a water bath (Polytherm, Dayton, OH). Then the enzyme reaction was initiated by adding 150 μL of 8.33 mM of the substrate, Hip-His-Leu, and terminated by adding 250 μL of 1M HCl after 30 min of incubation. The liberated hippuric acid was extracted with 1.5 mL of ethyl acetate. The mixture was then centrifuged at 2500 rpm (Sorvall Biofuge Fresco, Kendro Laboratory product, Osterode, Germany) for 2 min to separate the ethyl acetate layer. Exactly 1 mL of the extract in the ethyl acetate layer was transferred to another test tube and evaporated to dryness by a heat block (COD reactor, Hach company, Loveland, CO). The residue hippuric acid was re-dissolved in de-ionized water and its concentration was spectrophotometrically measured at 228 nm (Shimadzu Scientific Instruments, Baltimore, MD). The ACE inhibition was calculated and expressed as % inhibition.

ACE inhibition (%) = $[1-(A_s-A_{B2})/(A_c-A_{B1})] \times 100$

A_s = Absorbance of the sample

A_c = Absorbance of de-ionized water

A_{B1} = Absorbance of inactivated enzyme and de-ionized water

A_{B2} = Absorbance of inactivated enzyme and the sample

Anti-oxidative activity

Anti-oxidative activity of fish sauce was assayed according to the method of Chen and others (1996). For oxidation, 1.0 mL of 0.1 M sodium phosphate buffer (pH 7.0), 0.25 mL of fish sauce, 0.25 mL of de-ionized water, and 1.0 mL of 50 mM linoleic acid in ethanol (99.5% V/V) were mixed in a glass test tube. The tubes were sealed tightly with silicon rubber caps, wrapped with aluminum foil, and kept at 60 °C in the oven (Gravity Oven, VWR Scientific Company, West Chester, PA) for 48 hrs. Aliquots of the reaction mixture were taken to measure the peroxide value by the ferric thiocyanate method. The ferric thiocyanate analysis was performed as follows: To 50 uL of the reaction mixture, 2.35 mL of 75% ethanol, 50 uL of 30% ammonium thiocyanate, and 50 uL of 20 mM ferrous chloride solution in 3.5% HCl were added. The reaction mixture was left to stand at room temperature for 3 min. The absorbance was then spectrophotometrically measured at 500 nm. A blank (reference) sample was prepared by replacing the reaction mixture with 50 uL of de-ionized water while

keeping all other chemicals unchanged. The anti-oxidative activity was calculated from the following equation:

Anti-oxidative activity (%) = 100 x (1- absorbance of sample/ absorbance of reference)

Degree of hydrolysis

Trichloroacetic acid (TCA) (20%) and fish sauce were mixed 1:1 and centrifuged at 10,000x g for 30 min. TCA-soluble nitrogen in the supernatant and total nitrogen in the whole sample (mixture of fish paste and liquid) were analyzed using the Kjeldahl method (AOAC 1995). Degree of hydrolysis (DH) was calculated according to the method previously described by Hoyle and Merritt (1994):

DH = [(TCA-soluble nitrogen in sample)/ total nitrogen in whole sample] x 100.

Molecular weight profiles

Molecular weight profiles of proteins and peptides in fish sauce were investigated using gel chromatography. One mL of fish sauce was loaded onto a Superdex-30 column (Fast Protein Liquid Chromatography (FPLC), GradiFrac system with HiLoad pump-50, Pharmacia Biotech, UK) and eluted with de-ionized water. Chromatography was operated at a flow rate of 1 mL/min and a chart speed of 1 mm/min. Each 2.9 mL fraction was collected and the protein content was spectrophotometrically measured at 206 nm. Concentration of peptides was obtained

from the area under the peak of the elution diagram. The molecular weight of the peptides was identified according to the linear regression model ($y = 285221e^{-2.5108x}$, $R^2 = 0.9876$, when Y= molecular weight of peptides (dalton), X = ratio of elution volume (V_e) and void volume (V_o)) obtained from the standard proteins: blue dextran 2000 (MW 2000 kDa), cytochrome C (12.4 kDa), aprotinin (6.5 kDa), and vitamin B_{12} (1.36 kDa). The molecular weight profile was obtained from a plot of the percentage concentration of peptides versus their molecular weights (Determann 1968).

RESULTS AND DISCUSSION

ACE inhibition

Process conditions affected the ACE inhibitory action of Pacific whiting (PW) fish sauce. The greatest ACE inhibition (96.8%) was found in fish sauce fermented with 15% salt, at pH 5.0 for 30 days (Table 5.1). At the same pH and fermentation time, 91.2% and 82.1% inhibition was obtained at 20% and 25% salt, respectively. Our PW fish sauce showed greater ACE inhibitory activity than kimchi (62.0% inhibition) (Choi and others 2001), fermented soybean (64.9% inhibition) (Okamoto and others 1995b), water extract from turban shell protein (58.3% inhibition) (Kim and others 2000). However, our fish sauce showed less ACE inhibitory activity than a squid hydrolysate (100% inhibition) (Suh and others 1997). Okamoto and others

(1995a) compared various volumes of fish sauce per one mL of reaction mixture that give 50% ACE inhibition: 4.1 μL for salmon fish sauce, 2.7 μL for traditional sardine fish sauce, 9.5 μL for enzymatic sardine fish sauce, and 17.5 μL for anchovy fish sauce. Kohama and others (1988) found that the isolated peptide from tuna muscle also showed 50% ACE inhibition at a concentration of 2 μM.

According to Fig. 5.1, there was a good correlation ($R^2 = 0.73$) between the ACE inhibition of fish sauce and their DH. Similar to our results, Janitha and others (2002) reported that de-fibrinated bovine plasma (DBP) hydrolyzed with a protease showed greater ACE inhibition as the DH values improved. At 15% and 20% salt, the inhibitory action of PW fish sauce at pH 5.0 was greater than at pH 7.0 and 9.0 (Table 5.1). However, the inhibitory action of all fish sauce at pH 5.0 reached a maximum value at 30 days and then decreased (Table 5.1). This observation was likely due to the continuous progress of proteolysis, resulting in a higher concentration of amino acids. Thus, after 30 days the amount of short chain peptides reduced (Fig. 5.2) and the ACE inhibition activity decreased (Table 5.1).

Janitha and others (2002) reported that protein hydrolysis, by either alkali, acid, or enzyme, released bioactive peptides. Short chain peptides and organic compounds in fermented products exhibited inhibitory action on ACE (Kinoshita and others 1993; Okamoto and others 1995b). The shorter peptide fragments had greater ACE inhibition than the longer ones (Fujita and others 2000). Thus, the optimum degree of hydrolysis providing a great amount of short chain peptides could result in the greatest ACE inhibition.

At pH 5.0, cathepsin-B-like and L-like activity were probably the major enzymes involved with the proteolysis of Pacific whiting fish sauce (Tungkawachara and Park 2003b). Due to the fact that cathepsin B is a peptidyldipeptidase that cleaves dipeptides from the C terminus of protein and its optimum pH is 3.0-4.5, a significant amount of dipeptides might have been produced in PW fish sauce processed at pH 5.0. According to Okamoto and others (1995c), dipeptides in salmon fish sauce exhibited strong ACE inhibition. Thus, the great ACE inhibition of PW fish sauce processed at pH 5.0 could have been from dipeptides produced by cathepsin B in PW muscle (Eisen and Jeffrey 1969; Barrett and Kirschke 1981).

It was interesting to see the increase of ACE inhibition of fish sauce fermented at pH 9.0 (25% salt) when fermentation continued (Fig. 5.3). ACE inhibition of fish sauce processed for 60 days at pH 9.0 with 25% salt was greater (58.2%) than fish sauce processed under the same conditions, but with 15% and 20% salt (33.14% and 43.11%), respectively (Table 5.1). Nevertheless, there was no relationship between ACE inhibition and peptide size (MW) in fish sauce processed at pH 9.0 (25% salt) (Table 5.1 and Fig. 5.4). This finding suggested that there is more than one compound affecting ACE inhibition in PW fish sauce.

Okamoto and others (1995b) reported that the strong ACE inhibitory activity of fish sauce was primarily caused by the combined action of many substances rather than by a single compound. Choi and others (2001) found the variation of ACE inhibition of kimchi during fermentation and the cell number of microorganisms in kimchi affected ACE inhibition. They stated that fermentation products such as lactic

Table 5.1: ACE inhibition, anti-oxidative activity, and degree of hydrolysis of Pacific whiting fish sauce

Fermentation	Salt concentration (%)	pH	ACE inhibition* (%)	Anti-oxidative activity** (%)	Degree of hydrolysis*(%)
3	15	5	84.10 ± 20.12	96.55 ± 2.77	30.09 ± 0.71
		7	35.36 ± 5.62	82.67 ± 4.06	11.57 ± 1.35
		9	20.74 ± 6.26	82.53 ± 2.17	13.58 ± 1.25
	20	5	88.57 ± 2.56	94.24 ± 0.62	25.22 ± 1.86
		7	7.36 ± 3.86	90.70 ± 1.48	11.25 ± 0.99
		9	0.00 ± 0.00	89.93 ± 2.59	8.98 ± 0.70
	25	5	71.60 ± 0.96	96.46 ± 0.31	15.58 ± 3.12
		7	13.32 ± 1.87	94.37 ± 2.36	10.63 ± 4.37
		9	18.60 ± 9.79	82.21 ± 3.27	7.83 ± 3.00
30	15	5	96.79 ± 0.14	92.79 ± 1.16	42.84 ± 3.72
		7	61.48 ± 0.41	85.07 ± 3.69	15.77 ± 1.05
		9	44.07 ± 4.40	82.67 ± 1.84	12.85 ± 0.32
	20	5	91.25 ± 2.48	93.56 ± 1.28	38.03 ± 1.41
		7	49.55 ± 1.05	92.60 ± 0.88	14.44 ± 1.85
		9	45.91 ± 1.65	90.79 ± 0.96	13.92 ± 1.09
	25	5	82.10 ± 1.51	94.37± 1.38	28.47 ± 3.41
		7	50.10 ± 2.89	95.83± 0.39	15.33 ± 0.63
		9	44.36 ± 15.96	98.00± 1.78	13.29 ± 4.05
60	15	5	85.61 ± 1.69	98.41 ± 1.49	39.54 ± 0.46
		7	68.93 ± 0.65	96.51 ± 0.91	18.67 ± 1.17
		9	33.14 ± 10.41	93.92 ± 2.54	17.76 ± 0.93
	20	5	85.88 ± 1.43	99.05 ± 0.92	37.48 ± 0.55
		7	52.80 ± 7.91	99.05 ± 1.10	15.84 ± 0.23
		9	43.11 ± 5.71	99.23 ± 0.92	17.99 ± 1.00
	25	5	82.40 ± 1.82	99.82 ± 0.36	28.99 ± 1.39
		7	45.37 ± 9.07	100.00 ± 0.00	16.23 ± 0.06
		9	58.20 ± 3.63	100.00 ± 0.00	20.89 ± 0.57

*Mean values ± standard deviations from 2 replications
**Mean values ± standard deviations from 4 replications

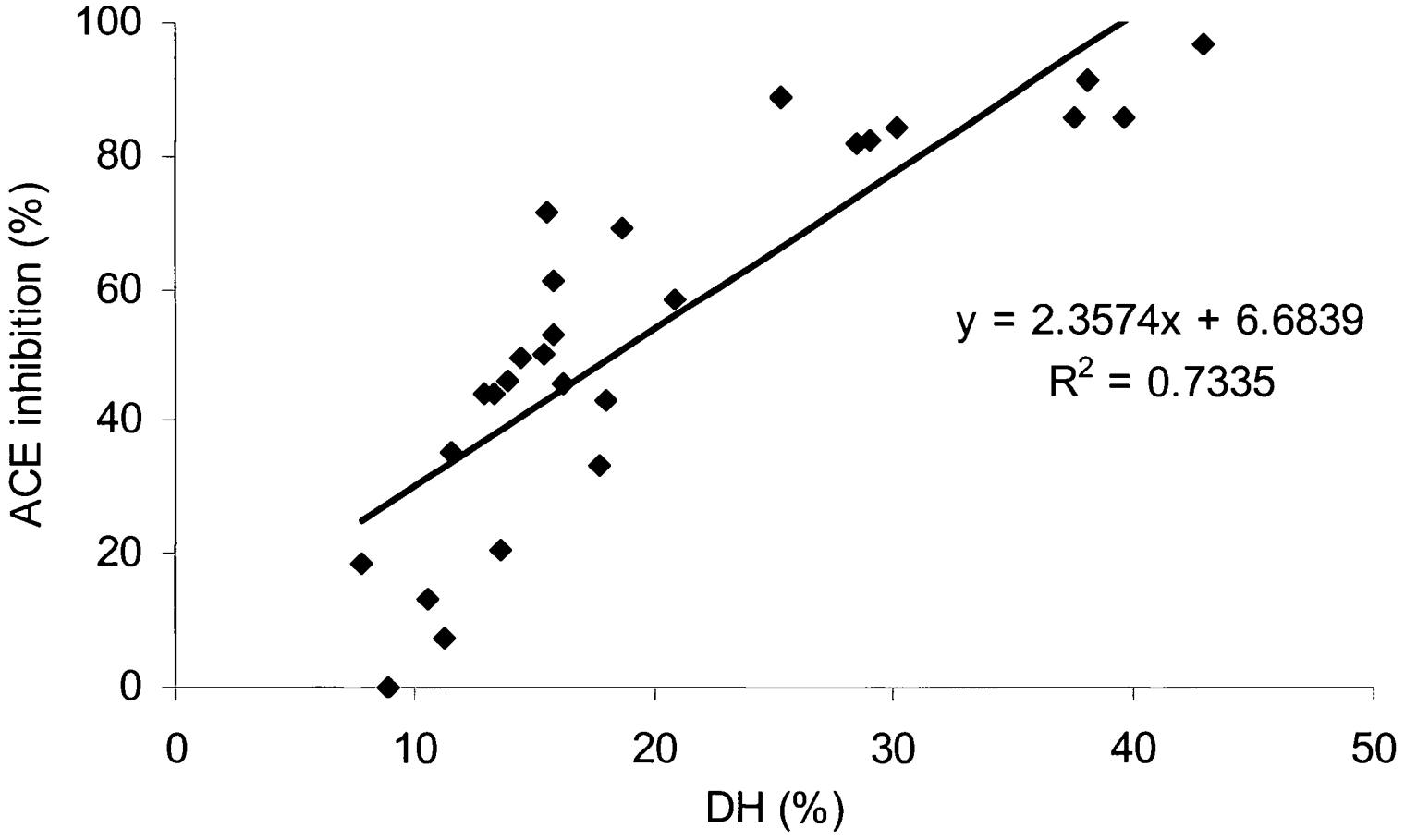

Figure 5.1: Correlation between ACE inhibition and degree of hydrolysis

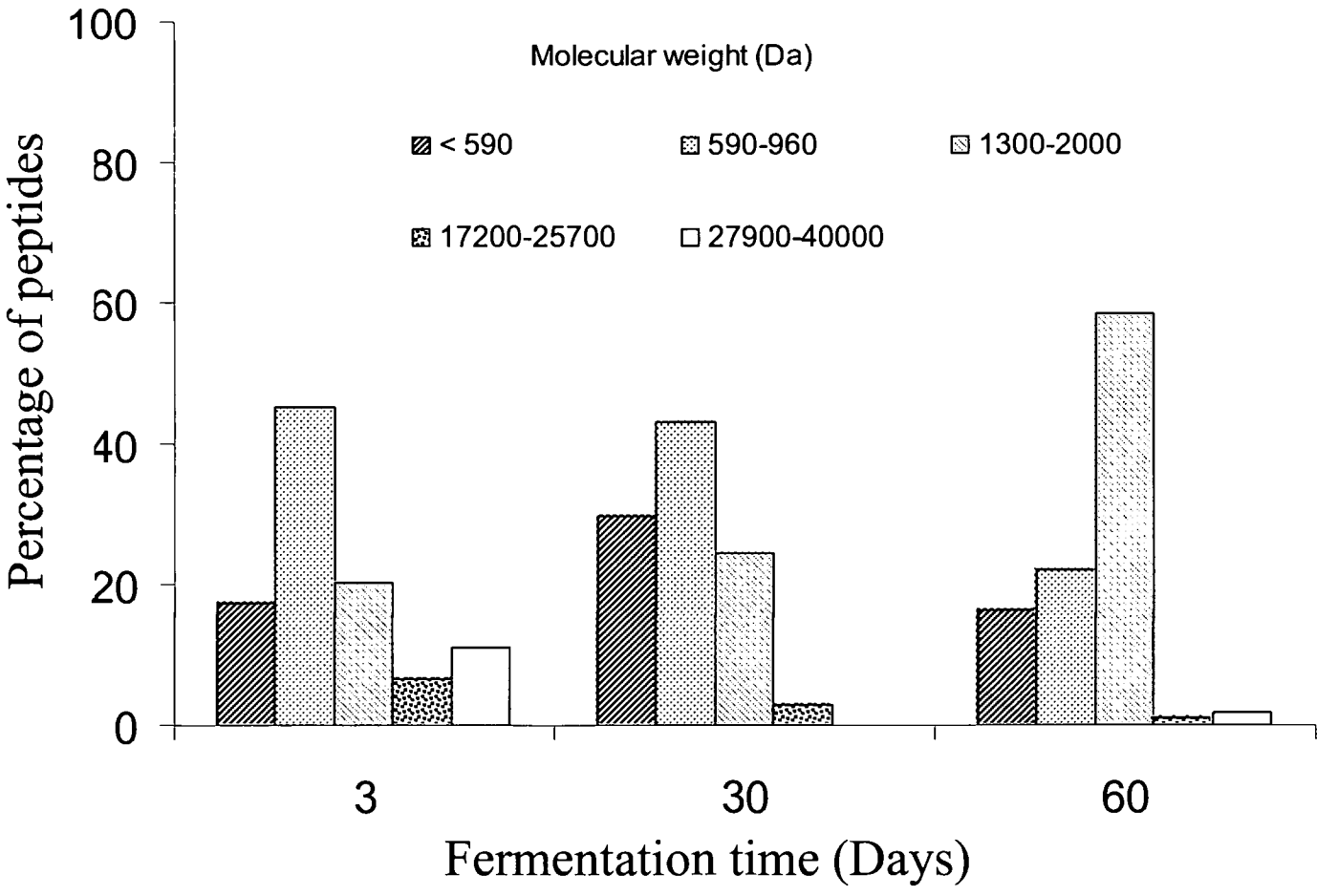

Figure 5.2: Molecular weight profile of peptides in fish sauce processed at pH 5.0 (15% salt) during 60-day fermentation

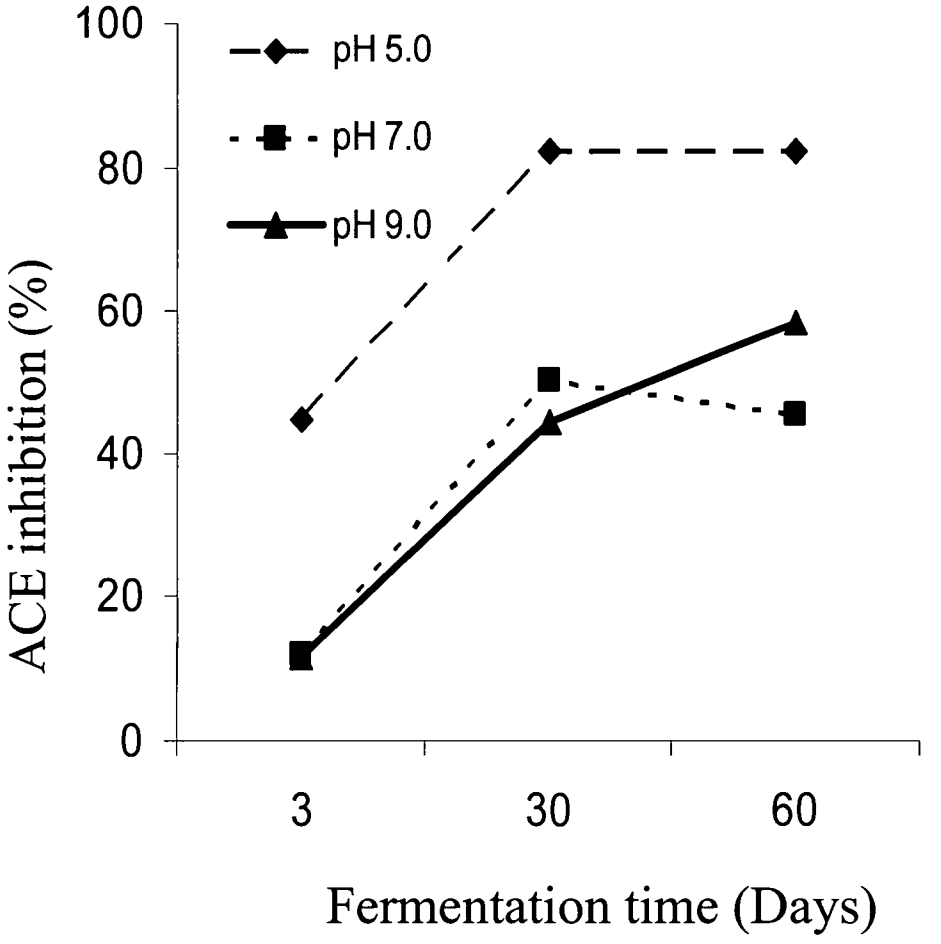

Figure 5.3: ACE inhibition of fish sauce (25% salt) during 60-day fermentation

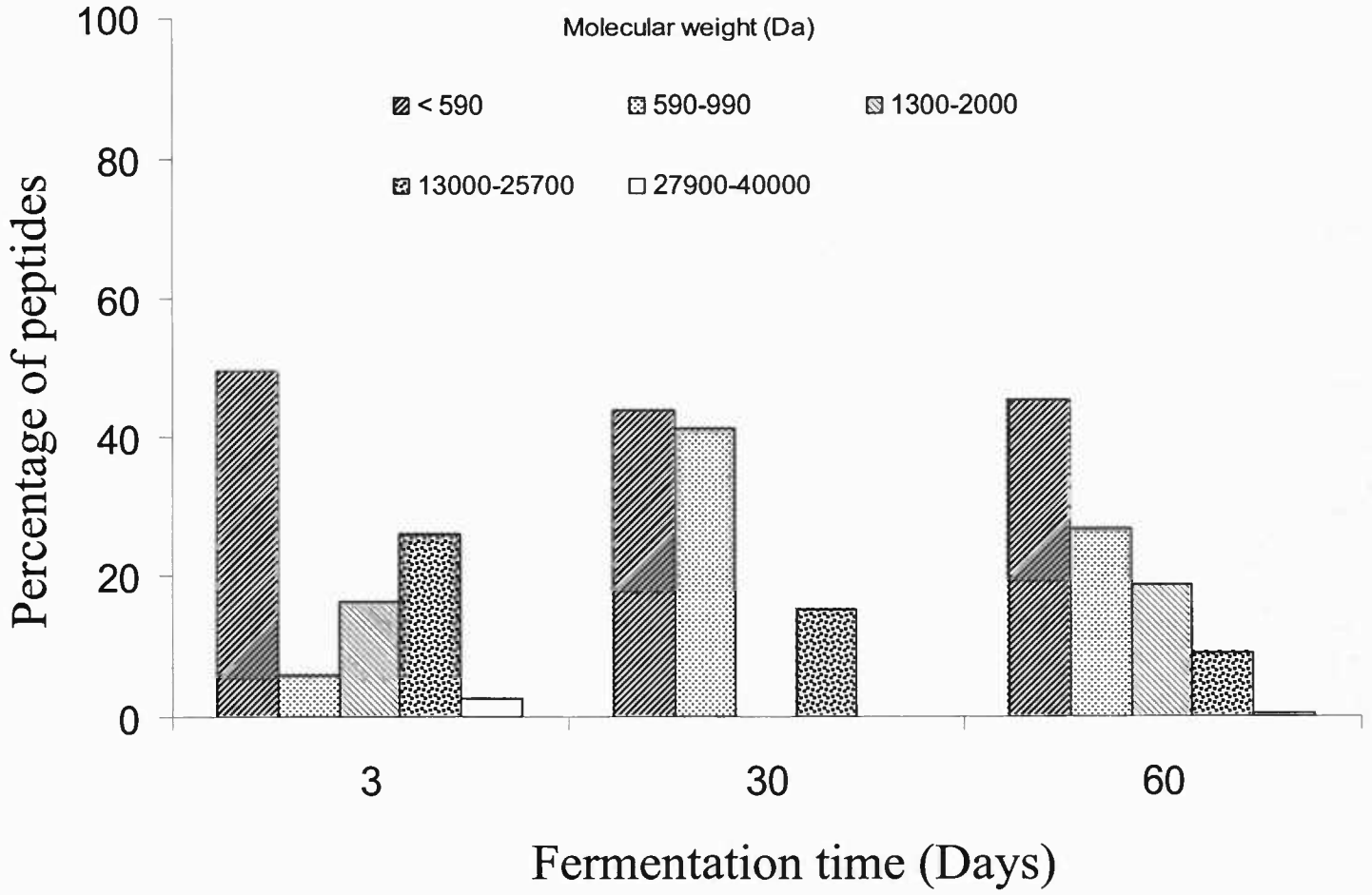

Figure 5.4: Molecular weight profile of peptides in fish sauce processed at pH 9.0 (25% salt) during 60-day fermentation

acid, ascorbic acid, and other organic acids, produced by microorganisms could directly affect the ACE inhibition of kimchi (Choi and others 2001).

Similarly, ACE inhibition in fish sauce processed at pH 9.0 was probably due to the coexisting serine proteases contained in fish intestines and/or those created from halophilic microbes that grow well at high salt concentration. Ishida and others (1994) reported that the serine proteinases found in salted anchovy had an optimum pH at pH >8.5 (anchovy from European countries). Okamoto and others (1995c) reported similar results that the coexisting serine protease in fermented soybean, natto, is directly related to ACE inhibition.

Anti-oxidative activity

Anti-oxidative activity of fish sauce was, in general, higher at high salt concentration and longer fermentation time (Table 5.1). The greatest anti-oxidative activity (100%) was found in fish sauce processed with 25% salt and pH 7 or 9 after 60-day fermentation (Table 5.1). The anti-oxidative effect of fish sauce was influenced by salt concentration and pH. The pH effect on anti-oxidative activity was more distinctive at low salt (15%), than at high salt concentration (20% and 25%) (Table 5.1 and Fig. 5.5).

Soybean protein hydrolysate exhibited anti-oxidative activity and a prolonged induction period as great as 4 times the autoxidation of linoleic acid in an aqueous system at pH 7.0 (Chen and others 1996). Our fish sauce exhibited higher anti-

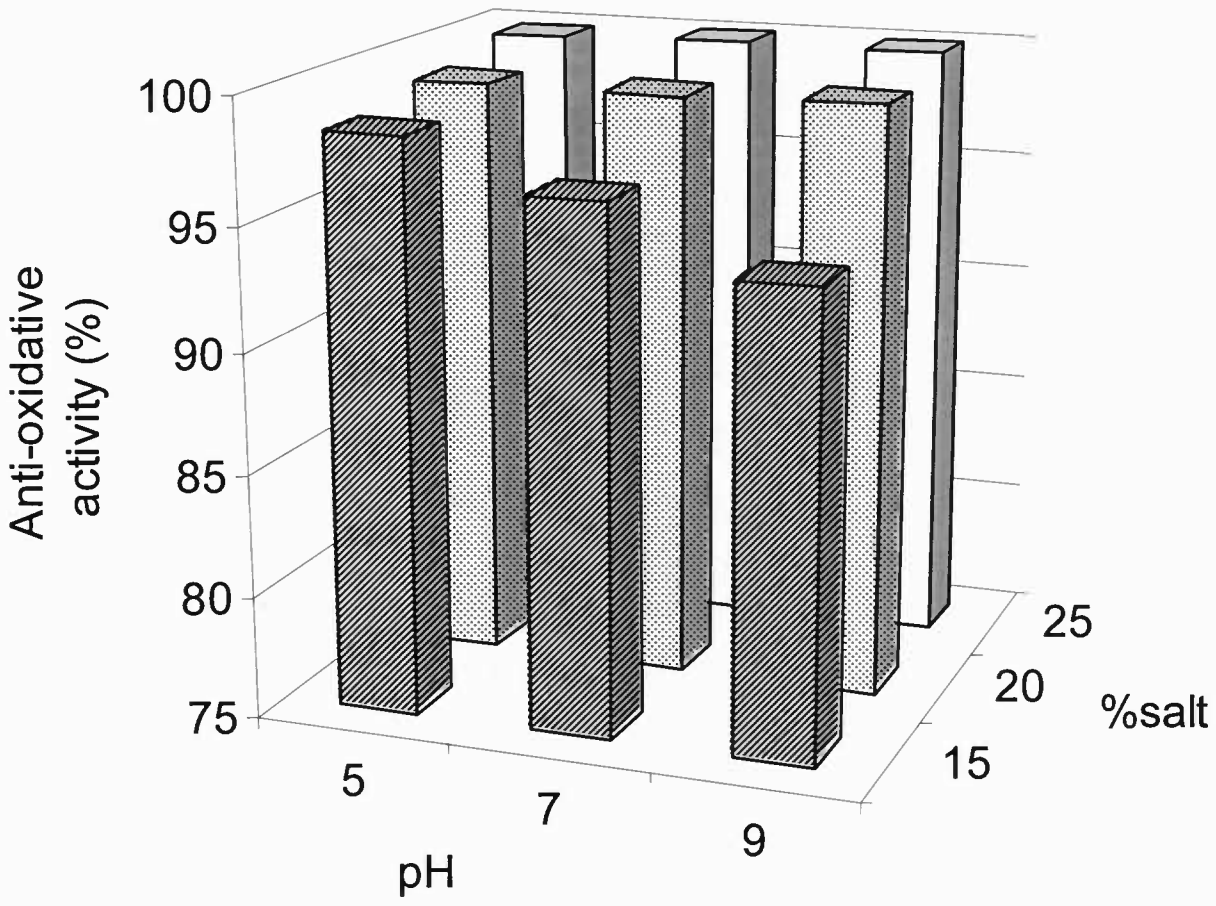

Figure 5.5: Anti-oxidative activity of fish sauce fermented for 60 days

oxidative activity than kimchi (83 %)(Choi and others 2001) and fermented Alaska Pollack (9.88%) (Cha and others 2002). The difference between the anti-oxidative activity of our PW fish sauce and fermented Alaska pollack was probably due to the composition of products, where the latter contained only 47% salted fish (7% salt) (Cha and others 2002). Strong anti-oxidative activity of fish sauce was probably due to fermentation products, for example, amino acids and peptides. Some amino acids such as histidine, alanine, methionine, and lysine exhibited anti-oxidative activity by decreasing the oxygen absorption by as much as 50-80% (Karel and others 1966). Dipeptides showed greater anti-oxidative activities than the constituent amino acid mixtures in an aqueous system (Chan and Decker 1994). The anti-oxidative activity of peptides is due to the hydrogen-donating ability, lipid peroxyradical trapping, and/or the metal ion-chelating ability of the imidazole group (Chan and Decker 1994; Chen and others 1996). Carlsen and others (2002) also confirmed the importance of buffering capacity of a dipeptide (carnosine) on its anti-oxidative activity. Moreover, Choi and others (2001) reported that the increase of microbe number in kimchi was also associated with the increase of anti-oxidative activity. Thus, in this experiment, besides peptides and amino acids, salt-tolerant microorganisms probably accounted for an increase in the anti-oxidative activity of fish sauce during fermentation, especially fish sauce processed at pH 7.0 and 9.0 (25% salt).

There was no correlation ($R^2 = 0.14$) between anti-oxidative effect and degree of hydrolysis (DH) (data not shown). The results suggested that the anti-oxidative effect of fish sauce depended on its composition rather than peptide size. Pena-Ramos

and Xiong (2002) also reported similar results that the anti-oxidative activity of protein hydrolysate was not influenced by the hydrolysis period. Hydrolysate produced at different pH and salt concentrations may have different amino acid compositions, various peptide sizes, and fermentation products. Chen and others (1995) stated that the anti-oxidative activity of hydrolysates was likely dependent on the characteristics of the amino acid sequence of peptides derived from proteins. Using Alaska pollock, which is a similar gadoid species to, Pacific whiting, Kim and others (2001) found strong anti-oxidative activity from purified peptides isolated from skin hydrolysate. In addition, the anti-oxidative activity of the dipeptides varied with the position of amino acids in the peptide (Yamaguchi and others 1975).

Degree of hydrolysis (DH)

DH of Pacific whiting fish sauce processed at pH 5.0 and 7.0 with low salt concentration (15% and 20%) was greater than fish sauce processed at high salt (25%)(Table 5.1). DH decreased as salt concentration increased. This is probably because proteases naturally found in muscle and viscera were partly inhibited at high salt. At acidic pH (5.0), fish sauce had greater DH values than at other pH (7 and 9) for all fermentation times (3, 30, and 60 days) (Table 5.1). Similar results were also reported by Beddows and Ardeshir (1979) that the presence of high salt concentration generally decreased the extent of proteolysis. Orejana and Liston (1982) concluded that endogenous enzymes are the major agents responsible for protein digestion during

the fish sauce process. Furthermore, it was determined that cathepsin L-like and B-like activities played important roles in the proteolysis of Pacific whiting fish sauce at acidic pH (Tungkawachara and Park 2003b). However, at pH 9.0 (60 days) fish sauce with high salt (25%) had greater DH (58.20%) than at low salt (15 and 20%)(33.14 and 43.11%, respectively)(Table 5.1). This was probably because halophilic bacteria and alkaline conditions accelerated the proteases to hydrolyze fish proteins, resulting in higher DH.

Molecular weight profile

The distribution of the molecular weight (MW) of peptides in fish sauce (15% salt) processed at pH 5.0 (Fig. 5.2) was in accordance with DH (Table 5.1). During proteolysis, protein molecules were hydrolyzed and resulted in smaller molecular sizes. Concentration of low MW peptides (<2000 Da) increased while concentrations of high MW peptides and proteins (>17200 Da) decreased when fermentation continued (Fig. 5.2). PW fish sauce fermented for 30 days (15% salt, pH 5.0) exhibited 42.8% DH (Table 5.1) and the predominant peptides (43.1%) had a molecular mass between 590 and 960 Da (Fig. 5.2). Our results were similar to Wanasundara and others (2002) that a hydrolysate of defibrinated bovine plasma with 43% DH was predominated (77.7%) with peptides having a molecular mass of <1040 Da. In addition, the isolated peptide (MW ~920 Da) from tuna muscle exhibited potent ACE inhibition (Komaha and others 1988).

It was interesting to discover that a change in the ACE inhibition of fish sauce processed at pH 5.0 (15% salt) at various fermentation times was closely related to DH and its molecular weight profile (Table 5.1 and Fig. 5.2). Fish sauce fermented for 30 days (15% salt, pH 5.0) had the greatest ACE inhibition (96.8%) and also contained the highest concentration of short chain peptides (<590 Da) (29.57 % of total peptides) (Table 5.1 and Fig. 5.2). As for PW fish sauce at pH 5.0 (15 % salt), ACE inhibition activity and the quantity of low MW peptides (<590 Da) showed similar trends during fermentation (Table 5.1 and Fig. 5.2). It was therefore noted that short chain peptides (molecular weight <590 Da) played an important role in the ACE inhibition of PW fish sauce processed at pH 5.0 (15% salt).

However, there was no correlation between ACE inhibition and size of peptides in fish sauce processed at pH 9.0 (25% salt) (Table 5.1 and Fig. 5.4). High MW peptides decreased and low MW peptides increased as fermentation time extended. When fermentation continued, low MW peptides could have been completely hydrolyzed giving amino acids and amines. Thus, the ACE inhibition of fish sauce decreased at 60 days due to the lower concentration (16.31 %) of short chain peptides (<590 Da) (Table 5.1 and Fig. 5.2).

CONCLUSION

Pacific whiting fish sauce contained ACE inhibitory activity as well as anti-oxidative activity. This indicates that Pacific whiting fish sauce has great potential to be used as a functional and health-promoting ingredient.

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CONCLUSION

The best processing conditions for Pacific whiting fish sauce were pH 5 and salt 15-20%. Consumers found Pacific whiting fish sauce can replace imported anchovy fish sauce. Pacific whiting fish sauce showed some health benefits: high ACE inhibition and anti-oxidative activity. Pacific whiting and surimi byproducts can be a good source for fish sauce.

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APPENDICES

Appendix A: Importer questionnaire

Please evaluate each attribute by placing an "x" in the appropriate box.

1. Do you import fish sauce?

   ☐ Yes

   ☐ No

2. Where do you import fish sauce product?

   ☐ Thailand

   ☐ Malaysia

   ☐ Philippines

   ☐ Indonesia

   ☐ Burma

   ☐ Cambodia

   ☐ Vietnam

   ☐ Japan

   ☐ India

   ☐ Pakistan

   ☐ China

   ☐ Korea

   ☐ Others; Please specify.....

3. Where is your business located in USA?

   City: State:

4. In which way do you distribute fish sauce products within the US market?

☐ Wholesale broker

☐ Food manufacturer (i.e., flavor, seasoning, sauce)

☐ Direct sales to groceries

☐ Others; Please specify.....

5. According to the US government report, about 15, 000 M/T of fish sauce products was imported to the US in 1998. The price of fish sauce products was about $1.10/kg. Do you think these numbers are practical?

Total quantity	Total dollar value
☐ Yes	☐ Yes
☐ No. It should be < this value.	☐ No. It should be < this value.
☐ No. It should be > this value.	☐ No. It should be > this value.
☐ I have no idea.	☐ I have no idea.

6. How much is the fish sauce proportionally among your entire imported food products? Based on dollar values,

☐ Less than 10 %

☐ 11-20 %

☐ 21-30 %

☐ 31-40 %

☐ 41-50 %

☐ More than 50 %

7. What are the typical sizes of fish sauce containers that the fish sauce manufacturer uses?

☐ Retail size: 500-1000 ml. or please specify volume.....

☐ Medium size: Gallon bottle or please specify volume.....

☐ Large size: 5 gallon or 50 gallon container or please specify volume.....

8. What is the species origin of your fish sauce?

____ Anchovy

____ Squid

____ Krill/shrimp

____ Other; Please specify.....

If you buy a variety of fish sauce, please indicate their proportions (%) by species.....

9. Which is the most important attribute that you consider to import fish sauce. Please rank the attribute from 1 to 6. If 1= the most important attribute; 6 = the least important attribute.

_____ Price

_____ Brand name

_____ Grade or quality of fish sauce; first grade, second grade, etc.

_____ Raw material; anchovy fish, or other kinds of fish

_____ Country of origin

_____ Others; Please specify.....

10. For the quality evaluation, do you or your staff personally evaluate (taste) the fish sauce? If so, what are the primary concerns? Please rank the attribute from 1 to 6. If 1= the most important attribute; 6 = the least important attribute.

_____ Color

_____ Aroma (Sweet)

_____ Off-odor (fishy, ammonia-like).

——— Sweet flavor (MSG-like): Most fish does not have MSG. But quality fish sauce gives MSG-like taste.

——— Saltiness: Is too salty product acceptable?

_____ Histamine content

——— Others; Please specify.....

11. Here at Oregon State University Seafood Laboratory, we have developed the quality fish sauce from Pacific whiting (white fish off the West coast). It gives much less fishy odor (than anchovy fish sauce), but gives still great taste. Do you know that scombroid fish like anchovies often creates health hazard toxin (histamine) when fish is not properly handled before processing? Therefore you may have paid attention to the level of histamine in fish sauce you bring to the US. However, Pacific whiting fish sauce can be manufactured without any problem of histamine.

Would you like to receive Pacific whiting fish sauce samples and participate in market and product evaluations?

Can I have your mailing address?

.....

All of our conversation shall be kept confidentially. The name of your business will not be disclosed anywhere in our study, but it is to be used only as a company A. Thank you very much. If you are interested in, we can provide you with the report.

Appendix B: Dealer questionnaire

Please evaluate each attribute by placing an "x" in the appropriate box.

1. Do you carry fish sauce?

   ☐ Yes

   ☐ No

2. Which country is your fish sauce manufactured from?

   ☐ Thailand

   ☐ Malaysia

   ☐ Philippines

   ☐ Indonesia

   ☐ Burma

   ☐ Cambodia

   ☐ Vietnam

   ☐ Japan

   ☐ India

   ☐ Pakistan

   ☐ China

   ☐ Korea

   ☐ Others; Please specify.....

3. Where is your business located in USA?

   City: State:

4. In which way do you distribute fish sauce products within the US market?

   ☐ Food manufacturer (i.e., flavor, seasoning, sauce)

   ☐ Direct sales to groceries

   ☐ Others; Please specify.....

5. How much is the fish sauce proportionally among your entire food products? Based on dollar values,

   ☐ Less than 10 %

   ☐ 11-20 %

   ☐ 21-30 %

   ☐ 31-40 %

   ☐ 41-50 %

   ☐ More than 50 %

6. Do you repackage fish sauce? If yes, what are the typical sizes of fish sauce containers used?

   ☐ Retail size: please specify volume.....

   ☐ Medium size: please specify volume .....

   ☐ Large size: please specify volume.....

7. What is the species origin of your fish sauce?

   ____ Anchovy

   ____ Squid

   ____ Krill/shrimp

   ____ Other; Please specify.....

   If you carry a variety of fish sauce, please indicate their proportions (%) by species.

8. Which is the most important attribute that you consider to carry fish sauce. Please rank the attribute from 1 to 6. If 1= the most important attribute; 6 = the least important attribute.

_____ Price

_____ Brand name

_____ Grade or quality of fish sauce; first grade, second grade, etc.

_____ Raw material; anchovy fish, or other kinds of fish

_____ Country of origin

_____ Others; Please specify.....

10. For the quality evaluation, do you or your staff personally evaluate (taste) the fish sauce? If so, what are the primary concerns? Please rank the attribute from 1 to 6. If 1= the most important attribute; 6 = the least important attribute.

_____ Color

_____ Aroma (Sweet)

_____ Off-odor (fishy, ammonia-like).

_____ Sweet flavor (MSG-like): Most fish saucedoes not have MSG. But quality fish sauce gives MSG-like taste.

_____ Saltiness: Is too salty product acceptable?

_____ Histamine content

_____ Others; Please specify.....

Here at Oregon State University Seafood Laboratory, we have developed the quality fish sauce from Pacific whiting (white fish off the West coast). It gives much less fishy odor (than anchovy fish sauce), but gives still great taste. Do you know that scombroid fish like anchovies often creates health hazard toxin (histamine) when fish is not properly handled before processing? Therefore you may have paid attention to the level of histamine in fish sauce you bring to the US. However, Pacific whiting fish sauce can be manufactured without any problem of histamine.

Would you like to receive Pacific whiting fish sauce samples and participate in market and product evaluations?

Can I have your mailing address?

.....

All of our conversation shall be kept confidentially. The name of your business will not be disclosed anywhere in our study, but it is to be used only as a company A. Thank you very much. If you are interested in, we can provide you with the report.

Appendix C: Retailer questionnaire

Please evaluate each attribute by placing an "x" in the appropriate box.

1. Do you carry fish sauce?

   ☐ Yes

   ☐ No

1. Which country is your fish sauce manufactured from?

   ☐ Thailand

   ☐ Malaysia

   ☐ Philippines

   ☐ Indonesia

   ☐ Burma

   ☐ Cambodia

   ☐ Vietnam

   ☐ Japan

   ☐ India

   ☐ Pakistan

   ☐ China

   ☐ Korea

   ☐ Others; Please specify.....

2. Where is your business located in USA?

   City: State:

3. How much is the fish sauce proportionally among your entire food products? Based on dollar values,

   ☐ Less than 10 %

   ☐ 11-20 %

   ☐ 21-30 %

   ☐ 31-40 %

   ☐ 41-50 %

   ☐ More than 50 %

4. Do you repackage fish sauce? What are the typical sizes of fish sauce containers used?

   ☐ Retail size: please specify volume.....

   ☐ Medium size: please specify volume .....

   ☐ Large size: please specify volume.....

5. What is the species origin of your fish sauce?

   ____ Anchovy

   ____ Squid

   ____ Krill/shrimp

   ____ Other: Please specify.....

   If you carry a variety of fish sauce, please indicate their proportions (%) by species.

8. Which is the most important attribute that you consider to carry fish sauce. Please rank the attribute from 1 to 6. If 1= the most important attribute; 6 = the least important attribute.

   _____ Price

   _____ Brand name

——— Grade or quality of fish sauce; first grade, second grade, etc.

——— Raw material; anchovy fish, or other kinds of fish

——— Country of origin

——— Others; Please specify.....

9. For the quality evaluation, do you or your staff personally evaluate (taste) the fish sauce? If so, what are the primary concerns? Please rank the attribute from 1 to 6. If 1= the most important attribute; 6 = the least important attribute.

_____ Color

_____ Aroma (Sweet)

——— Off-odor (fishy, ammonia-like).

——— Sweet flavor (MSG-like): Most fish saucedoes not have MSG. But quality fish sauce gives MSG-like taste.

——— Saltiness: Is too salty product acceptable?

_____ Histamine content

——— Others; Please specify.....

Here at Oregon State University Seafood Laboratory, we have developed the quality fish sauce from Pacific whiting (white fish off the West coast). It gives much less fishy odor (than anchovy fish sauce), but gives still great taste. Do you know that scombroid fish like anchovies often creates health hazard toxin (histamine) when fish is not properly handled before processing? Therefore you may have paid attention to the level of histamine in fish sauce you bring to the US. However, Pacific whiting fish sauce can be manufactured without any problem of histamine.

Would you like to receive Pacific whiting fish sauce samples and participate in market and product evaluations?

Can I have your mailing address?

.....

All of our conversation shall be kept confidentially. The name of your business will not be disclosed anywhere in our study, but it is to be used only as a company A. Thank you very much. If you are interested in, we can provide you with the report.

Appendix D: Fish sauce questionnaire

Panel no..... Date.....

Instruction: Please fill out the code numbers of your sample from left to right as they appear on your tray. Taste the sample one by one and place an X on the line that best describes how well you like/dislike the coded sample. Please use slice of white bread and water for rinsing fish sauce flavor before tasting the next sample. Continue until all the samples have been evaluated.

Overall Acceptability

	Sample number			
Like extremely				
Like very much				
Like moderately				
Like slightly				
Neither like nor dislike				
Dislike slightly				
Dislike moderately				
Dislike very much				
Dislike extremely				

Color

	Sample number			
Like extremely				
Like very much				
Like moderately				
Like slightly				
Neither like nor dislike				
Dislike slightly				
Dislike moderately				
Dislike very much				
Dislike extremely				

Flavor

	Sample number			
Like extremely				
Like very much				
Like moderately				
Like slightly				
Neither like nor dislike				
Dislike slightly				
Dislike moderately				
Dislike very much				
Dislike extremely				

If these fish sauce samples were available in the market, would you consider to purchase?

☐ YES (If say yes, please answer next question)
☐ NO

Please select 2 sample numbers that you might consider to purchase.

Sample number.....and .....

Demographic Questionnaire

1. Please indicate your gender.

   ☐ Male

   ☐ Female

2. Please indicate your age category.

   ☐ Under 19 years

   ☐ 20-29 years

   ☐ 30-39 years

   ☐ 40-49 years

- ☐ 50-59 years
- ☐ 60-69 years
- ☐ Over 69 years

3. How often would you say that you use fish sauce?

- ☐ 4 or more times a month
- ☐ 3 times a month
- ☐ 2 times a month
- ☐ 1 time a month
- ☐ Never use fish sauce

4. What do you usually use (select one)?

- ☐ Anchovy fish sauce
- ☐ Oyster sauce
- ☐ Another kind of fish sauce
- ☐ Do not know exactly about the kind of fish sauce

Thank you very much for your participation