ACCELERATION OF FISH SAUCE FERMENTATION USING PROTEOLYTIC ENZYMES

by

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ABSTRACT

Fish sauce fermentation is widely used in Southeast Asia as a means of preservation and producing valued added products from underutilized fish species. First grade and second grade Nampla, commercially produced Thai fish sauces, were analyzed for their chemical and microbiological composition. First grade commercially produced Nampla contained higher amounts of total nitrogen, formol nitrogen, free and total amino acids compared to second grade sauce. Most of the essential amino acids were present in both grades of sauces. Low microbial counts of halotolerant microorganisms were observed in both sauces. The use of trypsin and chymotrypsin to accelerate the rate of fish sauce fermentation produced from herring, one of the underutilized fish species in Quebec, was investigated. Results showed that supplementation with trypsin and chymotrypsin increased significantly the rate of proteolysis, the amounts of total nitrogen, formol nitrogen and free amino acids in the final fish sauces ($p<0.05$). Fermentation time was also reduced from 6-12 months to 2 months. A significant increase in total nitrogen and free amino acid contents in the end products was observed when the enzyme concentration was increased from 0.3% to 0.6% ($p<0.05$). Supplementation with 0.6% of 25:75 trypsin:chymotrypsin showed the most satisfactory results in terms of total nitrogen, formol nitrogen and free amino acid contents. However, the levels of free amino acids and volatile bases in herring sauces produced with various proportions of 0.6% trypsin and chymotrypsin were significantly lower than in first grade commercially produced Nampla. Results of sensory evaluation indicated that supplementation with various proportions of 0.6% trypsin and chymotrypsin did not result in a significant

difference in the preference for color, aroma and flavor ($p>0.05$). The lighter color of herring sauces produced with 0.6% enzyme supplement was preferred to the darker color of first grade commercial produced Nampla. However, there was no significant difference in the preference for aroma and flavor among enzyme supplemented sauces and first grade commercially produced Nampla. Microbiological composition of herring sauces produced with enzyme supplementation was similar to that observed from commercially produced Nampla.

RESUME

La fermentation est largement utilisée dans le sud-est de l'Asie comme moyen de préservation et de production de la sauce de poisson, constituant un produit à valeur ajoutée fait à partir d'espèces de poisson sous-utilisées. Des échantillons de première et de deuxième qualité de "Nampla", des sauces de poisson thaïlandaises produites commercialement, furent analysés au niveau de leur composition chimique et microbiologique. Le "Nampla" de première qualité contient des quantités plus élevées d'azote total, d'azote formolé, d'acides aminés totaux et libres comparativement à la sauce de deuxième qualité. La plupart des acides aminés essentiels étaient présents dans les deux types de sauce étudiés. Les dénombrements bactériens d'organismes tolérants aux concentrations élevées en sels furent quantitativement peu élevés. L'utilisation de la trypsine et de la chymotrypsine afin d'augmenter le taux de fermentation de la sauce de poisson produite à partir du hareng, une des espèces sous-utilisée du Québec, fut étudiée. Les résultats montrent que l'addition de trypsine et de chymotrypsine augmente significativement le taux de protéolyse, la quantité d'azote total, d'azote formolé, et d'acides aminés libres dans le produit final ($p<0.05$). Le temps de fermentation a été réduit de 6-12 mois à 2 mois. Une augmentation significative du contenu en azote total, en azote formolé, et en acides aminés libres dans le produit final fut observée lorsque la concentration d'enzyme passait de 0.3% à 0.6% ($p<0.05$). L'addition de 0.6% d'un mélange de trypsine et de chymotrypsine (25:75 respectivement) a donné les résultats les plus satisfaisants au niveau des contenus en azote total, azote formolé, et acides aminés libres. Toutefois, les taux d'acides aminés libres et de bases volatiles dans les sauces

produites à partir de hareng avec différentes proportions de trypsine/chymotrypsine (0.6%) étaient significativement plus bas que ceux obtenus avec la sauce "Nampla" produite commercialement (première qualité). Les résultats de l'évaluation sensorielle indiquent que l'addition de différents mélanges à 0.6% de trypsine et de chymotrypsine ne permet pas de déterminer des différences significatives au niveau des préférences pour la couleur, l'arôme, et la saveur ($p>0.05$). La couleur plus pâle des sauces de hareng produites avec l'addition d'enzyme à 0.6% a été préférée à la couleur plus foncée de la sauce "Nampla" commerciale de première qualité. Cependant, il n'y a pas de différences significatives au niveau de la préférence pour l'arôme et la saveur entre les sauces produites avec l'addition d'enzyme et la sauce "Nampla" de première qualité. La composition bactérienne des sauces produites par addition d'enzyme était similaire à celle observée pour les sauces "Nampla" produites commercialement.

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CHAPTER 1

LITERATURE REVIEW

1.1. Introduction

To a large number of world's population, fish, both marine and fresh water varieties, is one of the most important sources of dietary protein. While fish is an excellent food, it is also an extremely perishable product due to its biochemical and biological composition. The combination of high water activity, moderate pH of 6-7 and availability of a range of nutrients (Shewan, 1962) makes fish an ideal substrate for both the rapid growth of spoilage bacteria and activities of endogenous enzymes. The chemical composition of fish is shown in Table 1.

Table 1. Typical proximate analyses of post rigor flesh fish

	Fish muscle	
	Fatty	Lean
Water activity	0.99	0.99
pH	6.2-6.5	6.2-6.5
Water (%)	68.6	81.8
Protein (%)	20.0	16.4
Lipid (%)	10.0	0.5
Carbohydrate (%)	0.3	0.3
Ash (%)	1.4	1.3

Source: Jacquot, 1961.

Due to its biological nature and limited shelf life, several methods can be used to extend the shelf life of fish including chilling in ice, freezing, drying, smoking, canning and modified atmosphere packaging. Another method of preserving or improving the quality of fish is through fermentation with endogenous fish or microbial enzymes or added proteolytic enzymes to convert fish to sauces, pastes, soup stocks and protein concentrates. Fermented fish products are very popular in Asian countries and in Europe. Processing of fish in this manner is usually done to reduce waste that arises because specific varieties of fish are not acceptable to the consumer or there is a seasonal glut of all varieties of fish. Equally important is the fact that, in most of Asian countries, proper fish handling and storage facilities are either lacking or inadequate and fermentation constitutes a major method of fish preservation and adding value to underutilized species which would otherwise be discarded. Elsewhere, fermented fish products serve mainly as delicacies (Van Veen, 1965; Subba Rao, 1967; Burkholder et al., 1968).

With the preparation of a vast number of possible fermented fish products, the term "fermentation" has to be defined in its widest sense. In the restricted sense, fermentation refers to a particular mode of energy-yielding metabolism in which a micro-organism uses an exogenous organic compound to generate ATP solely by substrate-level phosphorylation (Morris, 1983). Traditionally, however, it has been used to describe almost any process in which natural organic material undergoes extensive transformation into simpler compounds by either the action of micro-organisms or by the action of endogenous fish enzymes (Mackie et al., 1971).

According to Amano (1962) fermented fish products are grouped into three categories, based on the methods of fermentation :

(i) Traditional products in which fermentation is carried out by endogenous fish enzymes in the presence of high salt concentrations. Products belonging to this category include fish sauces and fish pastes ;

(ii) Traditional products where fermentation is carried out by the combined action of endogenous fish and microbial enzymes in the presence of salt. In this procedure, microbial enzymes are added as a starter culture and are usually micro-organisms growing on some form of cereal e.g. cooked rice or maize. Products belonging to this type of fermentation process include Buro (Philippines), Plara (Thailand) and Funasushi (Japan);

(iii) Non-traditional products obtained by accelerating the rate of fermentation either with enzymes or by chemical hydrolysis. An example of products belonging to this category of fermented products is fish silage.

1.2. Fish sauce

Fish sauce is a liquefied protein food produced from hydrolysis of fish in a high salt concentration. Generally, fish sauce is a clear, dark brown liquid with a sharp distinctive aroma and a salty taste. It is also rich in soluble proteins and amino acids and is commonly used as a food condiment to improve the flavor and aroma of bland dishes such as rice.

1.3. Fish sauce production

1.3.1. Types of sauce and methods of production

Fish sauces are known under a variety of names and vary according to both their nutritive and organoleptic qualities. Examples of indigenous fermented fish sauces, their methods of manufacture and their country of origin are shown in Table 2.

Generally, traditional fish sauce fermentation begins with mixing uneviscerated fish with salt in the ratio of 5:1-2:1 (fish:salt) (Figure 1). The mixture is then transferred to fermentation tanks constructed of concrete and built into the ground or placed in earthenware pots and buried into the ground. When the tank or pot is filled, it is sealed off to allow the fish to liquify. At the end of fermentation, the supernatant liquor is then drained off and filtered through sand filtering beds. The filtrate may be sun-ripened in the earthenware container for up to 3 months to obtain a sauce with improved color and aroma. This liquid constitutes first grade fish sauce. The residue, which still contains unhydrolyzed fish tissue, is subsequently hydrolyzed with saturated brine for up to 3 months. These extractions, which give poorer quality sauces, may be improved by blending with various amounts of the supernatant liquid or by the addition of caramel or molasses prior to extraction. This darkens the color of the sauce and also improves its keeping quality by the production of acids, such as butyric acid from molasses or caramel, which lower product pH. However, lower quality sauces have poorer keeping properties as they have lower salt concentrations. The finished products are a clear, brown liquid, rich in salt and have a specific characteristic aroma and flavor. The final residue, which consists mainly of bones, is used as a fertilizer (Van Veen, 1965; Saisithi et al., 1966; Subba Rao, 1967).

Table 2. Examples of indigenous fermented fish sauces

Country	Name	Fish species	Fish:Salt	Fermentation time (month)
France	Pissala	*Aphya pellucida* *Gobius spp.* *Engraulis spp.* *Atherina spp.* *Meletta spp.*	4:1	0.5-2
Greece	Garos	*Scomber colias*	9:1	0.25
Hong Kong	-	*Sardinella spp.* *Jelio spp.* *Carandidae spp.* *Engraulis spp.* *Teuthis spp.*	4:1	3-12
India and Pakistan	Columbo-cure	*Ristrelliger spp.* *cybium spp.* *Clupea spp.*	6:1	up to 12
Indonesia	Ketjap-Ikan	*Stolephorus spp.* *Clupea spp.* *Leiagnathus spp.*	5:1	6
Japan	Shotturu	*Astroscopus spp.* *Clupea spp.* *Omnastrephis spp.*	5:1	6
Korea	-	Shrimp	4:1	6
Malaysia	Budu	*Stoleporus spp.*	5:1-3:1	3-12
Philippines	Patis	*Stoleporus spp.* *Clupea spp.* *Decapterus spp.* *Leionathus spp.*	3:1-4:1	3-12
Thailand	Nampla	*Stoleporus spp.* *Ristrelliger spp.* *Cirrhinus spp.*	5:1-1:1	5-12
Vietnam	Nuoc-mam	*Stoleporus spp.* *Ristrelliger spp.* *Decapterus spp.* *Clupea spp.*	5:1-1:1	3-12

Source: Adapted from Beddows, 1985.

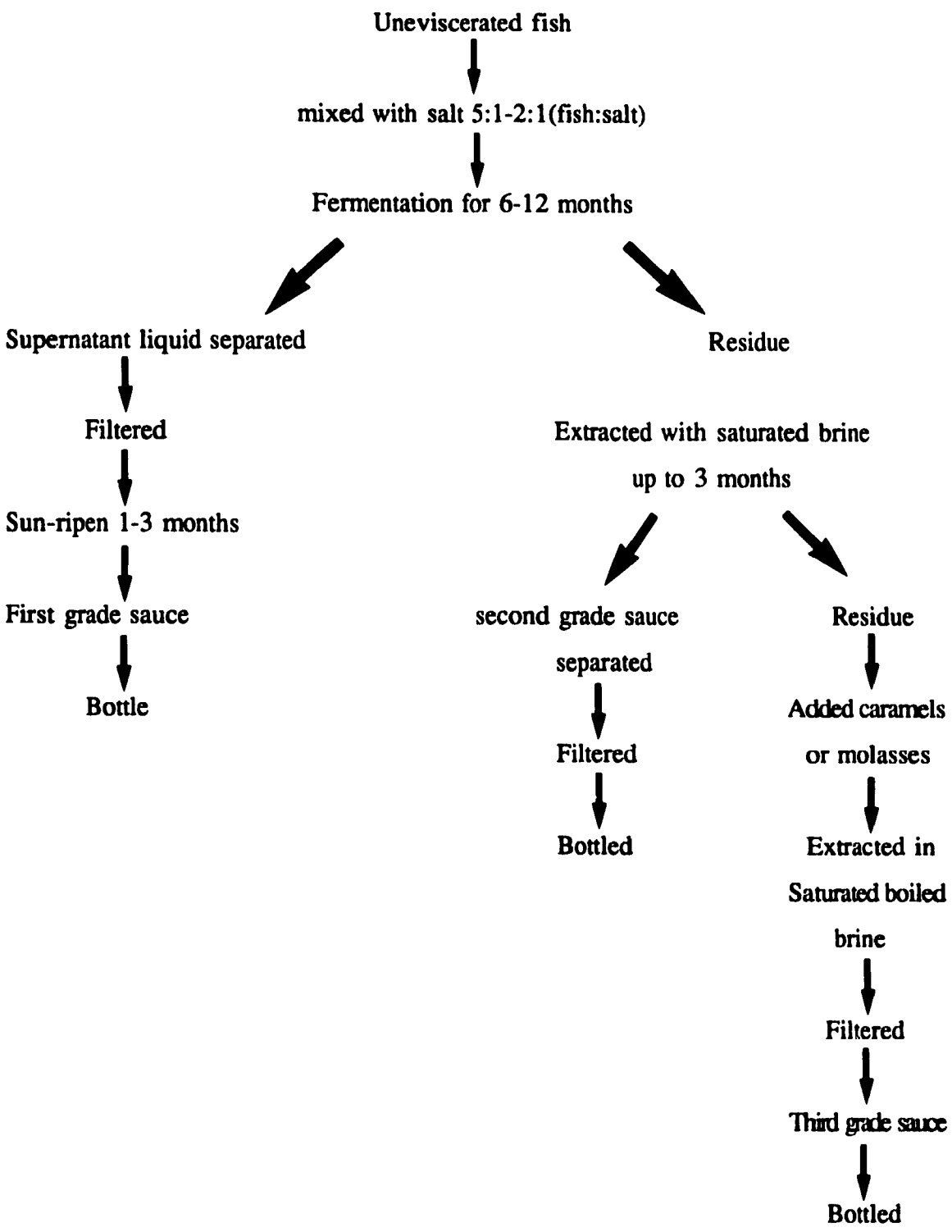

Figure 1. Fish sauce production.

In the production of Nouc-mam, an indigenous fermented sauce of Vietnam, the process is slightly different. The turbid and bloody liquid is drained off after three days of fermentation and then poured back over the fish until a 10 cm layer of brine covers the top of the vat (Steinkraus, 1983).

In Greece, fish sauce known as Garos, is made from fish viscera, particularly the liver, of *Scomber colias*. It has a short fermentation time (1 week), probably due to high concentration of proteolytic enzymes present (Beddows, 1985).

A Japanese fish sauce, called Shotturu, is prepared from sardines, anchovy, mollusc or sandfish (Subba Rao, 1967). Following fermentation, the liquid is filtered, boiled and can be stored for several years. Shotturu can also be processed into Shyoyu through the addition of a fermented wheat (Koji).

1.3.2. Raw materials

The major raw materials for fish sauce production are mainly fish and salt. A wide variety of fish, especially marine fish and shellfish, are used to prepare fish sauce examples of which are shown in Table 3. Marine salt, produced by sun-drying sea water on salt beds, is usually used.

The moisture, ash, protein and fat content of the various types of fish and shellfish used in fish sauce manufacture in Southeast Asia are shown in Table 4. There is a wide variation in protein and fat content of fish used in the fermentation process and this may account for the variation in both the nutritive and organoleptic quality of various fermented sauces.

Table 3. Marine and fresh water fish and shellfish mainly used in fish sauce production.

Fish	Common name
Marine Fish	
Rastrelliger sp.	Mackerel
Stolephorus indicus	Anchovy
Engraulis spp.	Anchovy
Stolephorus commersonii	Anchovy
Sardinella longiceps	Herring
Sardinella perforata	Deep-bodies herring
Sardinella fimbriata	Fimbriated herring
Leiognathus equulus	Slipmouth
Decapterus macrosoma	Round scad
Fresh water Fish	
Opicephalus striatus	Mud fish
Trichogaster	Gourami
Anabas testudineus	Climbling perch
Clarias sp.	Cat fish
Cirrhinus	Carp
Shell Fish	
Ostrea/Crassostrea	Oyster
Atya sp.	Shrimp
Mytilus	Mussel
Omnastrephes sp.	Squid

Source: Orejana,1983.

Table 4. Moisture, ash and protein and fat content of various types of fish and shrimp used in fish sauce production.

Species	Moisture(%)	Protein(%)	Fat(%)	Ash(%)
Mackerel, Herring,or Sardine	52.5-67.5	11.3-18.0	13.0-36.0	8.5-11.5
Anchovies (*Stolephorus sp.*)	52.5-73.7	11.3-13.4	13.0-36.0	8.5-11.5
Small whole herring(*Clupea*)	76.10	16.90	4.80	2.40
Shrimp	81.50	17.90	0.60	1.60
Slipmouth (*Leiognathus*)	79.50	17.32	1.82	-
Roundscad (*Decapterus*)	74.19	21.90	3.95	-

Source: Orejana, 1978.

1.4. Chemical and nutritional composition of fish sauce

The chemical composition of fish sauce are shown in Table 5. Nutritionally, fish sauce is an important protein supplement, supplying as much as 7.5 % of an individual's nitrogen intake (Amano, 1962). It is also a good source of mineral elements such as Ca, P, Fe and other organic nutrients and also the B-group of vitamins. However, the high salt concentration of fish sauce limits its consumption.

The distribution of nitrogenous compounds in fish sauce varies according to the types of raw material used and methods of production. Analysis of various fish sauces has shown that total organic nitrogen ranges from 1.7-2.3 % of which 40-60 % is in the form of free amino acids. Volatile nitrogen, mostly ammonia, comprises approximately 7-12 % of the total nitrogen (Uyenco et al., 1953; Saisithi et al., 1966; Beddows et al., 1979).

The amino acid profile of various commercially produced fish sauces are shown in Table 6. Both the bound and free amino acid content contribute to the nutritional quality of fish sauce. The essential amino acids are usually fairly well preserved (Orejana, 1978; Beddows et al., 1976; Amano, 1962) and the high level of lysine in fish sauce compensates for low levels of this amino acid in rice (Jansen and Howe, 1964).

Table 5. Chemical composition of fish sauce.

Component	Composition[1]
Moisture	60-70 %
Protein	28-33 g/100 g
Fat	1-3 g/100g
Carbohydrate	1-3 g/100g
Fiber	0
Ash	64-68 g/100g
NaCl	55-60 g/100g
Ca	30-130 mg/100g
P	90-130 mg/100g
Fe	3-30 mg/100g
K	800 mg/100g
Thiamine	0.03 mg/100g
Riboflavin	0.3 mg/100g
Niacin	6-12 mg/100g
Retinol	30 microgram/100g
Biotin	30 microgram/100g

1 Dry weight basis

Source: Campbell-Platt, 1987.

Table 6. Amino acid profiles of various commercial fish sauces.

Amino acids (mg/ml)	Fish sauce			
	Thai[1]	Malaysian[2]	Philippines[3]	Vietnam[4]
Glutamic acid	15.80	1.78	14.48	4.00
Alanine	5.38	1.53	6.74	4.20
Aspartic acid	5.52	1.10	6.59	2.40
Lysine*	7.34	0.40	6.02	4.00
Valine*	4.29	4.29	5.87	3.00
Leucine*	4.65	1.64	5.72	4.00
Glycine	4.14	0.44	5.61	2.40
Threonine*	2.79	0.70	4.90	2.00
Proline	5.41	0.26	4.37	0.50
Isoleucine*	3.86	0.98	4.01	4.00
Phenylalanine*	2.78	0.00	3.02	1.50
Serine	1.84	0.16	2.97	0.80
Methionine*	1.94	0.48	2.98	0.80
Histidine*	2.55	1.66	2.82	0.30
Cystine	0.00	0.42	0.90	0.25
Tyrosine	0.42	0.32	0.53	0.80
Arginine	0.00	0.00	0.16	2.00

* Essential amino acid.
1 Tongthai and Okada, 1981.
2 Beddows et al.,1976.
3 Orejana, 1978.
4 Subba Rao, 1967.

1.5. Role of salt

After the fish and salt mixture is packed into the fermentation tank, a brine is quickly formed due to the osmotic action of salt. The addition of salt is not only a means of extracting liquid, which is rich in soluble protein and other components from the fish, but it also controls the rate of fermentation and microbial spoilage. (Amano, 1962; Saisithi, 1967; Orejana,1978).

The concentration of salt is added at a level sufficient to prevent the growth of spoilage and pathogenic microorganisms and even results in a rapid decrease in microbial numbers. The bacteriostatic action of salt is due mainly to the reduction of water activity, which disrupts the osmotic balance in bacterial cells (Christian and Waltho, 1962).

However, too a high salt concentration may also slow down the activity of enzyme resulting in a slower rate of fermentation. While a low concentration of salt enhances rapid protein hydrolysis, it also increases the development of ammonia, which is considered undesirable in the final sauce (Amano, 1962). In addition, fish sauce with a low salt concentration will result in the growth of spoilage bacteria and a product with poor keeping quality. The minimum concentration of salt should be in the ratio of 4:1 (fish:salt) or 25% (w/w)(Rose, 1918; Amano, 1962; Raksakulthai, 1987). Salt, free from impurities, is always recommended for rapid hydrolysis of fish protein (Amano, 1962; Hamm and Clague, 1950).

1.6. Role of proteolytic enzymes in fish sauce fermentation

During the fermentation period, fish tissue is gradually hydrolyzed indicating the activity of proteolytic enzymes. The proteolytic enzymes responsible for the protein

degradation are either endogenous fish enzymes or enzymes from microorganisms.

1.6.1. Endogenous fish enzymes

Endogenous proteolytic enzymes in fish originate from the digestive tract, internal organs or muscle tissue. The properties and activity of endogenous proteolytic enzymes vary with fish species (Amano, 1962) and fishing season (Kashiwada, 1952).

1.6.1.1. Digestive enzymes

The contribution of digestive enzymes to fish sauce fermentation was studied by Uyenco et al., (1953). They observed a two fold increase in the amino nitrogen in fish sauce prepared from whole fish compared to sauce prepared from gutted fish. They concluded that digestive enzymes were the main agents for proteolysis and differences in the amino acid content of the end product.

Simpson and Haard (1987) found that the removal of viscera from herring prior to fermentation reduced the formation of brine-soluble proteins and peptides. Round herring contained approximately twice the amount of protein in the brine compared to gutted herring after 48 days of fermentation. Furthermore, the free amino acid concentration in conventional fermented herring was approximately twice of that of the products prepared from eviscerated fish (Simpson and Haard, 1987).

Raksakulthai (1987) observed that digestive enzymes appeared to contribute in the fermentation of capelin fish sauce since the rate of protein hydrolysis of whole fish was significantly higher compared to that of gutted fish. However, there was no significant difference in the sensory qualities of fish sauce produced from whole or eviscerated fish.

Digestive proteases include pepsins, secreted by the gastric mucosal glands, trypsins and chymotrypsins secreted by the pancreatic tissue or pyrolic caeca.

Pepsin is a gastric protease which is more active at acid pH values. Peptic activity depends on pH, temperature, and nature of the substrate (Kapoor et al., 1975).

Two acid proteases (I and II) have been isolated from the stomach of sardines (Noda et al., 1982). These enzymes were similar to mammalian cathepsin D and pepsin. The proteolytic activity of both enzymes was inhibited by 15-20% NaCl. However, acid protease type II was found to be stable for 3 months after the onset of fish sauce fermentation from sardines containing 22% NaCl (Noda et al., 1982).

Trypsin and chymotrypsin are endopeptidases. They are classified as serine proteases due to their requirement of a serine residue in the catalytic reaction. Kapoor et al., (1975) suggested the term "trypsin" be used for pancreatic proteolytic enzymes which are active between pH 7-11.

Noda et al., (1982) purified three alkaline proteinases (I, II and III) from pyrolic caeca of sardine. Type I was an alkaline proteinase, type II an anionic α-chymotrypsin like enzyme and type III was an anionic trypsin like enzyme. The proteolytic activity of these three enzymes was inhibited to varying degrees depending on the concentration of NaCl added. Alkaline proteinase type I was unstable when NaCl exceeded 15% while the trypsin like enzyme was still stable with 25% NaCl. Chymotrypsin like activity was inhibited by 20 % salt. The trypsin like enzyme was also found to be stable in fish sauce fermentation from sardine containing 22% NaCl after three months.

Orejana and Liston (1981) studied the enzymes responsible for proteolysis in Patis, a Philippine fish sauce, using specific proteinase inhibitors. They reported that a trypsin like enzyme developed within a few days after the onset of fermentation. Enzyme activity was maximal during the first month, then declined and remained at a low level throughout the remaining fermentation period. The decline in activity was suggested to be due mainly to end product inhibition by amino acids and small peptides. The initial low level of trypsin-like activity at the onset of fermentation was believed to be due to the presence of inhibitors in blood or substances produced by bacteria in fish. The trypsin like enzyme found in Patis produced from round scad was reported to behave like bovine trypsin.

Two aminopeptidases (I and II) have been isolated from the internal organs of sardine (Vo Van et al., 1983). Enzyme type I retained more than 70% of its original activity in 15% NaCl, which suggested that it may participate in the hydrolysis of fish proteins and peptides during fish sauce production. Vo Van et al., (1984) reported that the aminopeptidase in fish sauce produced from sardines was fairly stable and highly active during the initial 2 months of fermentation and then gradually lost its activity. The properties of the aminopeptidase in this study were similar to those previously isolated from sardines.

Vo Van et al., (1984) observed that carboxypeptidase activity disappeared almost completely within a few days after the onset of fermentation of fish sauce from sardines and concluded that this enzyme did not play a major role in the hydrolysis of fish protein into free amino acids and peptides in fish sauces.

1.6.1.2. Muscle tissue enzymes

Cathepsins, peptidases, transaminases, amidases, amino acid decarboxylases, glutamic dehydrogenases and related enzymes are all found in fish muscle tissue (Siebert and Schmitt, 1965) and these enzymes, particulary cathepsin, may play a role in fish sauce fermentation .

Cathepsins are intracellular enzymes which exist in animal tissues and catalyze hydrolysis of protein or synthetic substrates at acidic pH. These enzymes are located in the lysosomal fraction of the cell and are therefore distinguishable from digestive enzymes which are secreted by the cell (Mycek, 1970).

Voskresensky (1965) noted that the activity of cathepsins in the muscle tissues of salted herring decreased when salt concentrations exceeded 15% and that their activity in a saturated brine solution were inhibited to a higher degree compared to the activity of digestive tract enzymes. Furthermore, many of these enzymes were only active in the presence of certain reducing substances, such as cysteine, glutathione and hydrogen sulfide.

Rosario and Maldo (1984) studied the activity of cathepsins during the fermentation of Patis, a Philippine fish sauce. They reported that cathepsin A and C were important in proteolysis during fermentation. The activity of cathepsin A was found to be proportional to the amount of amino acids produced while the activity of cathepsin C was proportional to the amount of low molecular weight proteins in the fish sauce. The decrease in cathepsin D activity during the fermentation period corresponded to a decrease in the amount of high molecular weight proteins, which served as a substrate for cathepsin D.

Raksakulthai (1987) observed that cathepsin C contributed to proteolysis in fish sauce and the formation of the delicious fish sauce taste while enzymes associated with the fish viscera contributed only to proteolysis.

1.6.2. Microbial enzymes

In the presence of high salt concentrations, the activity of endogenous fish proteolytic enzymes are more significant than the proteolytic activity of microorganisms. Hamm and Clague (1950) proposed that bacterial growth was not important in the breakdown of fish protein since microbial counts decreased during fermentation. Furthermore, it has been shown that proteolysis occurred in the presence of bacteriostatic agents (Uyenco et al., 1953).

Although microorganisms do not appear to play an important role, enzymes from these organisms, which are present in fish prior to salting, may contribute to proteolysis of fish sauce fermentation (Amano, 1962; Saisithi, 1967).

Raksakulthai (1987) studied the contribution of bacterial enzymes in fish sauce fermentation from male capelin using a delayed-salting method. The results showed that there was no significant difference in proteolysis between 6-18 hr delayed-salting and the control, indicating that bacterial proteases were probably inactivated by salt and were therefore not important in fish sauce fermentation. Further studies using gentamicin sulfate a broad spectrum antibiotic, showed that antibiotic treatment did not significantly affect protein hydrolysis and protein solubilization in fish sauce. This would imply that microorganisms did not play a significant role in the fermentation of fish sauce using freshly caught capelin.

1.7. Proteolytic changes during the fermentation of fish sauce

Several studies have monitored the proteolytic changes during fish sauce fermentation. In nouc-mam, both organic and amino nitrogen increased up until 4-5 months at which time soluble nitrogen reached a maximum level (Uyenco et al., 1953). Soluble nitrogenous components were transformed into amino acids and ammonia. While the formation of amino acids eventually reached a maximum, ammoniacal formation continued at the expense of amino acid breakdown. Total nitrogen increased up to the fourth month and this period may be considered as the optimum fermentation time.

Studies on Thai fish sauce by Kasemsarn (1963) and Saisithi (1967) showed that total soluble nitrogen increased throughout the process while ammoniacal nitrogen increased during the first 6 months and then remained constant until the end of the process. Saisithi (1967) reported that while there were no differences between the types of amino acid present in Thai fish sauce, there were differences in the peptide fractions.

Proteolytic changes have been found to occur mainly in the first few months of fish sauce fermentation as shown by changes in amino nitrogen and soluble protein, and by increases in amino acids and peptides (Orejana, 1978). Ammonia and other volatile bases increased throughout the fermentation period and reached a maximum level in the latter stages of fermentation, indicating that the breakdown of protein degradation products continues throughout fermentation with a consequent loss of nutritional quality. Orejana (1978) concluded that the length of fermentation should not be prolonged after 5 months as fermentation beyond this time would enhance further breakdown of amino acids into ammonia.

Orejana and Liston (1979) also reported that amino nitrogen in fish sauce comprised mainly of peptides and not free amino acids. The molecular weight range of peptides, formed during the first 40 days, ranged between 700-1500 daltons. Orejana and Liston (1979) concluded that during the first 40 days of fermentation, endopeptidases of the trypsin type were the most active. During the period of 70-140 days, small molecular weight fractions increased significantly, indicating a higher activity of exopeptidases.

In the fermentation of capelin fish sauce at ambient temperature, changes in protein composition occurred mostly during the first four weeks as shown by an increases in free amino acids, formol nitrogen and soluble protein (Raksakulthai, 1987).

Uyenco et al., (1953), Orejana (1978) and Tongthai and Okada (1981) reported that the level of amino acid nitrogen in fish sauce decreased when the fermentation time was prolonged. However, Raksakulthai (1987), in studies on capelin fish sauce fermentation, found that free amino acids increased throughout the fermentation period and ripening, although this increase was reduced after the fourth week of fermentation.

1.8. Browning reaction and color of fish sauce

The color of fish sauce becomes darker as fermentation progresses. This is due mainly to non-enzymatic browning, a reaction which is desirable in fish sauce. The non-enzymatic browning reaction in fish sauce has been classified by Jones (1962) into 2 types: (i) Sugar-amino reaction or Maillard reaction and (ii) Lipid-amino reaction.

Saisithi (1967) in his studies with Nampla, considered the Maillard amino-sugar reaction to be the major contributor of color in Thai fish sauce.

However, Orejana (1978) observed an increase in free fatty acids in fish sauce fermentation as a result of lipolysis. She also reported that all fish sauce samples showed a linear increase in browning over a 40-140 day period. Orejana (1978) suggested that this increase in color was due to lipid-amino reactions since the concentration of sugar present in fish is very low and is unlikely to be a source of carbonyl compounds for the non-enzymatic browning reaction.

According to Jones (1962), lipid-amino reactions can be classified into three main types: (i) a reaction between carbonyl groups and the basic nitrogenous constituents (ii) oxypolymerization and (iii) oxidation of unsaturated lipids.

The role of fat in browning was further studied by the preparation of patis from defatted anchovies and the addition of fat to Patis made from defatted anchovies (Orejana, 1978). Browning was observed to decrease in patis made from defatted fish samples compared to the control sample while there was an increase in browning resulting from the addition of fat to Patis prepared from defatted fish. Orejana (1978) concluded that lipids and their degradation products (carbonyl compounds and peroxide) appear to be important sources of carbonyl groups for the non-enzymatic browning reaction in fish sauce.

1.9. Development of flavor and aroma

Color is a significant factor in consumer acceptability of traditionally produced fish sauce. However, flavor and aroma are the most important factors influencing consumer acceptability of the end product since a desirable color can readily be obtained by the addition of caramel or molasses (Beddows, 1985). Although flavor and aroma

varies according to the country of origin, fish sauce generally has a predominantly salty taste while amino acids and small peptides also contribute to the overall flavor.

Kasemsarn (1963) reported that the flavor of fish sauce was due to microbial action on proteins, or protein degradation products. Raksakulthai (1987) suggested that most bacterial species did not attack protein but utilized more readily available protein-degradation products. Therefore, the typical flavors of fish sauce are most probably developed during the latter stages of fermentation.

Fermentation of squid muscle in brine resulted in an increase in taste-active compounds which were identified as free amino acids. Lee and Sung (1977) showed that the fermentation of squid muscle resulted in a x 1.8 increase in free amino acids. The major taste-active amino acids in brined squid were proline, leucine, serine, lysine, arginine and alanine (Lee and Sung, 1977).

Raksakulthai (1987) studied the relationship between sensory preference score and chemical components in fish sauce and found that there was a strong correlation between free amino acid content and preference score. Larger molecular weight components present in fish sauce appeared to contribute significantly to the typical flavor of fish sauce since removal of these components resulted in a lower preference score. Raksakulthai (1987) concluded that the typical flavor of fish sauce was influenced by a combination of oligopeptides and free amino acids, especially glutamic acid and glycine.

Dougan and Howard (1975) classified the aroma of Nampla, a Thai fish sauce, into 3 major groups: (i) A cheesy aroma produced from low molecular weight volatile fatty acids, specifically n-butyric acid (ii) An ammoniacal aroma derived from the production of ammonia and amines and (iii) a meaty aroma produced by oxidation of

precursors present in the mature sauce by atmosphere oxygen.

Dougan and Howard (1975) suggested that fatty acids were more likely to be formed by the oxidation of fat than by any other mechanism. Saisithi et al., (1966) reported that certain bacteria isolated from fish sauce could also produce fatty acids from amino acids, but they did not identify the acids produced. However, Beddows et al., (1980) investigated the origin and mechanism of formation of volatile fatty acids present in Budu, a Malaysian fish sauce. They concluded that fatty acids did not appear to arise from the breakdown of fish lipids. Using labelled carbon 14 protein hydrolysate, it was shown that amino acids were the precursors of n-butanoic and n-pentanoic acids and also contributed to the formation of other acids. However, the metabolic route by which fatty acids were produced was not elucidated. Ooshiro et al., (1981) also confirmed that volatile fatty acids were an important component in the development of fish sauce aroma.

McIver et al., (1982) fractionated the solvent extract of Nampla, a Thai fish sauce, into acidic, neutral and basic fractions. The acidic fraction composed of acetic acid (29% of fraction), propionic (14%), iso-butyric (3%), 4-hydroxyvaleric acid lactone (12%), n-butyric (17%), iso-valeric (6%), levulinic (10%), phenylacetic acid and 3-phenylpropionic acid (3%). The proportion of short chain volatile fatty acids has been found to vary depending according to the type of sauce (Budu, Nampla or Patis) as well as the quality of the product. A product containing less volatile fatty acids is less cheesy and more ammoniacal compared to sauces with greater amounts of volatile fatty acids.

Fish sauce prepared from flounder and trout contained isovaleric acid in the highest concentration followed by acetic and iso-butyric acids (Chayovan et al., 1983a). The total volatile fatty acids (C-2 to C-6) in sauce made from trout, a fatty fish, (9.2%

fat) were approximately 3 times higher compared to sauce made from flounder, a lean fish, (1.6% fat) after 9 months fermentation. Non-volatile fatty acids (C-8 to C-18) were found in very low concentration compared to volatile fatty acids. It was concluded that the flavor of fish sauce could be due to the overall effects of both volatile and non-volatile fatty acids along with other biochemical reactions which generally occurred during fermentation. It is also possible that the flavor of fish sauce is also influenced by some breakdown oxidation products of long-chain polyunsaturated fatty acids found in fish lipids.

Sanceda et al., (1983) fractionated the volatile distillate of commercial Patis, a Philippine fish sauce, into 4 fractions-neutral, basic, acidic and phenolic fractions. They reported that the acidic fraction appeared to play a major role in the aroma of Patis since any other fraction, in combination with this fraction, produced an aroma similar to that of the whole concentrate. Five major acids were isolated from the acidic fraction: n-butyric, propionic, iso-butyric, valeric and acetic acids. These acids accounted for 98% of the total free fatty acids with n-butyric acid being the most abundant and accounting for about 50% of the total fatty acids (Sanceda et al., 1984). However, they reported that contribution of n-butyric acid to the overall of fish sauce aroma was not well pronounced, suggesting that other volatile compounds present in the neutral, basic and phenolic fractions may also play a major role in the development of aroma.

Sanceda et al., (1986) examined the formation of volatile acids at various stages of Patis fermentation. They observed that most of the acids identified in commercial Patis were found in low concentrations after only 24 hr fermentation suggesting that these acids might have been formed prior to salting during the period between harvesting,

transportation and salting. They also reported that fatty acids increased quantitatively and qualitatively with fermentation time and that almost all the acids identified in the sauce were formed within 3 months period.

An ammoniacal aroma was found in the basic fraction of solvent extracted fish sauce (McIver et al., 1982). This fraction contained mainly ammonia and trimethylamine while dimethylamine and 2,3-butanediol were found in small amounts. Sanceda et al., (1984) also reported nitrogen containing compounds, including trimethylamine, in the basic fraction of solvent extracted fish sauce.

The formation of an ammoniacal aroma may not involve bacterial action, as both ammonia and trimethylamine can be formed under aseptic processing conditions. However, it is possible that bacteria, when present, could increase the amount formed and hence contribute towards the intensity of aroma (Beddows, 1985).

The meaty aroma of fish sauce was found mainly in the neutral fraction of solvent extracted sauce (McIver et al., 1982). The major compounds isolated were 3 lactones (γ-butyrolactone, γ-caprolactone and 4-hydroxyvaleric acid lactone), 3-(methylthio)propanol and 2,3-butanediol. However, Sanceda et al., (1984) reported that the compounds found in the neutral fraction of fish sauce comprised mainly of alcohols and methyl ketones.

1.10. Microbiology of fish sauce

A wide variety of microorganisms have been found in fish sauce and the raw ingredients in fish sauce production. The possible types and sources of microorganisms involved in fish sauce fermentation are summarized by Orejana (1983): (i) organisms

naturally present in fish, predominantly, *Pseudomonas* and *Achromobacter* species (ii) organisms associated with the water and environment such as *Clostridium* and *Escherichia* species (iii) terrestrial organisms not normally associated with the marine environment and (iv) organisms associated with marine salt and other additives e.g. *Bacillus*, *Halobacterium* and *Micrococcus* species.

Saisithi et al., (1966) investigated the microbial changes during the fermentation of Nampla, a Thai fish sauce. They isolated 10 *Bacillus* species, 1 *Coryneform* species, 2 *Streptococci* species and 1 *Micrococcus* and *Staphylococcus* species. The microorganisms isolated could have been derived from the solar salt since the salt used in the process was found to contain an average of 2.73×10^4 bacteria per gram of salt with the principal organisms being halophilic *Bacillus* and *Micrococcus* species (Bain et al., 1957).

Crisan and Sands (1975) examined the microflora of four fermented fish sauces : Nampla, Patis, Koami and Onago and isolated 11 species of bacteria, 1 species of yeast, and 3 species of filamentous fungi. Isolates from Nampla, at various fermentation times, comprised mainly of *Bacillus* species while yeast, fungi and obligately anaerobic bacteria were not isolated. In Patis, the predominant isolates comprised of *Bacillus* species, *Micrococcus* and a yeast, *Candida clausenii*. Koami, a Japanese sauce produced from shrimp, contained *Bacillus* species and a strain of *Penicillium notatum* while *Bacillus* species and two molds, *Cladosporium herbarum* and *Aspergillus fumigatus* were found in Onago, a Japanese fermented sauce product. Crisan and Sand (1975) concluded that *Bacillus* species were the predominant microorganisms in fish sauce probably due to the salt resistant nature of these spore forming bacteria since they were isolated throughout

the fermentation period. The isolates appeared to be halotolerant rather than halophilic since they grew on media containing 10% salt, but not in media containing 20% salt. Molds isolated from Japanese sauce were observed only in the finished product. This was probably due to surface contamination during the aging process.

The microbiological changes in Patis, a Philippine fish sauce prepared from *Stolphorus sp.* and mixed species consisting of mackerel (*Rastrilliger sp.* and *Sardinella sp.*) were studied by Sanchez and Klitsaneephaiboon (1983). The predominant organisms throughout the fermentation period in both types of Patis again comprised mainly of *Bacillus* species. *Bacillus coagulans*, *Bacillus megatarium* and *Bacillus subtilis* were found during the early stage while *Bacillus licheniformis*, *Micrococcus colpogenes* and *Staphylococcus epidermis* were observed in the middle stages of the fermentation process. The predominant organisms in the latter stages of fermentation comprised mainly of salt resistant strains of *Micrococcus roseus*, *Micrococcus varians* and *Staphylococcus saprophyticus*.

The dominant microorganisms isolated from Shotturu, a Japanese fish sauce, were *Vibrio* and *Bacillus* species in medium containing 2.5% NaCl. However, when the salt concentration was increased to 20%, *Halobacterium, Bacillus* species and unidentified cocci were the predominant isolates (Fujii and Sakai, 1984).

Ok et al., (1982a) studied protease formation by a moderately halophilic strain of *Bacillus* isolated from Burmese and Chinese fish sauce. The isolated bacteria were capable of growth in a medium containing 4M NaCl. Maximal protease formation was observed in media containing 4M and 1M NaCl. The pH and temperature optima for enzyme production were pH 7 and 44^{0}C i.e. similar to the fermentation conditions for fish

sauce production in Southeast Asia.

It is believed that microorganisms do not play a major role in fish proteolysis since their numbers decrease steadily as fermentation progresses (Hamm and Clague, 1950; Saisithi et al., 1966; Orejana and Liston, 1979). However, they may be responsible for the development of fish sauce aroma.

Beddows et al., (1979 and 1980) reported that when fresh fish and salt were mixed and fermented without any time elapsing prior to salting, few of the volatile fatty acids, especially n-butyric acid, were formed. Addition of broad spectrum antibiotics also prevented the formation of the volatile fatty acids, suggesting that microbial action was involved in the production of these volatile acids components.

Sanceda et al., (1986) from studies on fish, with or without salt, concluded that bacteria must be involved in the formation of volatile acids since most of the acids found reached very high concentrations in fish incubated without salt.

However, due to the complex nature of aroma, it is difficult to ascertain any specific role of microorganisms in the production of fish sauce aroma.

1.11. Acceleration of fish sauce production

Traditional fish sauce production, which occurs mainly as a result of autolytic action by digestive proteases in the fish, usually takes 6-12 months to produce an acceptable end product. The fermentation method is time consuming and requires a large storage capacity, which is costly. To reduce this capital investment, it is desirable to accelerate the fermentation process. Many attempts have been made to accelerate the rate of fermentation. These include: (i) increasing the temperature of fermentation (ii) the use

of acid (iii) alkaline hydrolysis (iv) enzyme supplementation and (v) the use of bacteria and/or their enzymes

(i) Increasing temperature of fermentation

Fermentation of fish sauce at higher temperatures has been reported to increase the rate of protein hydrolysis and accumulation of free amino acids. However, temperature can only be increased to 37-45^{0}C since higher temperatures will denature essential proteolytic enzymes and also make processing relatively expensive if large tanks have to be heated for an extended time period.

Orejana (1978) reported that the level of free amino acids observed after 14 months at room temperature (23^{0}C) could be achieved after only 1 month if the fermentation temperaturewas increased to 37^{0}C.

Miyazawa et al., (1979) studied the effect of temperature on fish sauce produced from anchovy. They found that the percentage of liquefied protein after 150 days was 25.3, 17.6, 6.3 and 2.9% of the total protein at 50^{0}C, 30^{0}C, 10^{0}C and 2^{0}C, respectively.

Ooshiro et al., (1981) compared fish sauce fermentation at room temperature (24^{0}C), 37^{0}C and 50^{0}C. They observed that at the onset of fermentation at 37^{0}C, the concentration of amino acids and soluble nitrogen was higher compared to samples at room temperature. However, after 153 days similar levels were observed in all products. The concentration of volatile base nitrogen was greater at 24^{0}C than at 37^{0}C. Fish sauce fermented at 50^{0}C had the lowest amino nitrogen and the highest volatile nitrogen. Ooshiro et al., (1981) concluded that enzyme activity was inhibited at 50^{0}C and an increase in nitrogen could be attributed to a concentration effect as a result of moisture

loss.

Chayovan et al., (1983a) stated that the optimum temperature for commercial fish sauce production was 37^{0}C. However, the practice of placing the container with product directly in the sun, partly adopted by the Southeast Asia industry, results in a more rapid protein hydrolysis (Amano, 1962).

(ii) The use of acid

The addition of acid at ambient temperature encourages the activity of acidic proteolytic enzymes, such as pepsin, which are found abundantly in the fish stomach.

Beddows and Ardeshir (1979b) studied the use of hydrochloric acid in fish sauce production and reported that the optimum conditions were either pH 2.0 and 10% salt (w/w) or pH 3.0 and 15% salt (w/w). Acidic proteolysis occurred more rapidly compared to traditional methods of fermentation and the amount of soluble protein hydrolysed was comparable to traditional fermentation methods. However, eventhough similar concentrations of volatile nitrogen were observed, the end product had very little aroma.

Gildberg et al., (1984) studied the acceleration of fish sauce fermentation by adding acid and reducing salt content. Good quality fish sauce was prepared from minced anchovy which had been allowed to autolyse at pH 4.0 with a salt concentration as low as 5%. After the initial phase of rapid proteolysis, samples were neutralised and salt added to its normal concentration (25%). The increased proteolysis at low salt concentrations was due to the fact that pepsins are significantly inhibited by a salt concentration greater than 5%. This method was found to produce an acceptable sauce after 2 months while the traditional method required 6 months or longer. The product

produced was reported to have a better balance of essential amino acids than first grade commercially produced fish sauce although it had a lower level of volatile bases and acids.

(iii) Alkaline hydrolysis

It has been reported that hydrolysis of fish protein is enhanced if fish is adjusted to an alkaline pH and then neutralized. However, this method is not recommended for food processing because of the adverse effect on the nutritional quality of the end product (Hayashi and Kameda, 1980; Feron et al., 1978).

(iv) Enzyme supplementation

Commercial plant proteases such as bioprase, pronase, papain, bromelain and ficin have all been investigated as enzyme supplements to hasten the rate of fish sauce fermentation.

Murayama et al., (1962) reported that the addition of bioprase and pronase reduced the fermentation time to 70 days and produced a good quality fish sauce. Guevara et al., (1972) used papain in the production of Patis, a Philippine fish sauce, and claimed that the fermentation time was reduced to 4-7 days without any deleterious effect on the characteristic flavor of the sauce.

Beddows et al., (1976) reported a high degree of proteolysis was found in homogenized mackerel at 38^{0}C containing 0.2% bromelain. Conversion of insoluble protein into soluble protein was greater if a 24 hr pre-incubation period was employed prior to the addition of salt.

Beddows and Ardeshir (1979a) examined the effect of adding bromelain (0.8%), papain (2.75%) and ficin (2.5%) on the rate of protein hydrolysis using minced Ikanbilis. Bromelain was found to give more favorable results compared to papain or ficin with greater amounts of fish protein being solubilized in 18-21 days at 33°C. The distribution of nitrogen was similar to traditional fermentation methods but the end product lacked aroma.

Japanese anchovy was fermented with 20% salt and added "koji" (35%) or 0.5% pronase and stored at 30°C for 150 days (Miyazawa et al., 1979). The addition of "koji" had a pronounced effect on the liquefication of protein and the formation of free amino acids, while the effect of pronase on protein hydrolysis was neglible.

Ooshiro et al., (1981) also examined the use of papain, bromelain and trypsin to produce fish sauce from unminced sardines. Optimum results were obtained using 0.3% papain at pH 5.2, 37°C and 25% salt. The taste and color of the end product was satisfactory but it lacked the typical aroma of fish sauce. Furthermore, the fermentation took 340-350 days.

The visceral extract from sardine, which contains several proteases, was used in the preparation of fish sauce from sardine (Yoshinaka et al., 1983). The sardine flesh homogenate was incubated with the visceral extract for 5 hr at pH 8.0 and at 50°C. The mixture was clarified by centrifugation and 25% NaCl was added. The quality of fish sauce obtained was reported to be comparable with commercial Shotturu in both amino acid content and sensorial qualities.

Raksakulthai et al., (1986) examined the effect of proteolytic enzymes (fungal protease, pronase, trypsin, chymotrypsin, squid protease) or squid hepatopancreas on the

production of fish sauce from male capelin. They observed that an excellent quality fish sauce could be prepared from minced capelin when fermentation was carried out using a minced fish:salt ratio of 4:1 at 20-25^{0}C and 2.5% squid hepatopancreas as a fermentation aid. Addition of various enzyme preparations to minced capelin increased the initial rate of protein hydrolysis but did not yield a product with a greater free amino acid content. Analysis of capelin sauce indicated that the product, supplemented with squid hepatopancreas, was highly acceptable and more preferable than commercially produced Patis. However, supplementation with squid hepatopancreas did not improve the fermentation time since the process, including the aging time period, took 33 months.

(v) The use of bacteria and/or their enzymes

Acceleration of fish sauce fermentation by the addition of halophilic bacteria was studied by Ok et al., (1982b). When *Bacillus* C1 and C6, isolated from Chinese fish sauce, were added to a mixture of sardine and salt and stored at 30^{0}C, the fermentation time was reduced to 3 months. Partially purified protease from a moderately halophilic marine bacterium *Pseudomonas sp.* was used to reduce the fish sauce fermentation process (Nakano et al., 1986). Results indicated that the enzyme could reduce the fermentation time from 1.5-3 years to 3-6 months and the product contained nutritionally valuable amino acids in a high concentration. Sensory tests showed that the product had a fairly good taste and good quality compared to commercial products.

1.12. Rationale for this study

One of the major problems facing the world today, particularly in the developing countries, is the provision of adequate supplies of high quality protein. With respect to this objective, fish is regarded as a good source of protein in terms of quality, supply and cost. However, many fish species are rejected by consumers on the basis of their physical appearance, textural qualities and unacceptable taste and are discarded as waste products. This problem also faces developed countries such as Canada, specifically, Quebec where herring is an underutilized fish species and is often discarded as waste (Boudreau, 1990).

One viable method for the conversion of underutilized fish species into value added secondary products is through fermentation. Although fish sauce fermentation is widely practiced in Asian countries, it is very time consuming. Furthermore, little is known about fish sauce fermentation technology in North America. Detailed studies of the changes occurring in fish sauce fermentation using enzymes to accelerate the rate of fermentation would be of great economic value, not only in Asian countries, but also in Quebec which could produce its own fish sauce from underutilized fish species.

1.13. Objectives of study

In a view of these comments, the overall objectives of this study are:

(1) To analyze the chemical and microbiological composition of commercially produced first grade and second grade Thai fish sauces as a reference for laboratory produced fish sauce.

(2) To compare the chemical and microbiological composition of commercially produced first grade and second grade Thai fish sauce (Nampla) with regulatory Thai specifications for both grades of sauce.

(3) To produce an acceptable fish sauce from underutilized fish species, e.g. herring.

(4) To determine the effect of enzyme supplementation using trypsin and chymotrypsin to accelerate fish sauce fermentation.

(5) To monitor the physical, chemical and microbiological changes during fermentation of the enzyme supplemented fish sauce.

(6) To compare the chemical and microbiological composition and organoleptic quality of the enzyme supplemented fish sauce with first grade commercially produced Nampla.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

First grade and second grade Thai fish sauces (Nampla) were obtained from commercial suppliers in Chinatown, Montreal, Canada.

In the production of fish sauce, herring (*Clupea harengus harengus*) was used as raw material throughout the study. Fish were obtained fresh from Marché Centrale, Montreal and transported to the laboratory under refrigerated storage conditions and kept at ~4^{0}C until use.

Bovine pancreatic trypsin type III (activity: 10,000-13,000 BAEE units per mg protein) and chymotrypsin type II (activity: 40-60 units per mg protein) were obtained from Sigma Chemical Company (St.Louis, Missouri).

2.2. Analysis of commercial products

First grade and second grade Thai fish sauces (Nampla) were analyzed for chemical and microbiological composition as described in analytical procedures (2.4) and compared to Thai regulatory standards (Anon, 1983).

2.3. Preparation of fish sauce

In the first study, whole uneviscerated herrings were ground in a meat grinder to a uniform size. Approximately 100 gram amounts of minced herring were mixed thoroughly with 25% salt (w/w) and the following amounts of enzyme added to duplicate

batches: (i) no enzyme (control) (ii) 0.3% w/w of trypsin and chymotrypsin in the proportions of 100:0, 50:50 and 0:100. The mixtures were transferred to fermentation glass jars, sealed and stored at 37^{0}C. Liquid was removed from each treatment after day 1, 7, 14, 21, 28, 35 and 42 and analyzed for pH and degree of hydrolysis. At the end of fermentation (day 42), samples were filtered and the filtrates were analyzed for their pH, NaCl content, formol nitrogen, total nitrogen, volatile nitrogen, soluble protein and amino acid composition. Microbiological analysis was done using TSA as the growth medium.

In the second batch of fish sauce, duplicate (900 g) of minced herring were mixed thoroughly with 25% salt and 0.6% (w/w) of trypsin and chymotrypsin in the proportions of 100:0, 75:25, 50:50, 25:75 and 0:100 added to each mixture. The mixtures were placed in the 1 L glass jars, sealed and stored at 37^{0}C. On day 7, 14, 21, 28, 35 and 42 of fermentation, pH, degree of protein hydrolysis, total nitrogen, volatile nitrogen, free amino acid composition and soluble protein of each treatment were measured. Total aerobic and anaerobic counts were done using TSA as the growth medium. Analyses of the end products (day 42) were compared with first grade commercially produced Thai fish sauce (Nampla).

2.4. Analytical procedures

2.4.1. Chemical analysis

2.4.1.1. pH

The pH of all fish sauces were measured using a previously calibrated Corning Model 220 pH meter. Duplicate measurements were made by immersing the electrode directly into 5 ml samples in a beaker.

2.4.1.2. Sodium chloride

The sodium chloride content of all sauces was determined using a Horiba compact salt meter Model C-121 (Horiba Instruments, Inc., Irvine, California). Samples were prepared by diluting 1 ml of fish sauce to 25 ml with deionized water. The Horiba compact salt meter was calibrated with 0.5% and 5% NaCl standard solution and all readings were accurate to within $\pm$0.05%.

2.4.1.3. Total nitrogen

Total nitrogen was determined according to A.O.A.C. (1980) procedures. Approximately 0.5 ml of fish sauce were placed in the Kjeldahl digestion tube. Eight ml of concentrated H_2SO_4 and a Kjeldahl catalyst tablet (3.5 g K_2SO_4 and 175 mg MgO, Fisher Scientific) were added to the sample and digested in a digester at 410^{0}C until a clear solution was obtained. Distilled water (55 ml) was added to the tube and the mixture steam distilled using a Labconco Rapid Still III System (model 65300, Labconco, Chicago) while 85 ml of a NaOH (50%)/$Na_2S_20_3$ (5%) solution were added automatically via a built in dispensing system. The distillate was collected in a 50 ml saturated boric acid with indicator (Fisher Scientific). Titration was done using 0.05 N HCl until the color changed to pink-red. The results were expressed as mg nitrogen per ml of fish sauce using the following formula:

$$\text{Total N (mgN/ml)} = \frac{\text{Volume of HCl x Normality of HCl x 14.007}}{\text{Volume of fish sauce (ml)}}$$

2.4.1.4. Formol nitrogen

Formol nitrogen, a convenient index of the extent of protein hydrolysis, was determined by the formol titration method of Beddows et al., (1976). One ml of fish sauce was mixed with 40 ml of deionized water and titrated to pH 7.0 with 0.1 N NaOH and then 10 ml of formalin solution (38% v/v) added to the neutralized sauce. Titration was continued to pH 8.5 with 0.1 N NaOH. Test results were expressed as mg nitrogen per ml fish sauce using the formula below:

mg formol nitrogen = ml NaOH (pH 7-8.5) x Normality of NaOH x 14

2.4.1.5. Soluble protein

Soluble protein was estimated using the Biuret method of Cooper (1977). Approximately 0.2 ml of fish sauce was diluted to 1 ml with deionized water. To 1 ml of sample, 4 ml of biuret reagent was added, mixed and incubated for 20 minutes at 37^{0}C. The biuret solution was prepared by adding 1.5 g $CuSO_4.5H_2O$ and 6 g $(NaKC_4O_6.H_2O)_4$ to 500 ml deionized water. Three hundred ml of 10% (w/v) NaOH was then added and the mixture diluted to 1 L with water. A standard curve, with a concentration range of 1-10 mg/ml protein, was prepared by adding appropriate amounts of a stock Bovine Serum Albumin containing 10 mg protein/ml (Sigma Chemical Company) to deionized water. The absorbance of test and standard solution was read at 540 nm using 1 ml deionized water and 4 ml biuret reagent as a blank solution. Test results were expressed as mg protein per ml fish sauce.

2.4.1.6. Volatile nitrogen

Volatile nitrogen was determined by the method of Chayovan et al., (1983b). A 0.7 ml of fish sauce was pipetted into a Labconco Kjeldahl steam distillation apparatus and 4 ml of 0.2% $Mg(OH)_2$ solution added. The sample was distilled to release volatile nitrogen into 50 ml saturated boric acid containing indicator. The titration method for total nitrogen determination was then used as described previously. Results were expressed as mg nitrogen per ml fish sauce.

2.4.1.7. Amino acid analysis

Free and total amino acid composition were determined by Reversed Phase High Performance Liquid Chromatography (HPLC) (System Gold, Beckman Instruments Inc., U.S.A.), using dimethylaminoazobenzene sulfonyl chloride (dabsyl chloride) as the derivatizing agent. All samples were detected in the visible range at 436 nm.

2.4.1.7.1. Sample preparation

(i) Free amino acids

Fish sauce (3 ml) was deproteinized with 4 volumes of 20% sulfosalicylic acid (Lee et al., 1982b) and centrifuged at 15,600 *g* for 30 minutes. One ml of clear supernatant was diluted to 100 ml with ultra pure water obtained from a Millipore Milli-Q Plus System (Millipore Canada Ltd.). A 20-µl diluted sample was placed in a sample tube (5 x 50 mm) and freeze-dried for the dabsylation procedure.

Twenty µl of dabsylation buffer (50 mM sodium bicarbonate pH 8.1, Beckman Instruments Inc.) was added to each of the dried test, blank and standard. Forty µl of the

reconstituted dabsylation reagent (1 mg of dabsyl chloride in 1 ml acetonitrile, Beckman Instruments Inc.) was then added to each tube which was sealed with a stopper and heated at 70°C in a heating block for 12 minutes. All tubes were vortexed after 1, 4, 12 minutes during the heating period. The tubes were then cooled and 440 µl of dilution buffer (50 mM sodium phosphate pH 7.0/ethanol 1:1 v/v, Beckman Instrument Inc.) was added and mixed gently. The derivatized samples were then stored at 4°C until analysis by Reversed Phase HPLC.

(ii) Total amino acid

For analysis of the total amino acid composition, 20 µl of each diluted fish sauce (1:199) was pipetted into a sample tube, freeze-dried and then placed in a reaction vial. Four hundred µl of 6 N HCl (Beckman Instruments Inc.) was then added to each reaction vial and vials placed in a PICO TAG System (Waters Division, Millipore Ltd.) set at 110°C for 24 hours to permit gas phase hydrolysis of the sample. On completion of hydrolysis, samples were then freeze-dried prior to the dabsylation procedure as described previously.

2.4.1.7.2. Reversed Phase HPLC Chromatography

The chromatographic analysis was performed by a Beckman HPLC System using an ultrasphere C-18 HPLC column (4.6 x 250 mm, Beckman Instruments Inc.). The compositions of the mobile phases are shown in Table 7. The gradient program used for the amino acid analysis is shown in Table 8. All eluents were degassed by ultrasonication before use. Integration of the peak areas from the chromatograms, obtained at 436 nm, was done using the System Gold program. Results were expressed as µmol per ml sauce.

Table 7. Composition of the mobile phases used in amino acid analysis

Mobile phase	Composition
A	100 ml of 100 mM sodium citrate
	860 ml of ultra pure water
	40 ml of dimethylformamide (DMF)
B	300 ml of mobile phase A
	700 ml of 4% DMF in acetonitrile

Source: Beckman, 1989.

Table 8. The gradient program used for amino acid analysis by Reversed Phase HPLC-System Gold

Time (min)	Flow rate (ml/min)	%A	%B
Initial	1.4	75	25
0.00	1.4	44	56
17.20	1.4	14	86
23.20	1.4	0	100
29.20	1.4	75	25
39.90	1.4	75	25

Source: Beckman, 1989.

2.4.2. Microbiological analysis

Total aerobic and anaerobic plate counts were done using a pour plate technique (A.P.H.A., 1984). Trypticase soy agar (TSA) (BBL, Becton Dickinson and Co., U.S.A.) and trypticase soy agar containing 10% NaCl were used as the growth medium.

Media were prepared by suspending 40 g of TSA in either 1 L of distilled water or 1 L of 10% NaCl solution, mixing thoroughly and heating with frequent agitation to boiling temperature. After boiling for 1 minute to completely dissolve the rehydrated media, the solution was sterilized by autoclaving at 121^{0}C for 15 minutes, cooled and kept at 4^{0}C until use. Prior to use, the media was melted and cooled to 45^{0}C.

Serial dilutions of samples were done according to the procedures outlined in A.P.H.A. (1984), using 0.1% Bacto peptone (Difco Laboratories, Detroit, Michigan) solution as diluent. Aerobic plate counts were incubated at 37^{0}C for 48-72 hours. Anaerobic plate counts were incubated at 37^{0}C in an BBL anaerobic jar containing a BBL Gas Pak Plus (hydrogen-carbon dioxide gas generation pack, Becton Dickinson and Co.) for the same time period.

Plates containing 25-250 colonies per plate were counted using a colony counter (A.P.H.A., 1984). Counts, expressed as CFU (Colony Forming Unit) per ml of fish sauce, were obtained by multiplying the total plate count by the reciprocal of the appropriate dilution.

2.5. Sensory evaluation

Sensory evaluation was conducted only on fish sauces prepared from various ratios of 0.6% enzyme supplements at the end of the fermentation period (Day 42) and on the

first grade commercial Thai fish sauce (Nampla).

A group of nine Southeast Asian students from McGill University, Macdonald Campus, whose diets usually included fish sauce, were asked to evaluate each sauce for the following preference attributes: (i) color, (ii) aroma and (iii) flavor. Evaluations were carried out using an unstructured 10-cm hedonic graphic rating scale ranging from dislike extremely to like extremely as described by Giovanni and Pangborn (1983). Samples were coded and were evaluated in a random order. The color, aroma and flavor tests were all done on samples in three separate sessions and, in each session, samples were presented to panellists at the same time. All sensory evaluations were carried out in duplicate.

2.6. Statistical analysis

Statistical analyses of the data (Analysis of variance and Duncan's multiple comparison of means) were carried out as described by Steel and Torrie (1980) using a Statistical Analysis System (SAS, 1985).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Chemical and microbiological composition of first grade and second grade commercially produced Thai fish sauce (Nampla)

3.1.1. Chemical composition

The results of the chemical composition for both grades of Nampla are shown in Table 9. The pH values and the sodium chloride contents were similar with both grades of sauce having pH values of 5.3-5.5 and sodium chloride concentrations of 24-25%. First grade sauce contained approximately 1.6 times more total nitrogen, 1.5 times more formol nitrogen, 1.3 times more soluble protein, 2.6 times more free amino acid content, and 1.8 times more total amino acid content compared to the second grade sauce (Table 9). However, volatile nitrogen (mainly ammonia) was comparable in both sauces.

3.1.2. Nitrogen distribution in commercially produced Nampla

The nitrogen distribution for both grades of commercially produced Nampla are shown in Table 10. The nitrogen content of first grade sauce comprised of 53% in the form of free amino acids, 17% as protein and peptides and 13.5% as volatile nitrogen. However, in second grade sauce, only 31.5% of the total nitrogen was in the form of free amino acids with more nitrogen being distributed as protein, peptides and volatile nitrogen, which is indicative of lower quality sauce.

Table 9. Chemical composition of first grade and second grade commercially produced Nampla

	First grade	Second grade
pH	5.51	5.26
NaCl (%)	24.50	25.00
Total N (mgN/ml)	23.57	15.10
Formol N (mgN/ml)	13.79	9.07
Ammonia N (mgN/ml)	3.18	2.41
Soluble protein (mg/ml)	24.45	18.77
Free AA[1] content (µmol/ml)	628.40	237.23
Total AA[1] content (µmol/ml)	774.00	437.11

1 AA = Amino acid

Table 10. Distribution of nitrogenous compounds in first grade and second grade commercially produced Nampla

Sauce grade	Total N (mgN/ml)	% of total N			
		Volatile N	Amino acid N[1]	Biuret N[2]	Other N[3]
First	23.57	13.49	52.62	16.59	17.30
Second	15.10	15.96	31.46	19.89	32.69

1 From amino acid analysis data
2 From biuret soluble protein data (Biuret N = Biuret protein/6.25)
3 Determined by total N - volatile N - amino acid N - biuret N and could include nucleic acid and other breakdown products.

3.1.3. Amino acid composition

The free and total amino acid profiles of both grades of commercially produced Nampla are shown in Figures 2 and 3, respectively. Both first grade and second grade sauce contained high levels of histidine, lysine, glutamic acid, alanine, glycine and valine, and very low level of arginine, cysteine (cystine) and tyrosine. The essential amino acids such as lysine, histidine, phenylalanine, valine, leucine, isoleucine, methionine and threonine were all present in both grades of sauce. Negligible amounts of cysteine (cystine), arginine and tyrosine were present, probably due to the action of microorganisms or chemical oxidation and/or deamination of these amino acids. Arginine, for example is used as a nitrogen source by some microorganisms (Jonsson et al., 1983). These results are in agreement with the observations of Rosario and Basaran (1984) who found high levels of essential amino acids in Philippines fish sauce. They are also in agreement with the observations of Kurokawa (1986) who reported that glutamic acid, lysine and alanine were the predominant free amino acids in Chinese fish sauce. Furthermore, the level of free amino acids was greater in first grade Chinese sauce compared to second grade and lower grade sauce.

The high level of glutamic acid found in most fish sauces may be due to the addition of artificial flvouring agents, such as monosodium glutamate (Tongthai and Okada, 1981; Rosario and Basaran, 1984). However, this is controlled in regulatory Thai standards by the ratio of glutamic acid/total nitrogen which must be in the range of 0.4-0.6.

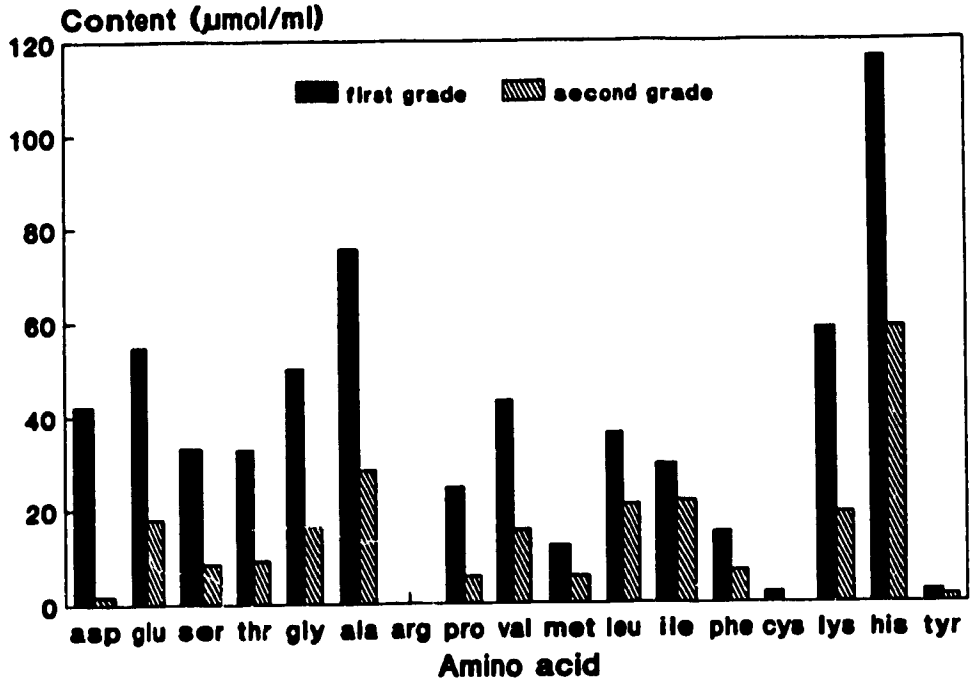

Figure 2. **Free amino acid profile of commercially produced Nampla**

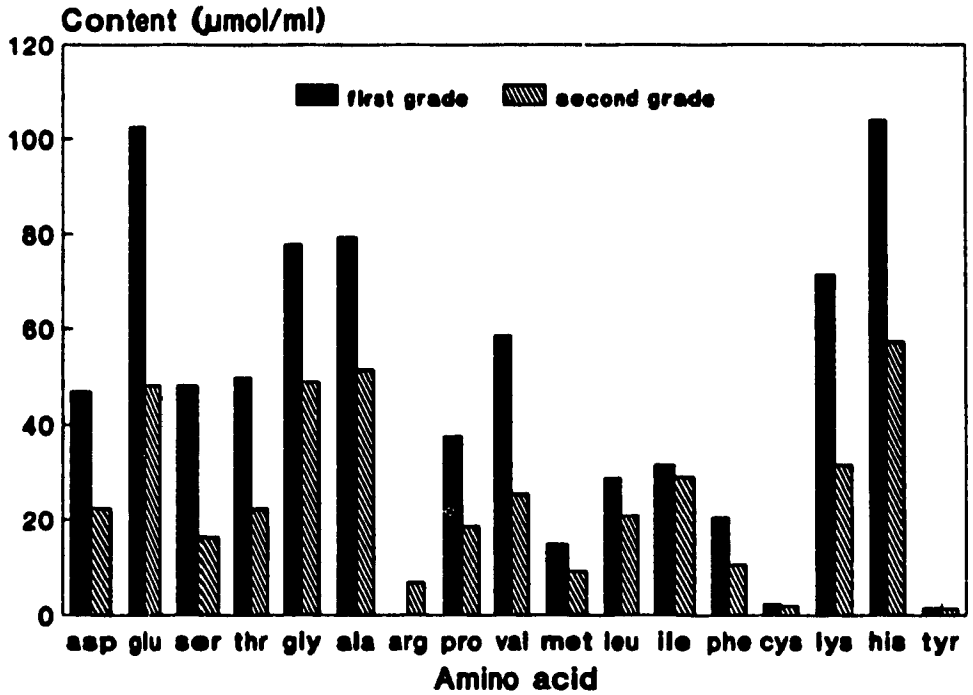

Figure 3. **Total amino acid profile of commercially produced Nampla**

3.1.4. Comparison of commercially produced Nampla with the regulatory Thai specifications

It is evident from Table 11 that the pH, sodium chloride content, total nitrogen and ratio of glutamic acid to total nitrogen for both grades of fish sauce products complied with Thailand regulatory specifications (Anon, 1983). However, while the amino acid nitrogen in the first grade sauce complied with specifications, the amino acid nitrogen was less than the regulatory specifications for second grade sauce.

3.1.5. Microbiological composition

The results of microbiological analysis for both grades of sauce are shown in Table 12. Both aerobic and anaerobic plate counts were less than 10^3 CFU/ml. The low microbial counts observed are probably due to the high salt concentration in these products and the inhibitory effect of salt on microbial growth. Slightly higher microbial counts were obtained with TSA media compared to TSA containing 10% NaCl. Therefore, the residual microorganisms could be classified as halotolerant rather than halophilic, which is in agreement with the results observed by Crisan and Sands (1975) and Raksakulthai (1987). However, Saisithi et al., (1966) isolated many halophilic strains from Nampla while Fujii and Sakai (1984) isolated some halophilic bacteria from Japanese fish sauce. Several acid producing halotolerant and halophilic bacteria were also isolated from fish sauces produced in Southeast Asia (Itoh et al., 1985a,b). While no regulatory microbiological specifications could be found for Nampla, the low microbial counts observed for both grades of Nampla in this study were similar to those observed for other indigenous fish sauces (Fujii et al.,1980; Itoh et al.,1985a; Kurokawa, 1986).

Table 11. Comparison of first grade and second grade commercially produced Nampla with regulatory Thai standards

	Sauce grade			
	First		Second	
	Standard[1]	Nampla	Standard[1]	Nampla
pH	5-6	5.51	5-6	5.26
Sodium chloride (mg/ml)	>230	245	>230	250
Total N (mgN/ml)	>20	23.57	>15	15.10
Amino acid N (mgN/ml)[2]	>10	10.60	>7.5	6.66
Glutamic acid/Total N	0.4-0.6	0.62	0.4-0.6	0.46

1 Anon, 1983.
2 determined by formol N - Ammonia N

Table 12. Total aerobic and anaerobic plate counts in first grade and second grade commercially produced Nampla (CFU/ml)

	First grade	Second grade
Aerobic counts		
TSA	90	115
TSA + 10% NaCl	<10	<10
Anaerobic counts		
TSA	210	210
TSA + 10% NaCl	<10	<10

3.2. Production of fish sauce from herring and using 0.3% of trypsin and chymotrypsin

3.2.1. Physical changes

As the fermentation progressed, the texture of all 0.3% enzyme supplemented herring sauces was more liquefied compared to sauces produced without enzyme (control). The color of sauces produced with added enzymes was also darker than the control sauces. However, the control sauce had a more fishy odor compared to sauces prepared with enzyme supplementation.

3.2.2. pH change

The pH changes for all herring sauces produced with various proportions of 0.3% enzyme supplementation during the fermentation period ranged from 5.8-5.9 and did not vary with time (Figure 4). The constant pH values may be attributed to the buffering effect of peptides, free amino acids and ammonia in the fermented sauces.

3.2.3. Changes in degree of protein hydrolysis

Changes in the degree of protein hydrolysis of herring fish sauces throughout the fermentation period are shown in Figure 5. During the first two weeks, the degree of protein hydrolysis increased rapidly in all sauces followed by a more gradual and constant increase until day 42. The reduction in the rate of the degree of protein hydrolysis after day 21 is probably due to the accumulation of amino acids and peptides (Orejana and Liston, 1981), enzyme inactivation, or the substrate becoming limiting with time. The addition of various proportions of 0.3% trypsin and chymotrypsin resulted in significantly

higher protein hydrolysis throughout the fermentation period ($p<0.01$) indicating the potential of trypsin and chymotrypsin to accelerate the rate of fermentation. Based on these results, a fermentation time of 42 days was used in all subsequent studies.

3.2.4. Chemical composition

The final chemical composition of herring sauces produced from various proportions of 0.3% trypsin and chymotrypsin (after day 42) are summarized in Table 13.

The final pH value of all sauces ranged from 5.82-5.85. Herring sauce produced with trypsin alone had a slightly higher salt content compared to the salt content of other sauces.

Fish sauces prepared with the addition of enzymes contained significantly more total nitrogen, more formol nitrogen and more soluble protein than the control sauce ($p<0.05$) indicating increased proteolysis. Statistical analysis of enzyme treated sauces indicated that trypsin and chymotrypsin added in the proportions of 50:50 and 0:100 (trypsin:chymotrypsin) contained more total nitrogen, more formol nitrogen and more soluble protein than the sauce treated with trypsin alone ($p<0.05$). However, volatile nitrogen was not significantly different in any treatments ($p>0.05$).

Addition of enzymes at various proportions also resulted in significant increase in free amino acid content of the sauces compared to the control sauce ($p<0.05$). However, there was no statistical difference in the concentrations of free and total amino acids between enzyme treated sauces ($p>0.05$).

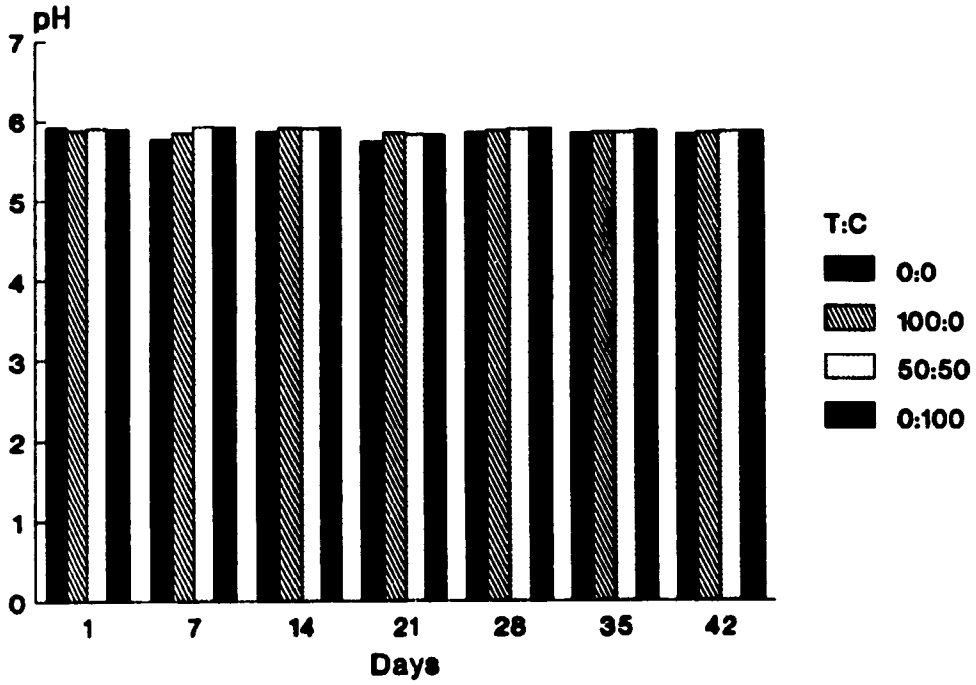

Figure 4. pH changes during fermentation of herring sauces produced with various proportions of 0.3% trypsin and chymotrypsin

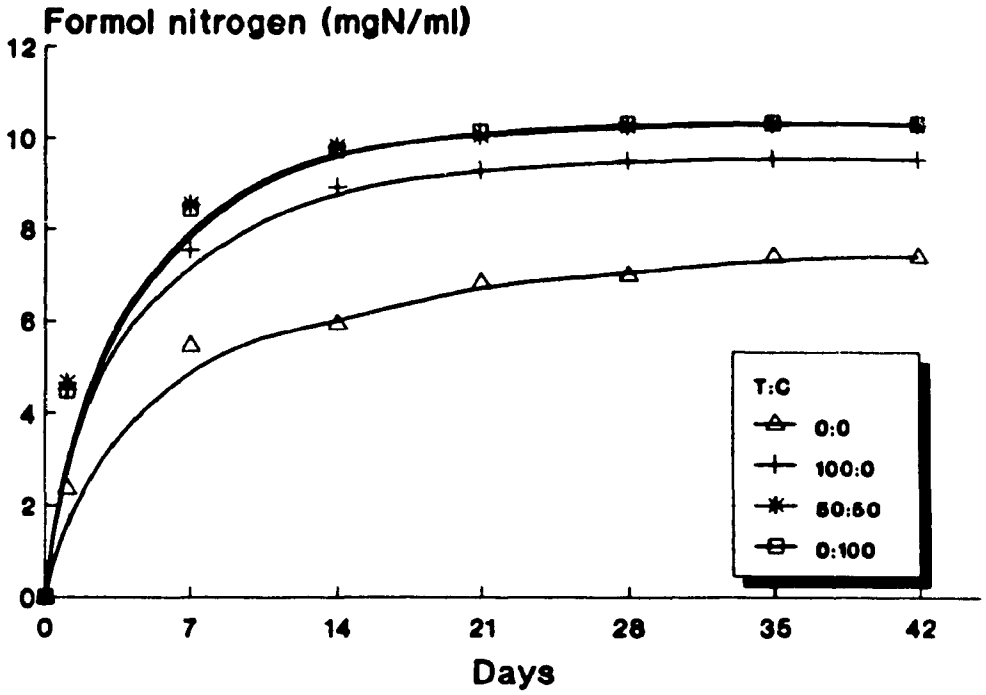

Figure 5. **Changes in degree of protein hydrolysis during fermentation of herring sauces produced with various proportions of 0.3% trypsin and chymotrypsin**

Table 13. Chemical composition of herring sauces produced with various proportions of 0.3% trypsin and chymotrypsin

	Sauce type (trypsin:chymotrypsin)			
	0:0	100:0	50:50	0:100
pH	5.82[a]	5.83[a]	5.83[a]	5.85[a]
NaCl (%)	23.25[b]	24.38[a]	23.45[b]	23.25[b]
Total N (mgN/ml)	16.52[c]	23.71[b]	25.77[a]	26.98[a]
Formol N (mgN/ml)	7.43[c]	9.58[b]	10.33[a]	10.36[a]
Volatile N (mgN/ml)	0.97[a]	0.99[a]	1.03[a]	1.08[a]
Soluble protein (mg/ml)	26.80[c]	42.62[b]	47.17[a]	49.52[a]
Free AA[1] (µmol/ml)	250.55[b]	377.85[a]	361.07[a]	340.62[a]
Total AA[1] (µmol/ml)	ND	904.68[a]	776.01[a]	790.01[a]

Values in the same row follow by the same letter are not significantly different at the 5% level.
Values are averages of duplicated batches.
1 AA = amino acid
ND = not determined

3.2.5. Amino acid composition

The free amino acid composition of the herring sauces produced from various proportions of 0.3% enzyme supplementation after day 42 are shown in figures 6 and 7. The level of free amino acids in most sauces produced with added enzyme was higher, by comparison to the control sauce. Analysis of variance of the amino acid composition of the various sauces indicated that sauces produced with enzyme supplement contained significantly higher levels of aspartic acid, threonine, alanine, arginine, leucine, isoleucine and phenylalanine compared to the control sauce ($p<0.05$). The distribution of free amino acids in all sauces produced with enzyme supplementation were similar i.e. they contained high levels of lysine, arginine, valine, isoleucine and alanine in varying amounts. Most sauces contained low levels of cysteine, histidine, proline and tyrosine.

The total amino acid composition of herring sauces produced with 0.3% enzyme supplementation are shown in Figures 8 and 9. All sauces produced with added enzymes contained similar levels of total amino acids with glycine , alanine, valine, lysine and isoleucine being the predominant amino acids.

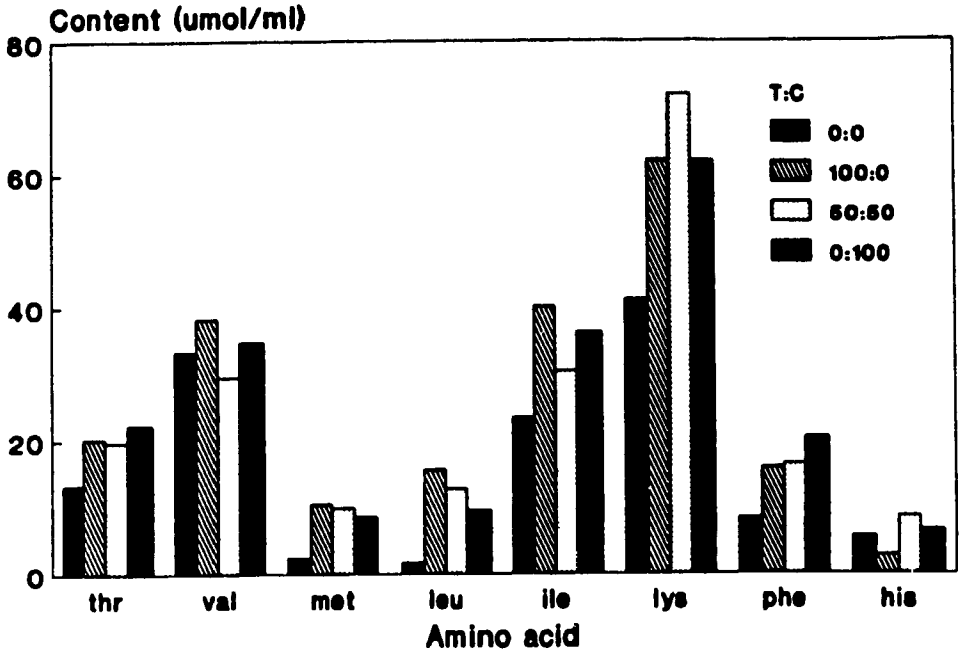

Figure 6. **Essential free amino acid profile of herring sauces produced with various proportions of 0.3% trypsin and chymotrypsin**

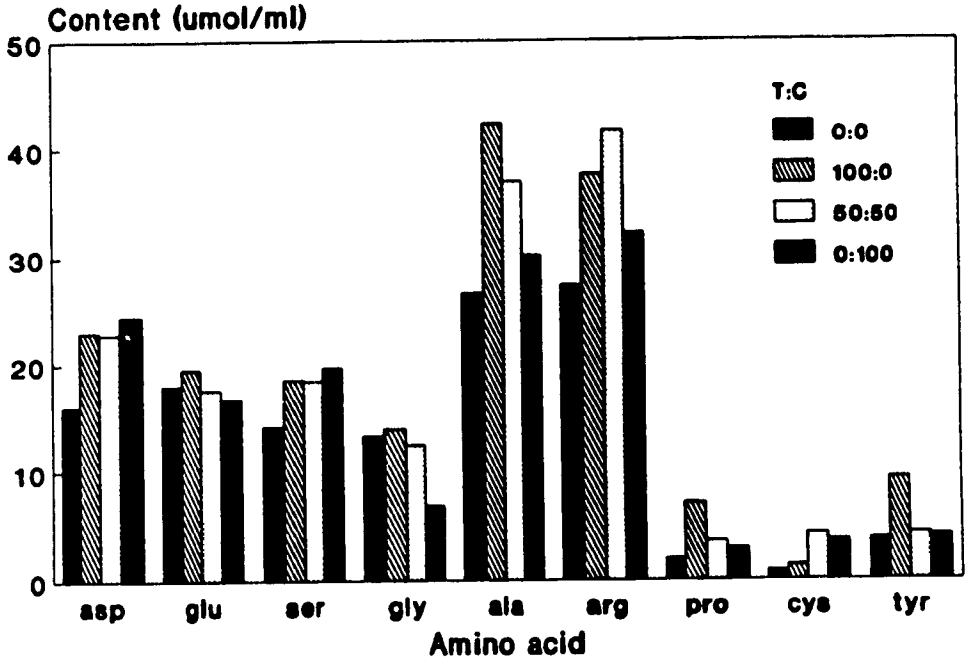

Figure 7. **Non-essential free amino acid profile of herring sauces produced with various proportions of 0.3% trypsin and chymotrypsin**

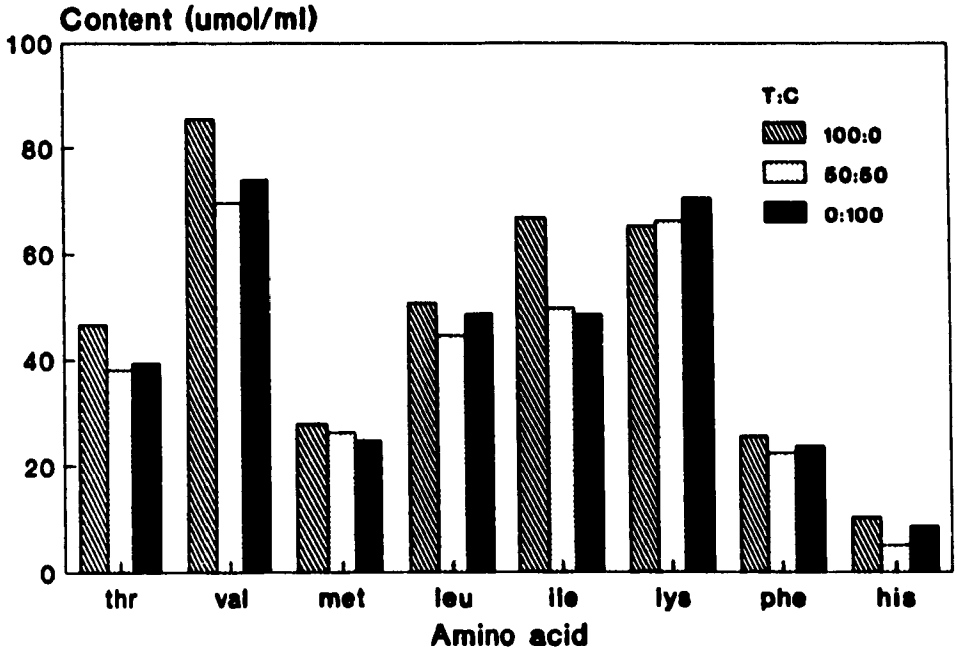

Figure 8. Essential total amino acid profile of herring sauces produced with various proportions of 0.3% trypsin and chymotrypsin

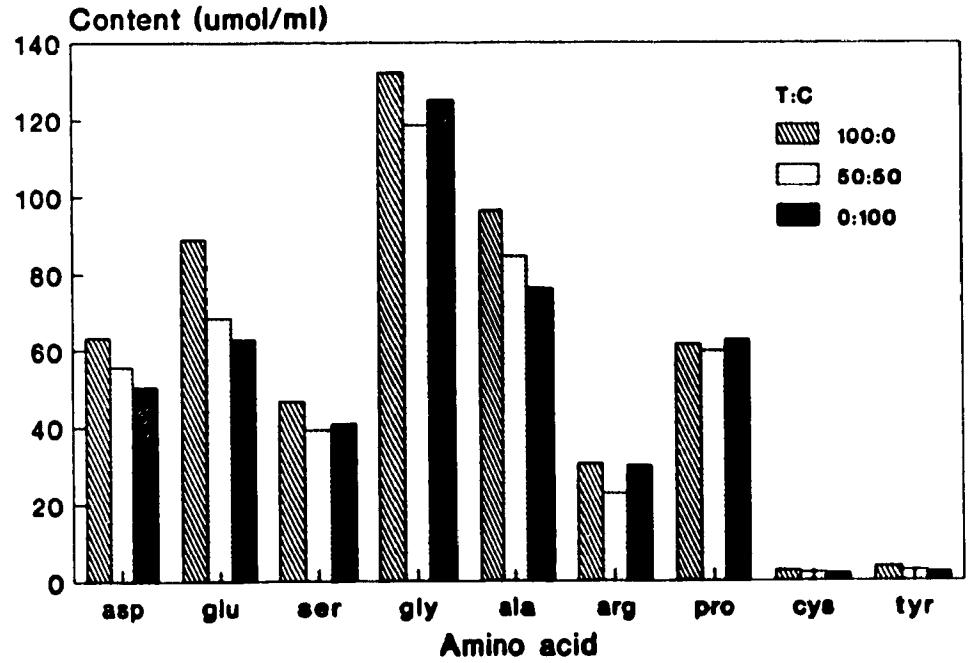

Figure 9. Non-essential total amino acid profile of herring sauces produced with various proportions of 0.3% trypsin and chymotrypsin

3.2.6. Microbiological composition

The total aerobic and anaerobic plate counts of herring sauces produced with 0.3% enzyme supplementation at the end of fermentation (day 42) are shown in Table 14. Plate counts in all sauces were very low ($<10^3$ CFU/ml). No differences were observed in aerobic and anaerobic plate counts between sauces with the exception of the control sauces which had higher aerobic plate counts.

Table 14. Total aerobic and anaerobic plate counts in herring sauces produced sauces with various proportions of 0.3% trypsin and chymotrypsin (CFU/ml)

	Sauce type (trysin:chymotrypsin)			
	0:0	100:0	50:50	0:100
Aerobic counts	<100	<100	<100	90
Anaerobic counts	400	<100	<100	210

3.3. Production of fish sauce from herring and using various proportions of 0.6% trypsin and chymotrypsin

Addition of enzymes has been shown to reduce the fermentation time for fish sauce production using herring. In the preliminary study, 0.3% of three proportions of trypsin and chymotrypsin (100:0, 50:50 and 0:100) were evaluated. In this study, the enzyme concentration was increased to 0.6% and five proportions of trypsin and chymotrypsin (100:0, 75:25, 50:50, 25:75 and 0:100) were used.

3.3.1. pH change

The pH values of all herring sauces produced with 0.6% enzyme supplementation increased gradually throughout fermentation period (Figure 10). This increase in pH can be attributed to the formation of ammonia and amines, such as di- or trimethylamine (Orejana and Liston, 1979).

3.3.2. Formation of nitrogenous compounds

The degree of protein hydrolysis, measured as formol nitrogen, during the fermentation of herring sauces prepared with various proportions of 0.6% trypsin and chymotrypsin are shown in Figure 11. As observed previously with 0.3% enzyme supplementation, the degree of protein hydrolysis in all sauces produced with 0.6% enzyme supplementation increased rapidly during the first two weeks and followed by a more gradual and constant increase until day 42. Statistical analysis showed that the increase in enzyme concentration from 0.3% to 0.6% resulted in an increase in degree of protein hydrolysis during the first two weeks ($p<0.05$) while it had no effect after day 14.

The formation of total nitrogen, free amino acids and volatile nitrogen in herring sauces produced with various proportions of 0.6% trypsin and chymotrypsin increased as fermentation progressed (Figures 12, 13 and 14). Initially, the formation of total nitrogen and free amino acids increased rapidly and then declined. However, the rate of increase in volatile nitrogen was consistent throughout the fermentation period. Furthermore, the formation of soluble protein and peptides increased and then decreased as the fermentation progressed (Figure 15). During fermentation, proteolysis resulted in an increase of smaller polypeptides as measured by biuret reaction. Biuret reagent reacts on the peptide bonds except for dipeptide. However, as fermentation progressed the concentration of peptides decreased while the concentration of free amino acids and volatile bases increased due to the action of proteolytic enzymes. These results are also similar to the observations of Raksakulthai (1987) in the fermentation of capelin fish sauce.

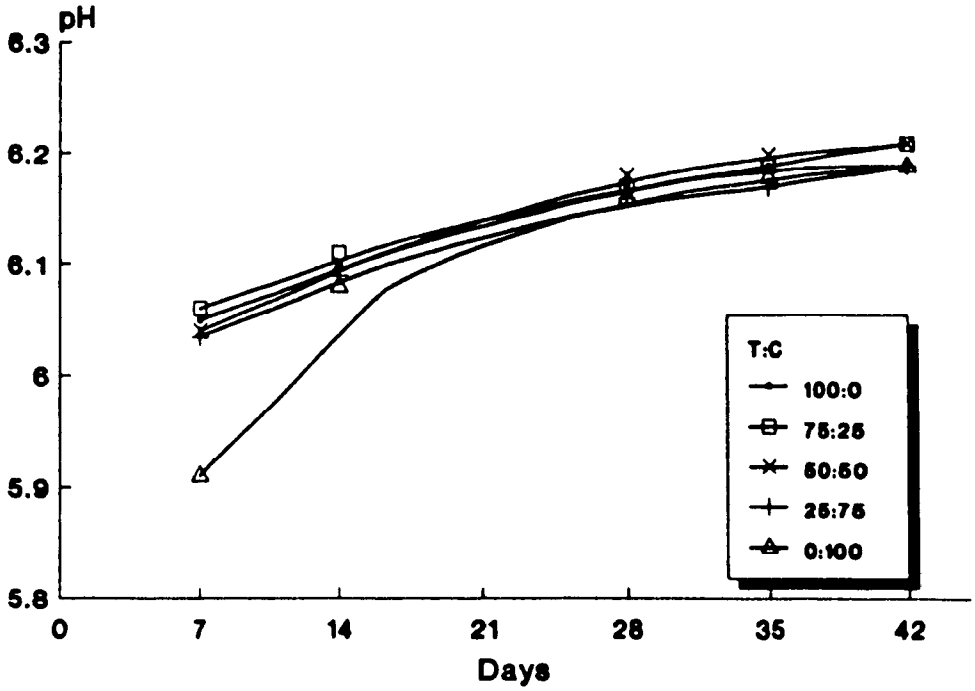

Figure 10. pH changes during fermentation of herring sauces produced with various proportions of 0.6% trypsin and chymotrypsin

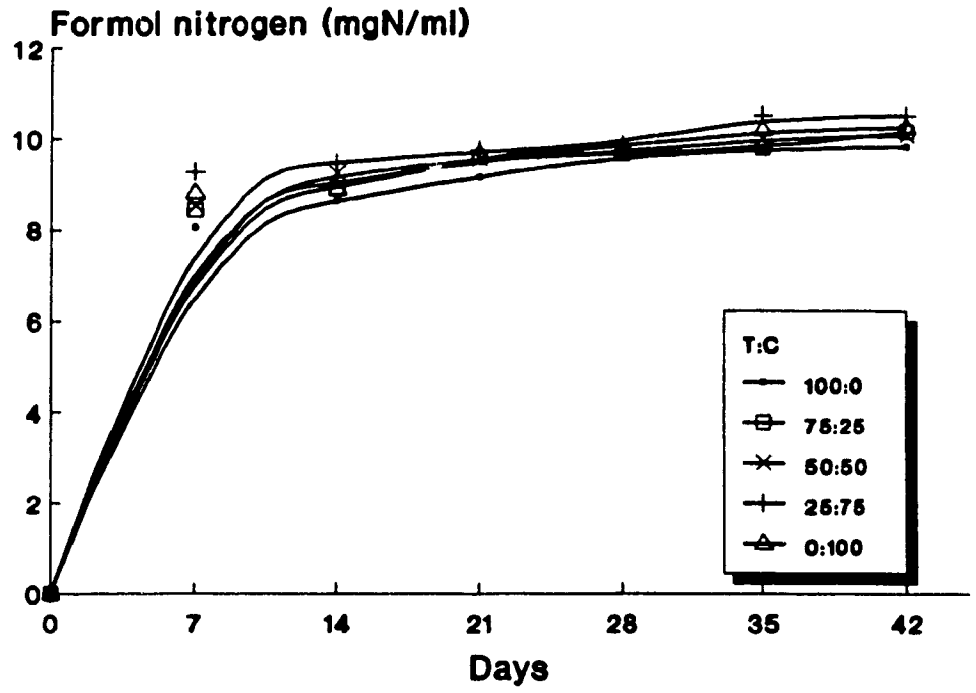

Figure 11. Changes in degree of protein hydrolysis during fermentation of herring sauces produced with various proportions of 0.6% trypsin and chymotrypsin

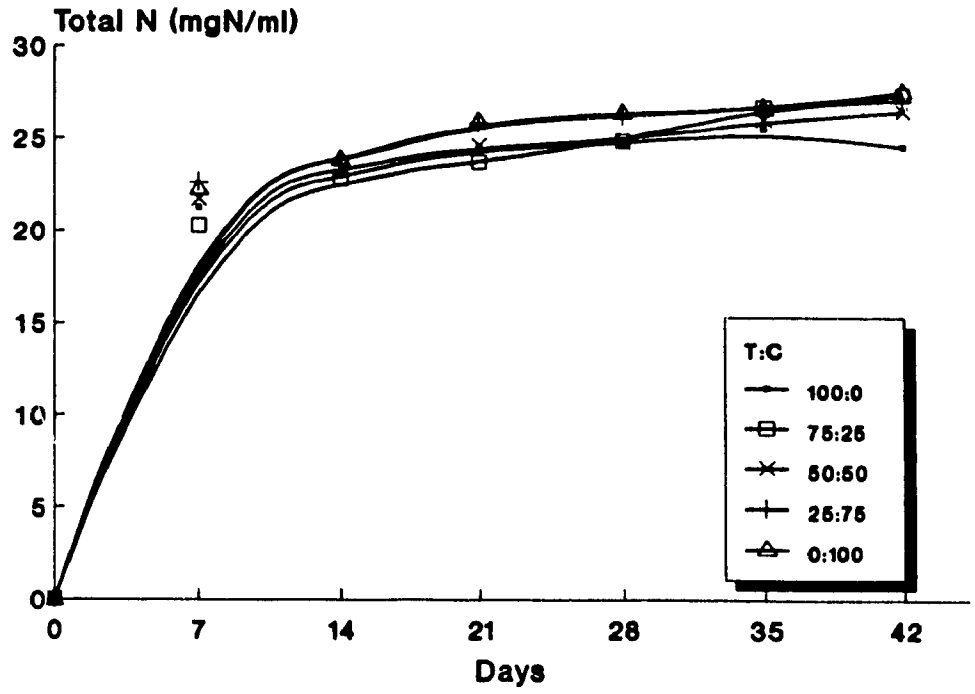

Figure 12. Changes in total nitrogen during fermentation of herring sauces produced with various proportions of 0.6% trypsin and chymotrypsin

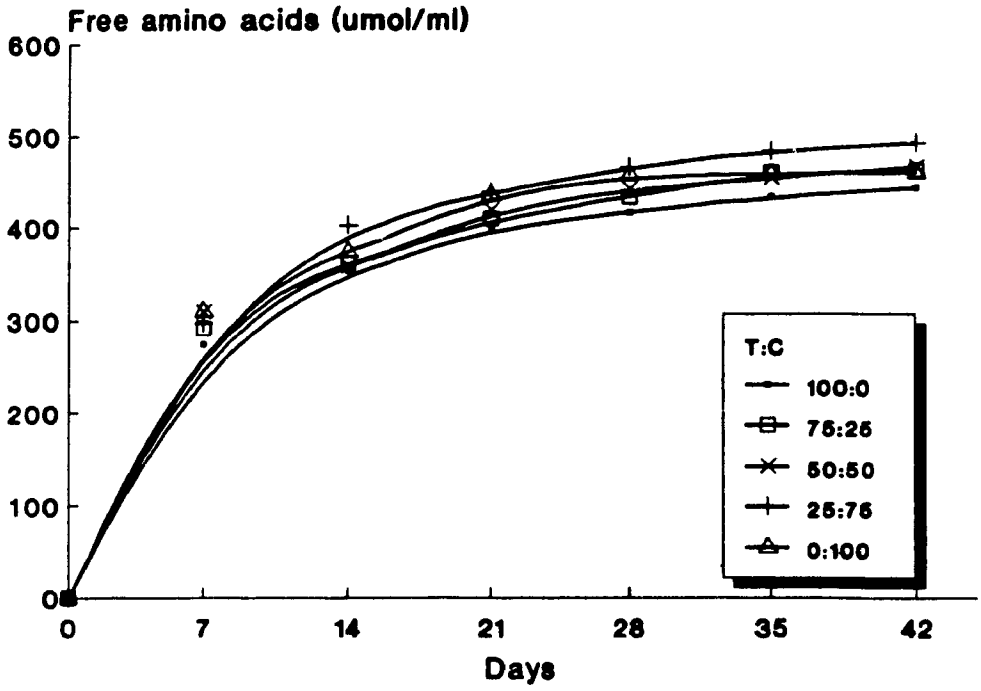

Figure 13. Changes in free amino acid co ent ing fermentation of herring sauces produced with various ep.tions of 0.6% trypsin and chymotrypsin

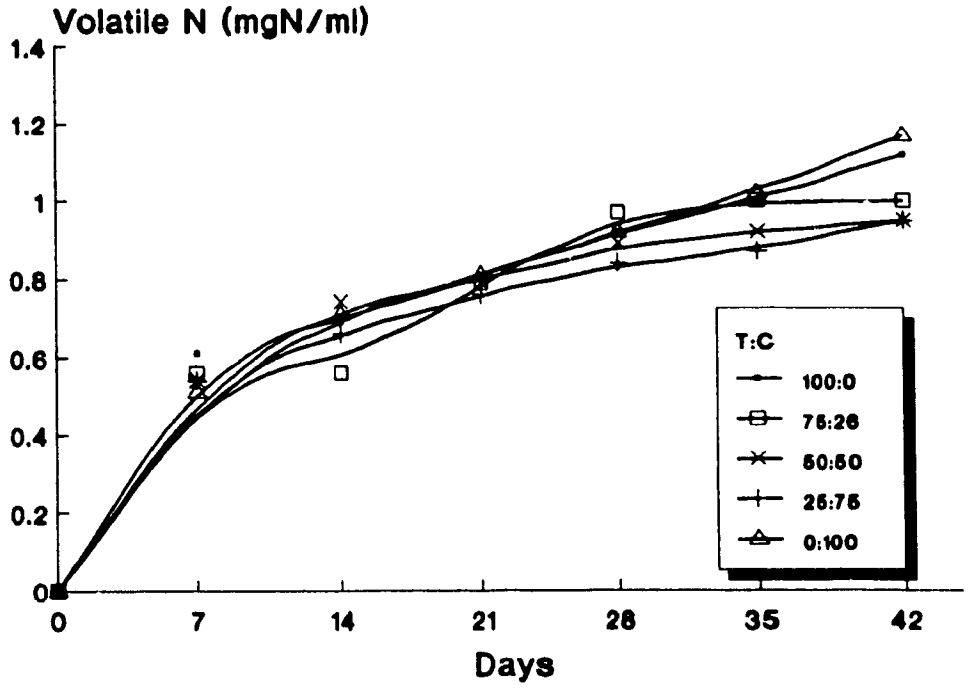

Figure 14. Changes in volatile nitrogen during fermentation of herring sauces produced with various proportions of 0.6% trypsin and chymotrypsin

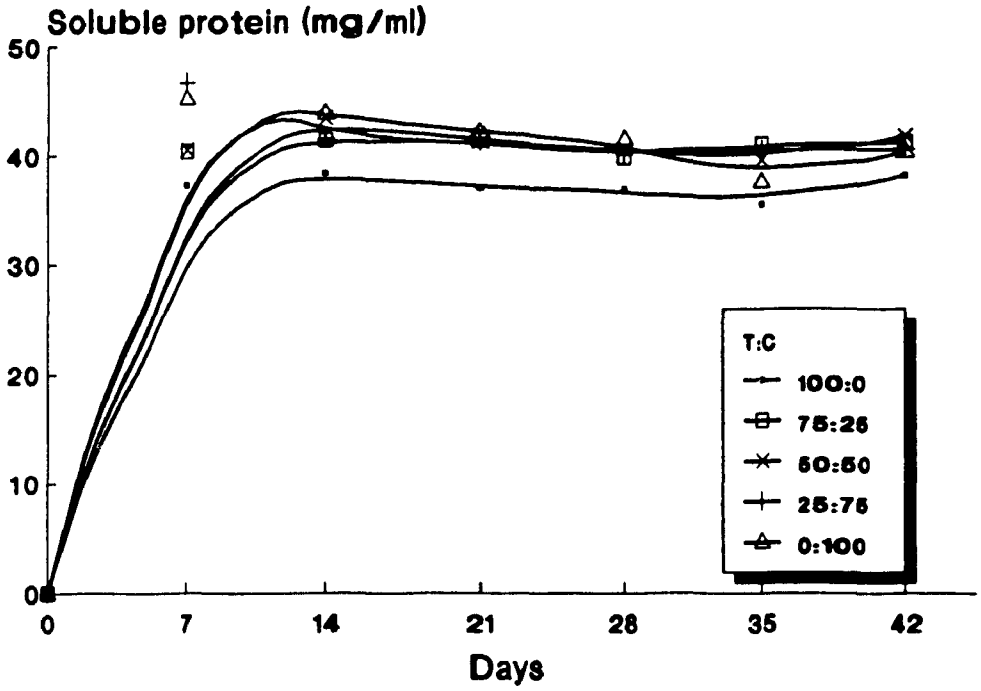

Figure 15. Changes in soluble protein during fermentation of herring sauces produced with various proportions of 0.6% trypsin and chymotrypsin

3.3.3. Chemical composition

The chemical composition of herring sauces produced with various proportions of 0.6% enzyme supplementation after day 42 are summarized in Table 15. The pH, sodium chloride content and volatile nitrogen were comparable in all sauces ($p>0.05$). The addition of 0.6% trypsin resulted in the lowest concentration of total nitrogen, formol nitrogen and soluble protein ($p<0.05$) while sauces treated with other enzyme proportions contained similar amounts of total nitrogen ($p>0.05$). The amount of formol nitrogen was greatest in sauce produced with an enzyme proportion of 25:75 while soluble protein was greatest in sauce produced with an enzyme proportion of 50:50. Laboratory herring sauce supplemented with an enzyme proportion of 25:75 contained significantly higher amount of free amino acids than other enzyme proportions used in herring fish sauce fermentation ($p<0.05$). However, there was no significant difference in the total amino acid concentration between treatments ($p>0.05$).

Table 15. Chemical composition of final herring sauces produced with various proportions of 0.6% trypsin and chymotrypsin

	Sauce type (trypsin:chymotrypsin)				
	100:0	75:25	50:50	25:75	0:100
pH	6.16[a]	6.22[a]	6.21[a]	6.19[a]	6.19[a]
NaCl (%)	23.25[a]	23.25[a]	23.38[a]	23.25[a]	23.38[a]
Total N[1]	24.61[b]	27.46[a]	26.57[a]	27.19[a]	27.62[a]
Formol N[1]	9.84[c]	10.15[bc]	10.06[bc]	10.48[a]	10.22[ab]
Volatile N[1]	1.12[a]	1.00[a]	0.95[a]	0.95[a]	1.17[a]
Sol. Protein[2]	38.08[c]	41.15[ab]	41.66[a]	40.51[ab]	40.29[b]
Free AA[3]	443.04[b]	462.22[b]	466.12[b]	492.35[a]	459.67[b]
Total AA[3]	794.30[a]	932.00[a]	920.20[a]	1008.80[a]	999.60[a]

Values in the same row follow by the same letter are not significantly different at the 5% level.
Values are averages of duplicated batches.
1 Express as mgN/ml
2 Express as mg protein/ml
3 Express as µmol/ml

3.3.4. Comparison of nitrogenous compounds of herring sauces produced with 0.3% and 0.6% trypsin and chymotrypsin at the proportions of 100:0, 50:50 and 0:100

The result of statistical analysis of the nitrogenous compounds of herring sauces produced with 0.3% and 0.6% enzyme concentration at the proportions of 100:0, 50:50 and 0:100 after day 42 are shown in Table 16. It is evident from Table 16 that an increase in enzyme concentration from 0.3% to 0.6% has a pronounced effect on the amount of nitrogenous compounds as shown by the increase in total nitrogen and free amino acid and the decrease in soluble protein ($p<0.05$). Since trypsin and chymotrypsin are very active during the early stage of fish sauce fermentation (Orejana, 1978), the addition of 0.6% enzyme concentration may enhance the hydrolysis of fish protein during the first 14 days of fermentation.

3.3.5. Comparison of the chemical composition of herring sauces produced with various proportions of 0.6% trypsin and chymotrypsin and first grade commercially produced Nampla

A comparison of the chemical composition of herring sauces prepared with various proportions of 0.6% enzyme concentration and first grade commercially produced Nampla is shown in Table 17. The results show that the pH value of commercially produced Nampla was significantly lower than laboratory produced herring sauces ($p<0.05$). This may be due to more volatile and non volatile fatty acids produced during the longer fermentation time period of 6 months or more used in commercial sauce production. These volatile and non volatile fatty acids contribute to the flavor and aroma of fish sauce

(Saisithi et al., 1966; Dougan and Howard, 1975; Chayovan et al., 1983a; Ooshiro et al., 1981).

The sodium chloride content of commercially produced Nampla was slightly higher than the laboratory produced sauces. This may attributed to a greater moisture loss during storage.

First grade commercially produced Nampla contained similar amounts of total nitrogen compared to the sauces produced with trypsin alone ($p>0.05$) but contained significantly less total nitrogen than herring sauces produced with other enzyme proportions ($p<0.05$). However, commercially produced Nampla contained significantly higher formol nitrogen compared to all laboratory produced herring sauces ($p<0.05$).

The volatile nitrogen in commercially produced Nampla was approximately three times higher than all laboratory produced herring sauces. Ammonia and other volatile bases were reported to contribute to the ammonia like aroma (Dougan and Howard, 1975). Hence, first grade commercially produced Nampla may have stronger ammoniacal aroma than herring sauces produced with various proportions of 0.6% trypsin and chymotrypsin.

Furthermore, first grade commercially produced Nampla contained significantly less soluble protein than all laboratory produced sauces ($p<0.05$). However, herring sauces supplemented with various proporations of 0.6% trypsin and chymotrypsin contained significantly lower concentrations of free amino acids ($p<0.05$) but comparable levels of total amino acids ($p>0.05$) compared to commercially produced Nampla.

Table 16. Nitrogenous compounds of herring sauces produced with 0.3% and 0.6% trypsin and chymotrypsin

	Enzyme concentration (% w/w)	
	0.3	0.6
Total N (mgN/ml)	25.48[b]	26.27[a]
Formol N (mgN/ml)	10.08[a]	10.03[a]
Volatile N (mgN/ml)	1.09[a]	1.08[a]
Soluble protein (mg/ml)	46.44[a]	40.01[b]
Free AA[1] (µmol/ml)	359.85[b]	456.27[a]
Total AA[1] (µmol/ml)	823.57[a]	904.69[a]

Values in the same row follow by the same letter are not significantly different at the 5% level.
Values are averages of 3 enzyme proportions: 100:0, 50:50 and 0:100.
1 AA = Amino acid

Table 17. Chemical composition of herring sauces produced with various proportions of 0.6% trypsin and chymotrypsin and first grade commercially produced Nampla

	Sauce type					
	100:0[1]	75:25[1]	50:50[1]	25:75[1]	0:100[1]	Nampla
pH	6.2[a]	6.2[a]	6.2[a]	6.2[a]	6.2[a]	5.5[b]
NaCl (%)	23.3[b]	23.3[b]	23.4[b]	23.4[b]	23.4[b]	24.5[a]
Total N[2]	24.6[b]	27.5[a]	26.6[a]	27.2[a]	27.6[a]	23.6[b]
Formol N[2]	9.8[c]	10.2[bc]	10.1[bc]	10.5[b]	10.2[bc]	13.8[a]
Volatile N[2]	1.1[b]	1.0[b]	1.0[b]	1.0[b]	1.2[b]	3.2[a]
Soluble protein[3]	38.1[c]	41.2[ab]	41.7[a]	40.5[b]	40.3[a]	24.4[d]
Free AA[4]	443.0[c]	462.2[bc]	466.1[bc]	492.4[b]	459.7[bc]	628.4[a]
Total AA[4]	794.3[a]	932.2[a]	920.2[a]	1008.8[a]	999.6[a]	774.0[a]

Values in the same row follow by the same letter are not significantly different at the 5% level.
1 trypsin:chymotrypsin
2 Express as mgN/ml
3 Express as mg protein/ml
4 Express as µmol/ml
AA = Amino acid

3.3.6. Distribution of nitrogenous compounds in herring sauces produced with various proportions of 0.6% trypsin and chymotrypsin and first grade commercially produced Nampla

The nitrogen distribution of all laboratory produced herring sauces with 0.6% enzyme supplementation and first grade commercially produced Nampla are shown in Table 18. The distribution of free amino acids, volatile nitrogen and soluble protein and peptides was highest in the sauces produced with the enzyme proportions of 25:75, 100:0 and 50:50 trypsin:chymotrypsin, respectively. Although total nitrogen was greater in the sauce produced with chymotrypsin alone, nitrogen was distributed less in the form of free amino acids compared to all other sauces. A comparison of nitrogenous compounds found in the herring sauces produced with various proportions of 0.6% enzyme supplementation and first grade commercially produced Nampla (Table 18) indicated that the longer fermentation/storage time period for commercially produced Nampla resulted in more soluble protein and peptides being broken down into free amino acids and volatile bases. This is evident by the presence of higher amounts of nitrogen in the form of free amino acids and volatile nitrogen and the lower concentration of protein and peptides in the commercial product than that found in all laboratory produced sauces. Generally, a low concentration of volatile nitrogen in herring sauces is indicative of less breakdown of amino acids to ammonia and other volatile bases. The presence of high volatile nitrogen (more than 25% of total nitrogen) indicates poorer quality fish sauce (Sanchez and Klitsaneephaiboon, 1983). High grade fish sauce contains not less than 40% amino nitrogen and not more than 60% of total nitrogen. However, the only enzyme treated sauces which contained these levels of amino nitrogen was the herring sauce

produced with 0.6% of 25:75 trypsin:chymotrypsin. The lower concentration of other nitrogenous compounds in commercial produced Nampla compared to laboratory produced sauce is probably due to deamination of these compounds to volatile nitrogen as shown by higher concentration of volatile nitrogen in Nampla. This can probably be accounted for by the longer fermentation/maturation period of commercial product.

Table 18. Nitrogen distribution in herring sauces produced with various proportions of 0.6% trypsin and chymotrypsin and first grade commercially produced Nampla

Sauce type	Total N (mgN/ml)	% of total N			
		Volatile N	Amino acid N[1]	Biuret N[2]	Other N[3]
100:0	24.61	4.55	37.59	24.75	33.11
75:25	27.46	3.64	35.36	23.96	37.04
50:50	26.57	3.58	36.66	25.10	34.66
25:75	27.19	3.49	38.07	24.42	34.02
0:100	27.63	4.23	34.85	23.34	37.58
Nampla	23.57	13.49	52.62	16.59	17.30

1 From amino acid analysis data
2 From biuret soluble protein data (Biuret N = Biuret protein/6.25)
3 Determined by total N - volatile N - amino acid N - biuret N and could include nucleic acid and other breakdown products.

3.3.7. Amino acid composition

3.3.7.1. Free amino acid

The free amino acid composition of laboratory produced herring sauces produced with various proportions of 0.6% enzyme supplementation at the end of fermentation (day 42) are shown in Figures 16 and 17. All herring sauces had similar free amino acid compositions. A high level of lysine, isoleucine, valine, arginine and alanine and a low level of proline, cysteine and tyrosine were observed in all sauces. These observations are in agreement with results obtained using 0.3% enzyme concentration. Analysis of variance of the free amino acid composition among herring sauces produced from 0.6% trypsin and chymotrypsin showed that the enzyme proportions of 100:0 and 25:75 (trypsin:chymotrypsin) resulted in a significantly higher levels of glutamic acid while the enzyme proportions of 25:75 and 0:100 gave a significantly higher level of proline and phenylalanine compared to all other enzyme proportions ($p<0.05$). In addition, supplementation with trypsin alone resulted in a significantly lower level of histidine in the end product than all other enzyme proportions ($p<0.05$).

The essential amino acids i.e. threonine, valine, methionine, leucine, isoleucine, proline, lysine and histidine, required for a balanced diet, were present in the free form at fairly high concentrations in all herring sauces produced with 0.6% enzyme supplementation.

Free amino acids not only contribute to the nutritive value of fish sauce but also to its flavor. Free amino acids and nucleotides were suggested by Lee et al., (1981; 1982a) to be the main taste active compounds in fermented sardine and anchovy. The taste active amino acids in fermented squid were glutamic acid, alanine, leucine, serine,

lysine, arginine and proline (Lee et al., 1982b). Chung and Lee (1976) reported that the most important taste compounds in fermented shrimp were the free amino acids, lysine, proline, alanine, glycine, serine, glutamic acid and leucine. Konosu (1979) identified free amino acids as taste-active compounds in squid, notably; proline, glycine, alanine and serine. Lee and Sung (1977) concluded that the major taste-active compounds of brine squid are the amino acids proline, leucine, serine, lysine, arginine and alanine. The free amino acids lysine arginine and alanine were abundantly present in all herring sauces produced with 0.6% enzyme supplementation and therefore may contribute to the taste of fish sauce produced from herring.

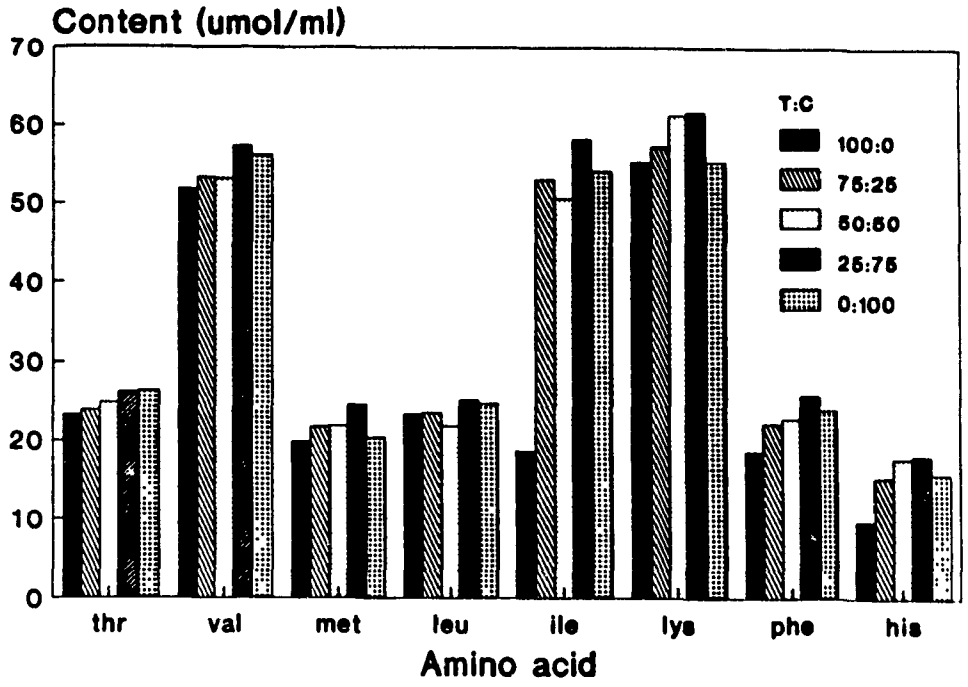

Figure 16. Essential free amino acid profile of final herring sauces produced with various proportions of 0.6% trypsin and chymotrypsin

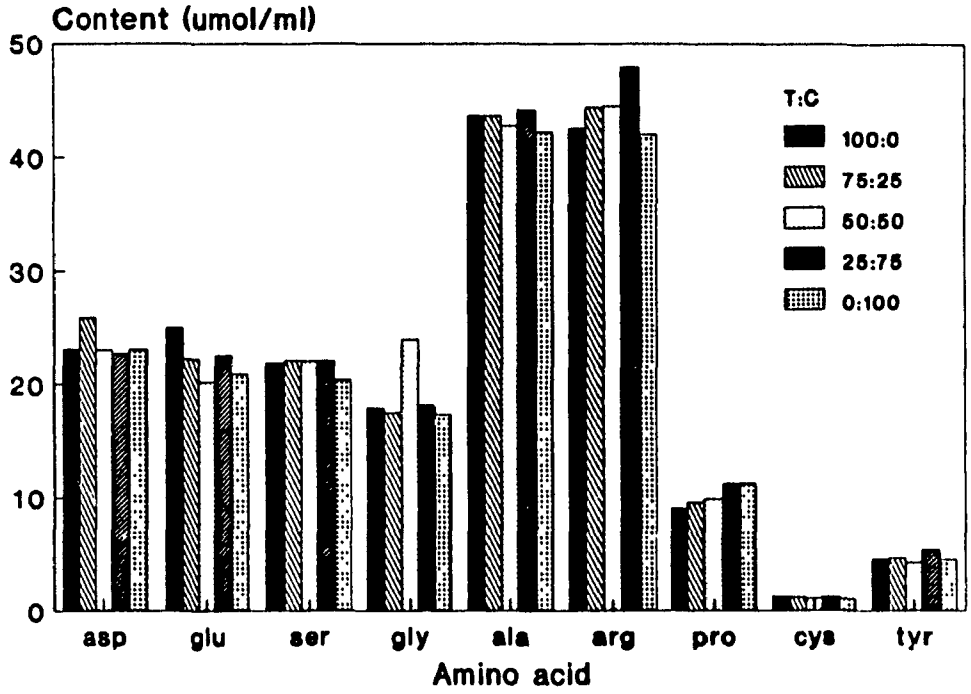

Figure 17. Non-essential free amino acid profile of final herring sauces produced with various proportions of 0.6% trypsin and chymotrypsin

3.3.7.2. Comparison of free amino acid composition of herring sauces produced with 0.3% and 0.6% trypsin and chymotrypsin at the proportions of 100:0, 50:50 and 0:100

A comparison of the free amino acid composition of the final herring sauces produced with 0.3 % and 0.6% enzyme concentration at the proportions of 100:0, 50:50 and 0:100 (trypsin:chymotrypsin) is shown in Table 19. It is evident that supplementation with 0.6% trypsin and chymotrypsin had a significant effect on the amount of the amino acids glutamic acid, glycine, alanine, arginine, valine, methionine, leucine, isoleucine, phenylalanine and histidine in the end products ($p<0.05$). Since trypsin and chymotrypsin are endopeptidases, the increase in enzyme concentration from 0.3% to 0.6% may encourage higher proteolysis and production of more peptides which would serve as substrates for other exopeptidases naturally present in fish.

3.3.7.3. Comparison of free amino acid composition of herring sauces produced with various proportions of 0.6% trypsin and chymotrypsin and the first grade commercially produced Nampla

A comparison of the free amino acid composition of final herring sauces produced with various proportions of 0.6% enzyme concentration and first grade commercially produced Nampla is shown in Table 20. The concentration of free amino acids, with the exception of arginine, valine, methionine, isoleucine, phenylalnine, lysine and tyrosine were significantly lower in laboratory produced sauces compared to first grade commercially produced Nampla ($p<0.05$). The lower level of glutamic acid, alanine, leucine, proline and serine, which are considered as taste active amino acids in fish sauce,

may result in a sauce with less flavor. Amino acids also contribute to the color of fish sauce, either through a sugar-amino reaction or a lipid-amino reaction (Jones, 1962). Hence, the lighter color of laboratory produced sauces compared to the first grade commercially produced Nampla may be attributed to the lower level of free amino acids.

3.3.7.4. Total amino acid composition

The total amino acid composition of herring sauces produced with various proportions of 0.6% trypsin and chymotrypsin and first grade commercially produced Nampla are shown in Figures 18 and 19. The major total amino acids in all laboratory produced herring sauces were glutamic acid, glycine, alanine, valine and isoleucine. Analysis of variance showed that all herring sauces, irrespective of the enzyme proportion used, had similar levels of total amino acids ($p>0.05$). All herring sauces also contained similar levels of total amino acids with the exception of arginine and histidine compared to first grade commercially produced Nampla. Arginine was present in trace amounts in commercially produced first grade sauce while histidine was present in a significantly higher level in commercially produced Nampla compared to herring sauces produced with various proportions of 0.6% enzyme supplementation ($p<0.05$).

Table 19. Free amino acid composition of herring sauces produced with 0.3% and 0.6% trypsin and chymotrypsin

Amino acid (μmol/ml)	Enzyme concentration (% w/w)	
	0.3	0.6
asp	23.38[a]	23.07[a]
glu	17.95[b]	22.04[a]
ser	18.89[a]	21.41[a]
thr	20.72[a]	24.76[a]
gly	11.06[b]	19.74[a]
ala	36.45[b]	42.88[a]
arg	37.17[b]	43.01[a]
pro	4.49[a]	10.02[a]
val	34.20[b]	53.76[a]
met	9.59[b]	20.75[a]
leu	12.55[b]	23.31[a]
ile	35.57[b]	52.22[a]
phe	17.58[b]	21.88[a]
cys	3.04[a]	1.14[b]
lys	65.42[a]	57.35[a]
his	5.89[b]	14.49[a]
tyr	5.88[a]	4.47[a]

Values in the same row follow by the same letter are not significantly different at the 5% level

Values are averages of 3 enzyme proportions: 100:0, 50:50 and 0:100.

Table 20. **Free amino acid composition of laboratory herring sauces produced with various proportions of 0.6% trypsin and chymotrypsin and commercially produced Nampla**

Amino acid	Sauce type					
	100:0[1]	75:25[1]	50:50[1]	25:75[1]	0:100[1]	Nampla
asp	23.09^{b}	25.86^{b}	23.05^{b}	22.71^{b}	23.09^{b}	46.79^{a}
glu	25.02^{b}	22.21^{c}	20.22^{c}	22.52^{c}	20.88^{c}	54.74^{a}
ser	21.84^{b}	22.08^{b}	22.03^{b}	22.03^{b}	20.36^{b}	33.20^{a}
thr	23.21^{b}	23.80^{b}	24.77^{b}	26.11^{b}	26.32^{b}	49.77^{a}
gly	17.92^{b}	17.52^{b}	23.98^{b}	18.12^{b}	17.32^{b}	49.90^{a}
ala	43.66^{b}	43.67^{b}	42.77^{b}	44.17^{b}	42.21^{b}	75.42^{a}
arg	42.50^{a}	44.41^{a}	44.55^{a}	47.97^{a}	41.98^{a}	trace^{b}
pro	8.99^{c}	9.55^{bc}	9.86^{bc}	11.17^{b}	11.21^{b}	24.73^{a}
val	51.90^{a}	53.31^{a}	53.14^{a}	57.37^{a}	56.26^{a}	58.53^{a}
met	19.94^{a}	21.78^{a}	21.89^{a}	24.50^{a}	20.41^{a}	14.76^{a}
leu	23.33^{b}	23.44^{b}	21.89^{b}	25.12^{b}	24.70^{b}	36.09^{a}
ile	51.95^{a}	53.10^{a}	50.62^{a}	58.28^{a}	54.09^{a}	31.41^{a}
phe	18.71^{c}	22.28^{abc}	22.89^{ab}	25.88^{a}	24.06^{ab}	20.40^{bc}
cys	1.32^{b}	1.16^{b}	1.08^{b}	1.24^{b}	1.04^{b}	2.22^{a}
lys	55.33^{a}	57.38^{a}	61.35^{a}	61.76^{a}	55.36^{a}	58.40^{a}
his	9.80^{b}	15.45^{b}	17.77^{b}	18.11^{b}	15.90^{b}	103.76^{a}
tyr	4.55^{ab}	4.67^{ab}	4.28^{b}	5.33^{a}	4.58^{ab}	1.43^{c}

Values (μmol/ml) in the same row follow by the same letter are not significantly different at the 5% level.

1 trypsin:chymotrypsin

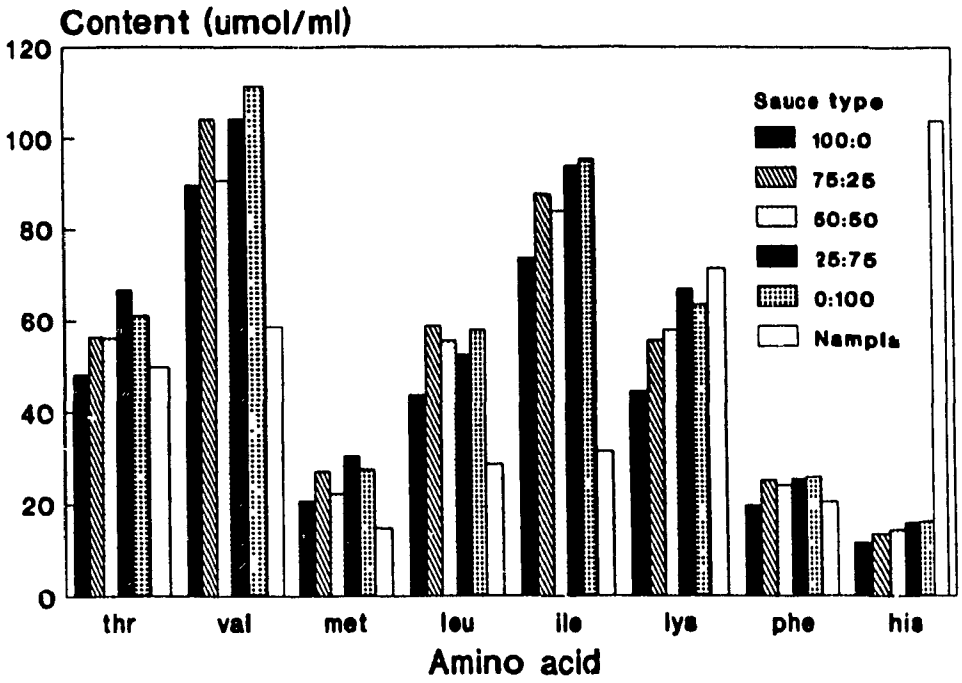

Figure 18. Essential total amino acid profile of final herring sauces produced with various proportions of 0.6% trypsin and chymotrypsin and first grade Nampla

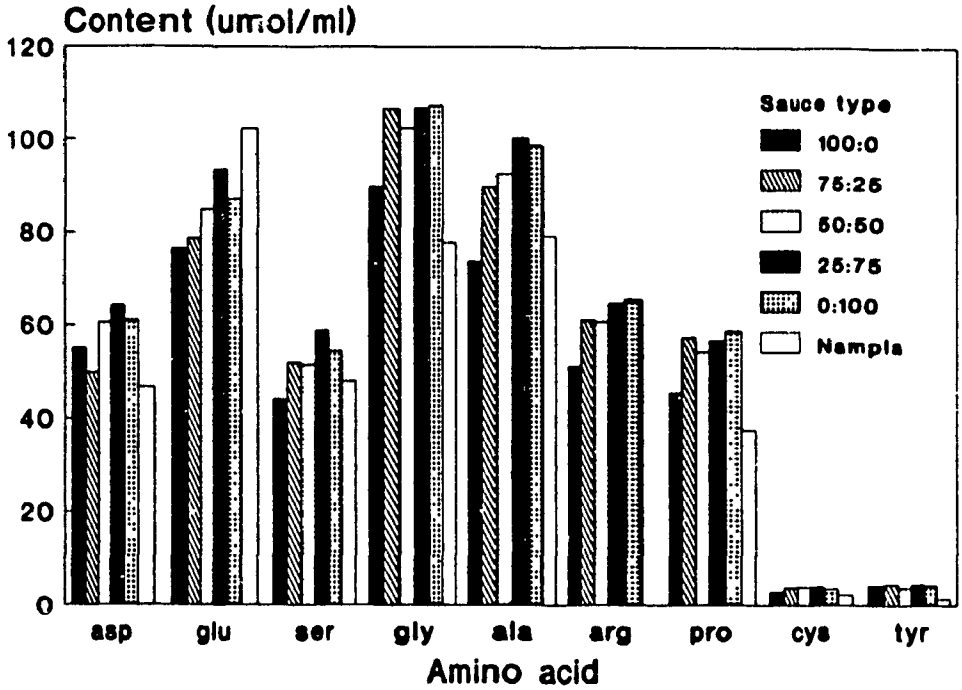

Figure 19. Non-essential total amino acid profile of final herring sauces produced with various proportions of 0.6% trypsin and chymotrypsin and first grade Nampla

3.3.8. Microbiological analysis

The total aerobic and anaerobic plate counts of herring sauces produced with various proportions of 0.6% trypsin and chymotrypsin are shown in Tables 21 and 22. At the onset of fermentation, anaerobic plate counts in all sauces were slightly higher compared to aerobic plate counts probably due to the closed system of the fermentation container which would favor the growth of anaerobic bacteria. However, both aerobic and anaerobic plate counts were less than 10^3 CFU/ml after 7 days fermentation. Total viable counts have been shown to decrease during fish sauce fermentation (Hamm and Clague, 1950; Orejana and Liston, 1979). At the end of fermentation of laboratory produced herring sauce (day 42), both aerobic and anaerobic plate counts were lower than 10 CFU/ml i.e. similar to counts observed for commercially produced Nampla. Since microbial counts were very low at the initial stages of fermentation when proteolysis was most rapid, it is unlikely that microorganisms contribute to proteolysis during fish sauce fermentation.

Table 21. Total aerobic plate counts during fermentation of herring sauces produced with various proportions of 0.6% trypsin and chymotrypsin (CFU/ml)

Time (day)	Sauce type (trypsin:chymotrypsin)				
	100:0	75:25	50:50	25:75	0:100
7	125	100	100	100	150
14	100	<100	<100	<100	<100
28	50	<10	<10	<10	<10
35	<10	<10	<10	<10	<10
42	<10	<10	<10	<10	<10

Table 22. Total anaerobic plate counts during fermentation of herring sauces produced with various proportions of 0.6% trypsin and chymotrypsin (CFU/ml)

Time (day)	Sauce type (trypsin:chymotrypsin)				
	100:0	75:25	50:50	25:75	0:100
7	400	125	750	250	750
14	250	100	200	100	300
28	175	<100	<100	<100	100
35	<10	<10	<10	<10	<10
42	<10	<10	<10	<10	<10

3.3.9. Sensory evaluation

The results of the sensory evaluation of final herring sauces produced with various proportions of 0.6% trypsin and chymotrypsin and first grade commercially produced Nampla are summarized in Tables 23 and 24. Statistical analysis of the sensory data shows that there was a significant difference in color score ($p<0.01$). Panellists preferred the clear, light brown color of laboratory produced fish sauces to the darker brown color of first grade commercially produced Nampla. While there was no significant difference in the means for aroma and flavor between fish sauces ($p>0.05$), there was, however, a highly significant difference between panellists ($p<0.01$). Three of the panellists preferred the stronger aroma and flavor of first grade commercially produced Nampla to the milder aroma and flavor of laboratory produced sauces. However, three panellists preferred the milder aroma and flavor of laboratory produced sauces compared to the stronger aroma and flavor of first grade commercially produced Nampla. The remaining three panellists had no preference for either sauce. The darker color and stronger aroma and flavor of first grade commercially produced Nampla is probably due to the higher level of free amino acids, volatile bases and volatile and non volatile fatty acids produced during the longer fermentation/aging period. No significant difference was observed in the color, aroma and flavor of laboratory produced sauces using various proportions of 0.6% trypsin and chymotrypsins ($p>0.05$).

Table 23. Analysis of variance on preference of color, aroma and flavor of herring sauces produced with various proportions of 0.6% trypsin and chymotrypsin and first grade commercially produced Nampla

Source	df	Color MS	Aroma MS	Flavor MS
Panelist	8	13.15**	14.56**	21.23**
Sauce type	5	38.75**	0.67ns	0.28ns
Error	40	4.14	1.26	6.18
Residual	54	0.27	1.45	6.18

MS = Mean Square
** significantly different at the 1% level
ns not significantly different at the 5% level

Table 24. Means of preference scores of color, aroma and flavor of herring sauces produced with various proportions of 0.6% trypsin and chymotrypsin and first grade commercially produced Nampla

Treatment	Color	Aroma	Flavor
100:0 (T:C)	7.40[a]	5.47[a]	6.79[a]
75:25 (T:C)	7.40[a]	5.26[a]	6.95[a]
50:50 (T:C)	7.29[a]	5.39[a]	6.62[a]
25:75 (T:C)	7.27[a]	5.43[a]	6.44[a]
0:100 (T:C)	7.24[a]	5.32[a]	6.90[a]
Nampla	3.73[b]	5.13[a]	6.88[a]

Means scores range from 0-dislike extremely to 10-like extremely.
Values in the same column follow by the same letter are not significantly different at the 5% level.
Values are averages of 18 observations.
T:C = trypsin:chymotrypsin

CHAPTER 4

SUMMARY AND CONCLUSION

The quality of first grade commercially produced Nampla was superior than second grade Nampla in terms of its higher total nitrogen, formol nitrogen, free and total amino acid content. Overall, the quality of first grade sauce met all the regulatory specifications of Thailand while second grade sauce contained only less amino nitrogen than the required regulatory specifications.

In the preparation of fish sauces using herring, enzyme supplementation with trypsin and chymotrypsin has the potential to accelerate the rate of fish sauce fermentation. The degree of protein hydrolysis increased significantly ($p<0.01$) compared to control sauces (no added enzyme) and the enzyme supplemented sauces contained higher amounts of formol nitrogen, total nitrogen, soluble protein and free amino acid compared to control sauces ($p<0.05$). An increase in enzyme concentration from 0.3% to 0.6% further increased the level of total nitrogen and free amino acids in the end products ($p<0.05$). Changes in the nitrogenous composition of sauces produced with various proportions of 0.3% and 0.6% enzyme supplementation occurred mainly during the initial stages of fermentation (14 days) as shown by the rapid increase in formol nitrogen, total nitrogen and free amino acids. Microbial counts were very low at the onset of fermentation, confirming that microorganisms did not appear to play a significant role in proteolysis of fish sauce fermentation.

The addition of trypsin alone produced less favorable results with increasing proportions of chymotrypsin to trypsin giving the most favorable results. With respect

to the contents of total nitrogen, formol nitrogen and free amino acids, the optimum results were obtained using 0.6% of 25:75 trypsin:chymotrypsin. In terms of cost, this proportion is also more viable since chymotrypsin is less expensive than trypsin.

A comparison of the chemical composition of herring fish sauces produced with various proportions of 0.6% trypsin and chymotrypsin and first grade commercially produced Nampla showed that laboratory produced fish sauce was comparable in terms of its total nitrogen and total amino acid content. However, it contained significantly less amounts of formol nitrogen, free amino acid and volatile bases ($p<0.05$). Analysis of the free amino acid profile indicated that herring sauces produced with various proportions of 0.6% enzyme supplementation contained significantly less of the free amino acids aspartic acid, glutamic acid, serine, threonine, glycine, alanine, proline, leucine, cysteine and histidine compared to the first grade commercially produced Nampla. This may contribute to the lighter color and less flavor and aroma of laboratory produced fish sauces in the sensory evaluation with first grade commercially produced sauce. However, the results of the sensory evaluation showed that panellists preferred the lighter color of herring sauces produced with various proportions of 0.6% trypsin and chymotrypsin to the darker color of first grade Nampla. Nampla had a stronger aroma and flavor than the laboratory produced herring sauces. However, there was no significant difference in the preference score of aroma and flavor ($p>0.05$). Furthermore, no significant difference was observed in the preference of color, aroma and flavor among herring sauces produced from various proportions of 0.6% enzyme suppiementation.

In conclusion, supplementation with trypsin and chymotrypsin has the potential to accelerate fish sauce fermentation. In this study, a consumer acceptable fish sauce was

produced within 2 months compared to 6-12 months used commercially. However, fish sauces produced by accelerating the rate of proteolysis did not give the typical aroma and flavor of the commercial product. This may be due to a shorter fermentation/aging time i.e. 2 months compared to 6-12 months or the type of fish used in this study i.e. herring. Further studies are needed to determine the optimum concentration and proportion of enzymes to accelerate fish sauce fermentation which have the desired aroma and flavor characteristics of the commercial product. A combination of endopeptidases (trypsin and chymotrypsin) and exopeptidases, such as cathepsin, warrants investigation. Such a combination may result in more protein breakdown and produce more flavor and aroma producing amino acids and nitrogenous compounds. Further studies are also required to determine the commercial viability of accelerating fish sauce fermentation with commercial enzymes due to the relatively high cost of both trypsin and chymotrypsin.

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