ROLE OF PROTEIN DEGRADATION IN FERMENTATION OF FISH SAUCE

NONGNUCH RAKSAKULTHAI

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by

©NONGNUCH RAKSAKULTHAI, B.Sc., M.Sc.

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Department of Biochemistry
Memorial University of Newfoundland

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ABSTRACT

Male inshore capelin were used to prepare fish sauce, a fermented liquid product used as a condiment in South East Asia. Fermentation of mince capelin with salt (4:1 w/w) alone did not give satisfactory results in terms of extractable soluble nitrogen, free amino acid formation or sensory evaluation score. Supplementation of the salted mince with 2.5% (w/w) squid hepatopancreas (SHP) significantly increased the degree of protein hydrolysis ($P<0.01$), the free amino acid content of the finished product (2.1-fold) and the sensory evaluation score ($P<0.05$). The free amino acid content in control and SHP-supplemented sauce were 242 and 520 mM, respectively. The failure of the heat-treated SHP (100°C, 30 min) to aid the fermentation process indicated that SHP aids the fermentation of fish sauce by virtue of its enzymes. Acidification of the salted mince containing 2.5% SHP with HCl to pH 4.5 gave a product having a lower sensory score, although the degree of protein hydrolysis significantly increased ($P<0.05$) and the free amino acid content increased to 1.5-fold the control (370 mM).

The results of fermentation of salted mince at initial pH values ranging from 3-8, at ambient temperature and at 37°C and at concentration of NaCl ranging from 15-30% indicated that the conditions recommended for fermentation of

capelin fish sauce were at 25% salt (w/w), at ambient temperature (20-25°C) and at natural pH of fermentation (pH 5-7).

Enzymes associated with the viscera were found to contribute to the hydrolysis of protein during fish sauce fermentation but the sensory evaluation score of the finished product prepared from round and gutted capelin did not show any significant difference. Enzymes from the viable bacteria in salted mince were of little importance in the fermentation process as indicated by insignificant difference between protein hydrolysis of the antibiotic-treated sample and the control. When minced capelin was held at ambient temperature for 24 h prior to addition of salt, the number of total viable bacteria increased significantly as did the amount of protein hydrolysis in salted mince ($P<0.05$). However, delayed salting for 24 h did not significantly improve the sensory quality of the fish sauce.

The importance of aging or ripening on quality of fish sauce was investigated by comparison of fish sauce kept at -20°C and at ambient temperature for 4-6 months after fermentation. It was found that ripening process had no significant effect on free amino acid content, color or sensory evaluation score of fish sauce although a trend toward darker color of the aged samples was resulted.

The contribution of free amino acids and peptides to flavor of fish sauce was examined. Fish sauce was filtered through an ultrafiltration unit, M.W. cut off 10,000. The results indicated that removal of larger molecules from fish sauce lowered the acceptability ($P<0.001$). Gel filtration chromatography indicated

that the apparent molecular size of peptides and amino acids in fish sauce ranged between 100-300 daltons. Regression analysis of preference score and free amino acid content indicated a significant correlation of 0.724 ($P<0.05$).

To characterize, partially, the enzymes retained in fish sauce after 6 months fermentation, fish sauce was concentrated 3.5-fold using an ultrafiltration unit, M.W. cut off 10,000. Protease activity of the enzymes retained in fish sauce prepared with SHP was greater than that of control (3-fold on azocasein and 5-fold on hide powder azure substrate). The pH optimum of the residual enzymes in low concentration (0.2 M) of NaCl was at pH 4. At salt concentration of 1.5 M, the optimal pH shifted to pH 5.0, and at 4.0 M NaCl the maximal activity was at pH 6. Salt partially inhibited the proteolytic activity, however, the residual enzymes in SHP-supplemented sauce appeared to be more tolerant to salt than those in the control sauce. Protease activity at low salt concentration on azocasein was partially inhibited by ethylenediaminetetraacetate, iodoacetate, p-chloromercuribenzoic acid, mercuric chloride and soybean trypsin inhibitor. In the presence of 4 M NaCl, inhibitors for sulfhydryl proteases appeared to be the only effective inhibitors against hydrolysis of azocasein. Hydrolase activity of cathepsin C was less inhibited by salt than was the azocasein hydrolysis. It was apparent that cathepsin C and other sulfhydryl proteases were of importance to the fish sauce fermentation.

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List of Abbreviations

ANOVA	Analysis of variance
BAPA	N-α-benzoylarginine-p-nitroanilide
CFU	Colony forming units
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetate
H-Cys	Cysteic acid
H-Lys	Hydroxylysine
H-Pro	Hydroxyproline
M.W.	Molecular weight
MT	Metric tonnes
PCMB	p-Chloromercuribenzoic acid
PMSF	Phenyl methyl sulfonyl fluoride
r.t.	Retention time
SBTI	Soybean trypsin inhibitor
SHP	Squid hepatopancreas
TAME	Tosyl arginine methyl ester
TCA	Trichloroacetic acid
TSA	Trypticase soy agar

Introduction

Capelin (*Mallotus villosus* Muller) is the most abundant fish in the North Atlantic. It is an important forage fish for marine mammals, marine birds and some fish e.g., cod, haddock, flounder, Atlantic salmon and herring (Carscadden, 1981). In Newfoundland, it has been estimated that the resource available can be measurable in millions of metric tonnes of which about 50,000 metric tonnes are caught each year to be used for bait, human food, dog food and fertilizer (Carscadden, 1981). During the spawning season, capelin schools migrate to the coast of Newfoundland and Labrador. Traditionally, capelin is harvested in Newfoundland when the schools are near or on the spawning beaches. Only the female capelin has high commercial value; the roe or fish containing roe are frozen and exported to Japan. The male capelin is not normally used for food export; however, some male capelin is used to manufacture fish meal and is used for domestic food.

The spawning capelin, caught during June - July in Newfoundland, has lower lipid content than winter capelin (MacCallum *et al.*, 1969). Summer capelin is more susceptible to autolysis due to the high activity of digestive enzymes when the fish is feeding heavily (Gildberg and Raa, 1980). Summer capelin would appear to be a desirable raw material for the preparation of fish sauce because of this tendency to undergo autolysis.

Fish sauce is a well-known fermented product used as a condiment in South East Asia, especially in the Philippines, Thailand and Vietnam. The North American market for fish sauce has grown considerably with the increasing population of individuals of South-East Asian descent (Raksakulthai *et al.*, 1986). Fish sauce is normally made by fermenting small fish such as sardine, anchovies or round scad in 15 - 30 % of salt. The use of high salt serves to prevent microbial spoilage of fish in the hot and damp climate of South East Asia. The time required to produce fish sauce varies from 6 months to 1 year or longer. At the end of fermentation, the liquid is drawn off, filtered and ripened in the sun for 1-4 months. The product is a clear liquid, rich in salt and soluble nitrogen compounds with a characteristic flavor and odor. The color of fish sauce varies from yellow-straw to amber or reddish brown.

A study undertaken to determine the suitability of male inshore capelin as raw material for fish sauce showed that mincing the fish accelerated the process (Raksakulthai *et al.*, 1986). Enzyme supplements, including fungal protease, pronase, trypsin, chymotrypsin and squid protease, resulted in an acceleration of protein hydrolysis during the early stages of fermentation; but somewhat lower yields of free amino acids in the final product. Supplementation of salted mince with squid hepatopancreas yielded a product with a very high free amino acid content and sensory acceptability better than a commercial product from the Philippines.

The present study was undertaken to determine the chemical change of

protein during fermentation of fish sauce; the contribution of fish, squid hepatopancreas and microbial enzymes in protein hydrolysis; the optimal conditions for fermentation of capelin fish sauce; and the contribution of amino acids and peptides to the characteristic flavor of fish sauce.

Chapter 1

Literature review

1.1. Capelin

Capelin (*Mallotus villosus Muller*), a relatively small fish, is classified in Class Osteichthyes, suborder Salmonoidae, family Osmeridae. The length of mature specimens is approximately 13-20 cm. At sexual maturity, the male is 1-2.5 cm larger than the female. The spawning male appears distinctly different from the female. The male capelin's fins are larger and project out from the body. Two pairs of spawning ridges develop in 4-5 weeks before the start of the spawning season. A prominent dorsal pair of ridges runs the length of the body above the lateral line and a smaller ventral pair extends from the pectoral fin back to the pelvic fin. These ridges disappear within a month after the spawning has ended. After the spawning season the appearances of the sexes are similar (Winters, 1969).

1.2. Distribution

Capelin has a circumpolar distribution in the northern region of the Atlantic and the Pacific (Jangaard, 1974a). In the eastern Atlantic, the species inhabits the region from western Norway to eastern Russia and is widely distributed throughout the Barents Sea and around Iceland and Greenland. On the east coast

of North America, the stocks are found from Hudson Bay to Nova Scotia but are most abundant around Newfoundland and Labrador. Small stocks exist on both sides of the North Pacific (Carscadden, 1981).

The largest stocks of capelin in Canada are found around Newfoundland and Labrador coast (Jangaard, 1974a). Capelin spawn on the beaches or inshore in most areas of Newfoundland and Labrador, starting early in June on the south coast and later along the north coast. In Labrador, the spawning often begins after mid-July. In northern Norway and Iceland, spawning occurs in March-April. It takes place on the beaches in some areas, but also in deep water. Most capelin die after spawning (Jangaard, 1974a).

Most capelin harvested in Canada are landed on the east and southeast coast of Newfoundland. Capelin have traditionally been caught by local fishermen employing traps, seines and dip net when they are near or on the spawning beaches. In Newfoundland, capelin have been used for bait, human food, dog food and fertilizer. During 1950-1960, harvesting declined probably due to the decrease in number of gardens and the use of dog teams. In 1971, 2,272 MT of capelin were harvested as compared to 13,636 MT in 1952 (Condon and Allan, 1979). However, since the mid-1970's, the catches have increased as a result of the demand for frozen roe or frozen fish containing roe by Japanese buyers (Anon., 1978).

The total landings and the utilization of capelin in Newfoundland from 1975-1984 are given in Tables 1-1 and 1-2.

Table 1-1: Capelin landings and landed values in Newfoundland, 1975-1984

Year	Quantity (MT)	Value ($)
1975	4,589	130,170
1976	9,613	286,433
1977	13,473	587,559
1978	18,252	1,121,659
1979	18,794	1,959,884
1980	20,638	3,216,706
1981	28,588	4,750,022
1982	31,601	6,936,452
1983	29,762	5,605,703
1984[1]	42,904	9,596,759

[1]1984 figures are preliminary and subjected to revision

Source : Fisheries Statistics and System Branch, St. John's, Nfld.

1.3. Chemical composition of capelin

Chemical components of capelin, especially fat and water, fluctuate seasonally (MacCallum *et al.*, 1969). For whole capelin, the fat content reaches a minimum just after spawning in spring or in early summer and increases until it reaches a maximum at the end of the feeding period in the Fall. The overwintering mature capelin from Trinity Bay, Newfoundland, has as high as 14% fat in February and March. The fat content declines gradually toward the spawning season in June, at which time the fat content can be as low as 1-2%. Immature fish have much lower fat content than adults. Apparently there is no seasonal variation in chemical composition among immature capelin (Winters, 1970).

Table 1-2: Capelin production in Newfoundland, 1975-1983

Year	MT Frozen Round	Frozen Bait	Fresh Round	Fresh Bait	Dried/ Smoked
1975	381	409	56	271	112
1976	472	148	129	224	93
1977	606	151	185	138	83
1978	1,633	74	5	168	12
1979	3,527	25	-	48	43
1980	7,708	39	35	112	52
1981	11,315	105	142	322	10
1982	15,166	139	47	336	-
1983	11,854	43	46	360	2

Source : Fisheries Statistics and System Branch, St. John's, Nfld.

According to Andrews (1954) and MacCallum *et al.* (1969), the fat content of capelin, caught on or near the shore in Newfoundland during June - July before or during spawning, ranged from 2-6%, with the large number around 3%. Fish caught in July on the Southeast Shoal of the Grand Bank had fat content as low as 1% (Hinds, 1972).

Protein content in beach spawning capelin was found to be lower than other fish of the same family e.g., Salmon, but their moisture content was slightly higher. Proximate analysis data collected over a period of 20 years showed a sharp decline in fat content (8.1-1.8%), a slight increase in moisture (77.1-82.3%) and a

small decrease in protein (15-12.9%) of the whole fish during the spawning period. For muscle tissue, similar results were obtained (MacCallum *et al.*, 1969).

Proximate composition of Newfoundland capelin is shown in Table 1-3.

Table 1-3: Proximate composition of whole spawning Newfoundland capelin (% wet weight) - Adapted from MacCallum *et al.*, 1969

	Moisture Range	Lipid Range	Crude Protein Range	Ash Range
Male	77.13-82.26	1.83-8.08	12.88-14.38	2.09-2.20
Female	77.96-84.10	2.00-6.42	12.94-15.30	1.84

A recent study on the composition of mature inshore spawning Newfoundland capelin (Montevecchi and Piatt, 1984) gave similar results. They reported the highest content of lipid was in late Fall and lowest during the summer spawning season. The protein levels were constant at 13-14% of body weight throughout the year. Avid females had higher lipid and protein content and lower water content than males and spent females. There was no significant difference between the chemical composition of spent females and males. Amino acid composition was found to be the same in both sexes (Montevecchi and Piatt, 1984).

1.4. Utilisation of capelin

With respect to quantity, capelin is one of the top 10 species landed in the world (Anon., 1981). In the past decade the production of meal and oil from capelin has increased, especially in Norway, Iceland and USSR, due to the decrease in herring stocks. More than 99 percent of the world catch was used for the production of meal and oil (Jangaard, 1974b). Capelin oil has been widely used in the production of margarine, shortenings or soups after hydrogenation and refining. It has a relatively low iodine value which varies with fishing area and season and could be used interchangeably with herring oil (Eaton *et al.*, 1975). A new form of utilization commenced in 1965 onward when Japan started importing capelin with roe from Iceland, Russia and Norway (Anon., 1978).

Smoked, dried and salted-dried capelin products are exported to Japan as well as the frozen roe or frozen fish containing roe. Canned capelin has been produced but there has been a limited market. Lantz (1966) reported that a lightly smoked product in vegetable oil was the most successful can pack tested and the Research Laboratory of the Norwegian Canning Industry indicated that smoked products of good quality can be made from capelin. Capelin with a high fat content, packed in salt and spice yielded a good quality anchovy - like product (Jangaard, 1974b).

1.5. Fish fermentation

Fermentation is normally defined as the transformation of organic substances into simpler compounds by the action of enzymes or microorganisms (Mackie *et al.*, 1971).

Fermentation of high protein foods such as milk, soy bean, fish and meat involves the hydrolysis or breakdown of protein into free amino acids and peptides. In most cases, the process of fermentation is carried out by microorganisms, and the extent of proteolysis is a function of the type of microorganism employed and the aging time. The characteristic taste and odor of fermented products usually depends upon the degree of proteolysis together with lipolysis and/or carbohydrate fermentation. Salt is normally used to suppress the growth of undesirable microorganisms, hence spoilage by undesirable microorganisms is prevented.

The production of fermented fish products goes back to the Graeco-Roman time (Badham, 1854; Radoliffe, 1921, as cited in Mackie *et al.*, 1971). A sauce made from the viscera and blood of mackerel, called garum, was popular with consumers at that time. Fermentation is practiced as a means of preserving and/or altering the flavor of fish products more so in the Orient than in Europe or North and South America (Liston, 1980). In South-East Asia, fermentation is still the most common method of fish preservation, although modern technologies such as chilling or freezing have been introduced. A number of factors have favored the continued use of fermentation to preserve or produce acceptable products from fish. These factors are :

- inexpensive cost of production
- no requirement for special processing apparatus
- no requirement for refrigeration
- relatively long shelflife
- highly acceptable products
- less problem with pathogenic microorganisms

There are three categories of fish fermentation according to the fermenting agents (Amano, 1962) :

1. Traditional products, fermented mainly by the action of endogenous enzymes in the presence of salt. The products of this category are, for example, fish sauce and fish paste.

2. Traditional products, fermented by combined effects of both endogenous enzymes and microbial enzymes supplemented as a starter in addition to salt. These products are mam-chau (Cambodia), pla-ra (Thailand) and funasushi (Japan) etc..

3. Non-traditional products, produced by an accelerated process either by added enzymes or by chemical hydrolysis e.g., fish silage and fish solubles.

Subba Rao (1967) classified fermented fish products on the basis of the consistency of the product into 3 types, namely :

1. Products in which the fish retain much of their original form or in which large chunks are preserved and may be dried or partially dried e.g., plara (Thailand), buro (Philippines).

2. Products in which the fish are reduced to a paste e.g., bagoong (Philippines), kapi (Thailand), belachan (Malaysia).

3. Products in which the fish are reduced into liquid e.g., fish sauce.

1.5.1. Fish sauce

Fish sauce is a fermented product well-known in South-East Asia under *different names* (Table 1-4). It is a hydrolyzed product of fish protein basically consisting of water, salt and solubic nitrogen compounds. Fish sauce normally has been used as a condiment in those areas where rice is the staple food. To the Vietnamese, Philippino or Thai, fish sauce is like soya sauce to the Chinese and the Japanese. Due to the very high salt content, the consumption of fish sauce is limited; however, because of the low level of nitrogen intake in these areas, fish sauce alone can provide 7.5% of the daily nitrogen intake (Amano, 1962). Fish sauce is also an important source of calcium for a population where diet may be low in this mineral (Beuchat, 1983).

The raw materials for manufacturing fish sauce in South-East Asia are usually of the genera *Stolephorus*, *Engraulis*, *Clupeoides*, *Decapterus* and *Dorosoma*. The best fish sauce is believed to be anchovy sauce (Anon., 1982b).

Table 1-4: Names of fish sauce in different countries

Country	Name
Burma	Ngam-pya-ye
Japan	Shotturu
Malaysia	Budu
Philippines	Patis
Thailand	Nam-pla
Vietnam	Nuoc-mam

The process of making fish sauce is simple (Fig 1-1); fish are mixed with salt in a large vat or cement tank and kept submerged under the brine which is formed. The ratio of fish to salt varies from 5:1 to 2:1. The fermenting time for small fish is shorter than for larger fish and varies from 6 to 36 months. It is believed that the longer the fermenting time, the better the quality of the sauce. However, the nutritive value of fish sauce declines during prolonged fermentation due to the breakdown of amino acids and loss of N as ammonia (Uyenco *et al.*, 1953; Orejana, 1978). At the end of fermentation, the liquid is drained off and left exposed to the sun for a period of 1-4 months before packing. The color of the end product varies from yellow straw to amber or dark reddish brown. The product has strong characteristic flavor and odor.

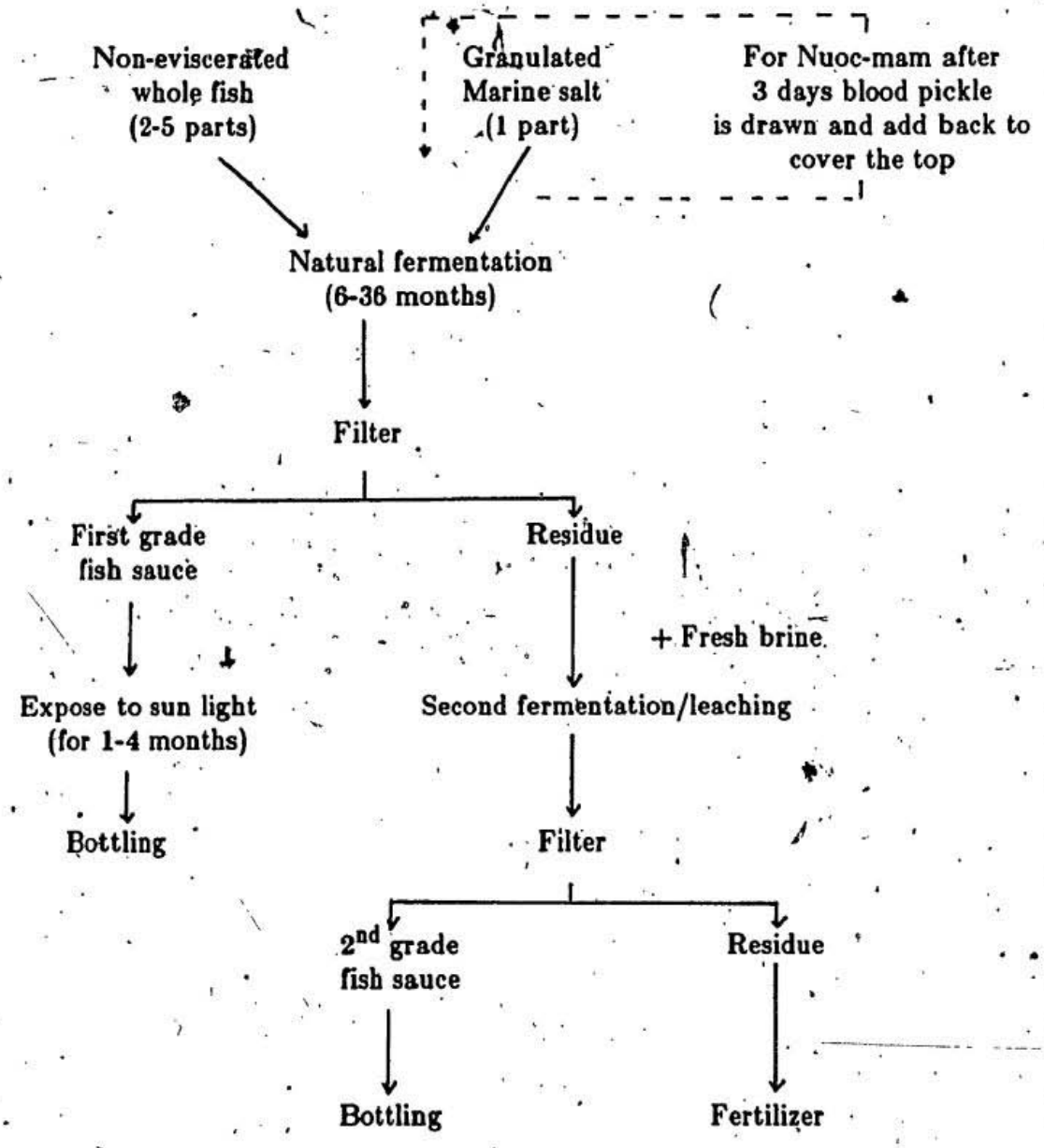


Figure 1-1: Flow diagram of the production of fish sauce

The process for nuoc-mam of Vietnam is different. The blood pickle is allowed to flow out slowly over a 3-day period and then it is poured back over the fish until about 10 cm layer of brine has covered the top of the vat (Steinkraus, 1983).

After the first quality fish sauce is obtained, fresh brine is added to the residue and the mixture is left for 1-4 months. The liquid drawn off is second grade fish sauce. The residual mass is normally used as fertilizer.

In Greece, fish sauce made from the liver of a scromboid fish called "garos" is still produced. In the south of France, fish sauce called "pissala" is made from larvae or small fish. The fish is fermented with salt for 10-12 days for small fish or 1-2 months for larger fish, the liquid is filtered off and is used without further aging (Mackie *et al.*, 1971).

A sauce called "shotturu" is produced in Japan from sand fish, sardine, anchovy and molluscs especially squid (Subba Rao, 1967). After fermentation the liquid is filtered, boiled and may be stored for years.

1.6. Proteolytic enzymes in fish fermentation

Apparently the use of high salt concentration (more than 20% w/w in general) to prevent the activity of spoilage organisms in the fermentation of fish sauce suppresses the activity of proteolytic enzymes, therefore the process requires a fairly long time. The proteolytic enzymes responsible for the proteolysis and ripening of fish sauce can be of endogenous, exogenous or microbial origin.

1.6.1. Endogenous enzymes

Endogenous enzymes in fish fermentation can originate from the digestive tract, internal organs (kidney, liver, etc.) or muscle tissue.

1.6.1.1. Digestive enzymes

Although the digestive organs and digestive enzymes of most fish are, in principle, similar to those of warm blooded vertebrates (Tarr, 1972), it is reported that the properties and activity of proteolytic enzymes vary with species of fish (Amano, 1962) and the harvest season (Kashiwada, 1952). Digestive proteases include pepsins, secreted by the gastric mucosal glands, and trypsins and chymotrypsins secreted by the pancreatic tissue or pyloric caeca. A number of exopeptidases are also secreted in the gut (Gildberg, 1982). It is well established that pyloric caeca are rich in digestive enzymes or their zymogens (Kalac, 1978a,b).

Pepsin

Pepsin is an acidic protease, originating from the mucosa of stomach lining in the form of pepsinogen. The high acidity of the stomach aids in the autocatalytic conversion of pepsinogen into pepsin. The conversion involves the splitting of several peptide fragments from the N-terminal end of pepsinogen (de Man, 1976). When feed or inert material enters the stomach, pepsin and hydrochloric acid secretion is stimulated (Norris *et al.*, 1973). Recent studies indicate that gastric proteases of fish include gastricsins as well as pepsin (Squires *et al.*, 1986a).

Norris and Mathies (1953) reported that fish pepsins were more susceptible to irreversible denaturation by neutral and alkaline conditions than those of mammalian pepsins. Haard *et al.* (1982) isolated pepsin from stomach lining of Arctic cod, Greenland cod and American smelt and found that fish pepsins had a more alkaline pH optimum than pepsins from mammals and pH optima were dependent on assay temperatures. Pepsins from mammals may exhibit two pH optima when hemoglobin is used as a substrate and it has been suggested that pepsin may have two active sites with optimum activity at two different pHs (Taylor, 1962). Recently, a double pH optimum was observed for a purified isoenzyme of porcine pepsin (Shamsuzzaman and Haard, 1986). Pepsins from salmon and tuna were found to have two pH optima (Norris and Mathies, 1953). However, when the ionic strength of the reaction was increased, the tuna pepsin showed only one optimum. Pepsin and gastricsin have broad specificity for peptide bonds but the incomplete solubilization of protein by pepsin hydrolysis has been observed (Backhoff, 1976; Raa and Gildberg, 1976).

Two proteases were purified from capelin stomach (Gildberg, 1982), namely Pepsin I and II. These two proteases had similar amino acid composition but different isoelectric points. The major stomach protease was Pepsin I which had almost a neutral isoelectric point and had a significantly higher pH optimum on hemoglobin than pepsin from mammalian species. This enzyme resembled cathepsin D more than mammalian pepsin on the basis of optimum pH and isoelectric properties, however, the inactivation at alkaline conditions was irreversible thus it was considered a true pepsin. Furthermore, the amino acid

composition showed that the polypeptide chain was more like pepsin than that of cathepsin D. The purified capelin pepsins had remarkably high activity at low temperatures which was consistent with the crude enzyme preparation. The reason for this low temperature activity may be due to the fact that the capelin habitat is at a low temperature environment. The capelin pepsins digested capelin muscle proteins at a lower rate than was observed for hemoglobin, however, the pepsin I is capable of digesting muscle protein at a significant rate even at pH 6 (Gildberg, 1982).

Noda *et al.* (1982) isolated two acid proteases from the stomach of sardine. These two enzymes, acid protease I and II, were similar to mammalian cathepsin D and pepsin but could hardly hydrolyze a synthetic pepsin substrate, N-acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine. Preincubation of these two acid proteases with 5-25% NaCl for 24 h at pH 5.0 before being assayed for proteolytic activity at pH 4.0 (acid protease I) and 2.0 (acid protease II) with hemoglobin substrate, at 37°C showed that acid protease I was not inactivated during preincubation in salt up to 20% NaCl, while preincubation in 25% NaCl resulted in 40% reduction in activity. Acid protease II was not inactivated during preincubation in 25% salt. In fish sauce prepared from sardine with 22% salt, activity of acid protease II was retained after 3 months of fermentation.

Squires *et al.* (1986a) isolated three gastric proteases from the stomach mucosa of the Greenland cod. The cod proteases had a more alkaline pH optimum and were active over a wider range of pH than porcine pepsin. The

activities of cod proteases 1 and 2, against hemoglobin at pH 2.0 were doubled in the presence of 25 mM NaCl, while the activities of cod protease 3 and porcine pepsin were not affected (Squires *et al.*, 1986b). It was suggested that salt activation of gastric protease is characteristic of gastricsins from fish.

Since fish sauce fermentation is usually at pH around 6-7, and in very high salt concentration, the contribution of acid proteases is assumed to be less important than that of the neutral proteases.

Trypsin and chymotrypsin

Trypsins and chymotrypsins, like pepsins, are endopeptidases. They have been classified, along with other similar enzymes, as serine proteases because of the involvement of a highly reactive serine residue in the catalytic reaction. Most studies of tryptic enzymes from fish have involved the use of crude preparations and do not discriminate between trypsin and chymotrypsin activity, thus the term "trypsin" was suggested to be used for pancreatic proteolytic enzymes which were active in the pH range from 7-11 (Kapoor *et al.*, 1975). According to Kiel (1971), trypsins show maximum activity toward their substrates within the pH range of 7-9. However, the specific pH optimum of trypsin may vary with different substrate and trypsins from different species may have different pH optima.

Partly purified trypsins from capelin or herring had the optimum activity at about pH 8.0 with tosyl arginine methyl ester, TAME (Kalac, 1978a). Chymotrypsin from herring had an optimum pH at 7.5 while capelin

chymotrypsin's optimum was at pH 7.8 (Kalac, 1978b). An extract of cod digestive organs were found to have a pH optimum for both trypsin and chymotrypsin between 8 and 9 (Overnell, 1973). Greenland cod trypsin had a pH optimum of 7.5 with N-α-benzoylarginine p-nitroanilide (BAPA) and pH 9.0-9.5 with casein substrate (Simpson and Haard, 1984). Hjelmeland and Raa (1982) purified two trypsin-type enzymes from digestive tract of capelin. Both enzymes had a pH optimum of 8-9 with BAPA and were not affected by $CaCl_2$. The apparent temperature optimum of the enzymes was 42°C. The isoelectric points of trypsin-like enzymes in capelin were 5.6-5.9 and 5.1-5.3 which were significantly different from 10.8 of mammalian trypsin but resemble the trypsin-like enzyme in shrimp, crayfish, and sardine.

Three alkaline proteases were isolated from pyloric caeca of sardine (Noda *et al.*, 1982). The first protease was an alkaline protease I, the second (alkaline protease II) was an anionic α-chymotrypsin-like enzyme and the third (alkaline protease III) was an anionic trypsin-like enzyme. Preincubation with 25% NaCl at pH 7.0 for 24 h prior to assaying at pH 10 with casein substrate at 37°C, the trypsin-like enzyme was active; the chymotrypsin-like and the alkaline protease I activities were 80 and 40% of the control, respectively. The proteolytic activity of these enzymes was inhibited in proportion to the concentration of NaCl present in the assay mixture. However, when sarcoplasmic protein from sardine muscle was used as substrate the inhibition by salt was less than that observed with milk casein or hemoglobin as substrates. For alkaline protease III, with small substrate, benzoyl arginine ethyl ester in the presence of 20% NaCl the inhibition of

protease activity was only 19% whereas a 60-90% inhibition was found when protein substrates were used.

Orejana and Liston (1982) reported the agents of proteolysis in patis (fish sauce) to be a trypsin-like enzyme. Enzymes in fish sauce at different fermentation times were precipitated by acetone. Activity of the precipitate was measured against BAPA at pH 8.2. The trypsin-like activity in samples fermented for a few days was low. Maximum activity was found in samples fermented for 1 month. After a 1-month fermentation, the activity declined rapidly and remained low throughout the remainder of the fermentation process. It was suggested that the decrease of the enzyme activity was due to end product inhibition, i.e. resulting from amino acids and small peptides which accumulate. The initial inhibition of trypsin-like activity could be due to inhibitors in fish blood or inhibition by substances produced by bacteria.

Sardine visceral extract was reported to contain several proteases probably of pancreatic origin (Yoshinaka *et al.*, 1983). The proteolytic activity was stabilized by calcium ions, the maximal rate of hydrolysis of the sardine flesh was at pH 8 and 50°C. The activity of the proteases was reduced with addition of NaCl. At 5, 10 and 20% NaCl in the assay mixtures, the proteolytic activity on the sardine muscle were 80, 75 and 64%, respectively, of the activity without addition of salt. Heat treatment of the sardine flesh at 100°C for 5 min, before addition to the visceral extract, also reduced the rate of hydrolysis by the visceral enzymes.

Two aminopeptidases were isolated from internal organs of sardine (Vo Van *et al.*, 1983). These two enzymes were inhibited by EDTA and activated by Co^{++}. Sardine aminopeptidase I was also inhibited by bestatin. Both enzymes can hydrolyze synthetic substrates containing alanine and leucine, di-, tri- and tetra-alanine. Sardine aminopeptidases resembled human alanine-aminopeptidase. It was found that enzyme I retained more than 70% of its original activity in the presence of 15% NaCl in the assay mixture. Later, Vo Van *et al.* (1984) determined the aminopeptidase activity of a sardine-salt mixture and reported that the aminopeptidase in fish sauce was similar to sardine aminopeptidase I and concluded that this aminopeptidase participated in the hydrolysis of fish protein and peptides during fish sauce fermentation.

Carboxypeptidases have been isolated from the viscera or pancreas of fish (Reeck and Neurath, 1972). Vo Van *et al.* (1984) measured carboxypeptidase activity during fish sauce production and found that the activity disappeared almost completely after a few days of fermentation. Thus, it is assumed that carboxypeptidase is inactivated by NaCl and for this reason carboxypeptidase is not important in the fermentation process of fish sauce.

The existence of proteinase and amylase in the viscera of squid (*Ommastrephes sloani pacificus*) has been reported (Takahashi, 1960a). Extract from the stomach and blind sac intestine of squid had an optimal pH for casein digestion in the range of 8.0-8.5, the pancreatic extract had a pH optimum of 6.5-7.0 and the liver extract had two optimal pH's of 2.5 and 5.0-6.0. The optimal

temperature for casein digestion of these enzyme extracts was in the range of 40-50°C (Takahashi, 1960b). It was concluded that the squid liver contained two kinds of cathepsin-like enzymes of similar nature, and each proteinase possessed sulfhydryl group(s) at the active center (Takahashi, 1961).

Inaba *et al.* (1976) purified cathepsin B from the liver of the squid *Dorythenthis bleekri*. The optimum pH for the hydrolysis of αN-benzoyl-L-argininamide and αN-benzoyl-DL-arginine-2-naphylamide was 4.5 and for hydrolysis of N-N-dimethyl hemoglobin was 4.2-4.7. LeBlanc and Gill (1982) reported that major proteolytic enzymes occurring in the hepatopancreas tissue of Atlantic short-finned squid *Illex illecebrosus* were cathepsin B, D and E. These cathepsins are acid proteases which are normally most active at pH 3-4.

Cathepsin C was isolated from the hepatopancreas of Atlantic short-finned squid, *Illex illecebrosus* (Hameed and Haard, 1985). This enzyme was activated by chloride ion and had an optimal pH for hydrolase and transferase activity at pH 5.6 and 7.0, respectively. Use of cathepsin C partially purified from squid hepatopancreas in the fermentation of squid was found to enhance the development of flavor (Lee *et al.*, 1982b).

1.6.1.2. Muscle tissue enzymes

Cathepsins, peptidases, transaminases, amidases, amino acid decarboxylases, glutamic dehydrogenase and related enzymes are found in fish muscle tissue (Siebert and Schmitt, 1965). The amount of proteolytic enzymes from muscle was enough to apparently justify the assumption that they play an important role in

fish spoilage by degrading the muscle protein resulting in peptides and amino acids for the growth of microorganisms (Siebert, 1962).

Cathepsins

Cathepsins are a group of intracellular enzymes which occur in animal tissues and catalyze hydrolysis of proteins or specific synthetic substrates. These enzymes are largely located in the lysosome within the cells and thereby differ from digestive enzymes which are secreted by cells (Mycek, 1970). Cathepsins are classified on the basis of their specificity toward substrate. The properties and cellular locations of the lysosomal peptide hydrolases were reported. Among these lysosomal peptide hydrolases, cathepsins A, B, C, D, E and carboxypeptidase B have been more thoroughly studied than cathepsins G, H, L, M, N, S and T. Cathepsin A is an exopeptidase (carboxypeptidase) which has optimum activity at pH 5.0-6.0. Cathepsins B, D and L are all endopeptidases with optimum activity at pH 4.0-6.5, 2.5-5.0 and 3.0-6.5, respectively, (Goll *et al.*, 1983). However, at pH 5.2 the activity of cathepsin D on native myosin was found to have 50% of the activity at pH 4.0 (Pearson *et al.*, 1983). Cathepsin H is both an aminopeptidase and an endopeptidase which has an optimum pH of 5.5-6.5 (Goll *et al.*, 1983). Cathepsin A, B and C are active on N-benzylcarbonyl-γ-L-glutamyl-L-tyrosine, benzoyl-L-arginamide and glycyl-L-phenylalaninamide, respectively, (Mycek, 1970). Cathepsins D and E were not found to act on synthetic substrates of cathepsin A, B and C but could act on proteins like hemoglobin, albumin and casein. According to Huang and Tappel (1971), cathepsin D is the most important enzyme of the cathepsins, since it

initiates protein hydrolysis resulting in the formation of peptides that are substrates for other cathepsins such as cathepsin C. The specificity of cathepsin D is similar to that of pepsin, but pepsin is active at more acid conditions. Cathepsin L has very little activity on small peptides including peptide substrates, but it has more potent ability to hydrolyze proteins than cathepsin B. It was estimated that cathepsin L has ten times and cathepsin H five times greater specific activity against myosin than cathepsin B (Bird and Carter, 1980). According to Pearson *et al.* (1983), cathepsins A, B, C, D, and L have optimal activity at acidic pH values, and thus, could play a role in post mortem meat tenderization during aging, especially after pH falls during completion of glycolysis.

Cathepsin C is a dipeptidyl hydrolase removing amino terminal dipeptides from polypeptides with a wide specificity and requires the presence of chloride ion for activity. Cathepsin C is also able to catalyze the transpeptidation reaction to form successive tetrapeptide, hexapeptide, and longer chains, depending on the initial concentration of the substrate and the solubility of the oligopeptide formed. The chain is lengthened by the successive addition of the dipeptide amide to the C-terminal of the growing chain (Fruton, 1982). Activity of cathepsin C was reported to be optimum at pH 5.0-7.0 (Goll *et al.*, 1983).

It was reported that fish muscle tissue contains cathepsins which are active at neutral pH (Makinonda and Ikeda, 1971), and at acidic pH (Huang and Tappel, 1971). Different species of fish showed different amounts of catheptic activities.

Siebert and Schmitt (1965) reported that the catheptic activity of fish muscle was about ten times greater than that of mammalian tissue. Reddi *et al.* (1972) reported that catheptic activity of winter flounder was maximal at pH 4 on hemoglobin substrate, and that 5% NaCl inhibited the activity of the enzyme by 95%.

Rosario and Maldo (1984) studied the activity of cathepsins during fermentation of fish sauce (patis). They reported that cathepsin A and C were important in proteolysis during fermentation. The cathepsin A activity in fish sauce at different fermentations periods was found to have a positive correlation with the amounts of amino nitrogen. A positive correlation was also found between the cathepsin C activity and the amount of TCA soluble nitrogen. Cathepsin D activity in patis prepared from fresh fish was greater than that prepared from stale fish. The decrease in cathepsin D activity during fermentation corresponded with the decrease in the amount of high molecular weight protein which served as substrate for cathepsin D.

Calcium-dependent protease

The calcium-dependent protease (calcium activated factor; CAF) is a neutral protease, located inside skeletal muscle cells. It is activated by calcium ion and exerts its optimal activity at pH 7.0-7.5 (Pearson *et al.*, 1983). CAF is unique in being unable to degrade myosin and actin. Goll *et al.* (1983) indicated that most of the proteolysis of the myofibrillar proteins that occurs during resolution of rigor may be due to CAF. The activity of CAF in muscle has previously been accepted

as being limited due to its high requirement (1-2 mM) for calcium (Pearson *et al.*, 1983). Dayton *et al.* (1981) suggested that CAF may have a low Ca^{2+} requiring form that is active at physiologically available levels of calcium in muscle. Thus, the tenderization of meat immediately post mortem, when the carcass temperature and pH are still high, may well be due to the effects of the low calcium-requiring form of CAF (Pearson *et al.*, 1983). However, according to Goll *et al.* (1983), it is not certain that post mortem proteolysis by CAF increases tenderness in aging of meat.

1.6.2. Exogenous enzymes

Starters are commonly used in some of the traditional fermented fish (Amano, 1962). The starters may be grown on cooked rice, roasted rice, or roasted rice bran. Examples are Koji or rice malt starter (a concentrate of fungal amylase, proteases and other enzymes obtained by overgrowing steamed rice with a selected strain of *Aspergillus oryzae*), Ragi (an Indonesian preparation of starter from yeast and rice), Angkak or red rice (rice fermented by a red yeast-like organism, *Monascus purpureus*). In this type of products, the fish is usually eviscerated and salted before the addition of the starter. The choice of starter depends on the type of the product as well as the production location.

Koji was used in the fermentation of fish sauce by Miyazawa *et al.* (1979). The flavor of the product was different from the ordinary fish sauce. The free amino acid content was higher than the control when the fermentation temperature was at 30°C, but at 50°C the control had a higher free amino acid content.

Use of commercial proteolytic enzymes such as papain, bromelain, ficin, pronase, bioprase, trypsin etc. in fish sauce fermentation has been reported (Murayama *et al.*, 1962; Guevara *et al.*, 1972; Beddows and Ardeshir, 1979a; Ooshiro *et al.*, 1981). However, none of these enzymes have been used commercially in the production of fish sauce. Use of pineapple peel and core to aid the fermentation of home-made fish sauce is practiced in the North of Thailand (Chauepohuk *et al.*, 1981).

1.6.3. Microbial enzymes

The role of microbial enzymes in fish sauce fermentation is still under investigation. There are conflicting reports as to the importance of microbial enzymes in the fermentation of fish sauce. Although, microorganisms do not appear to play a significant role in this fermentation process, enzymes from bacteria which are present on the fish prior to salting, may contribute to the process of autolysis (Amano, 1962; Saisithi, 1967; Beuchat, 1983).

Crisan and Sands (1975) isolated 39 microorganisms, comprising 11 species of bacteria, one yeast and three filamentous fungi from four fermented fish sauces namely, nampla, patis, koami and ounago. All of the isolates found in nampla at different times of fermentation were species of *Bacillus*. Yeast, fungi and obligate anaerobic bacteria were not found. In patis, the bacteria found were species of *Bacillus* and *Micrococcus* and the yeast; *Candida clasenii* was also found. In koami and ounago, which were prepared from shrimp and small fish, respectively, *Bacillus* was found together with strains of fungi *Penicillium notatum*, *Cladosporium herbarum* and *Aspergillus fumigatus*. They concluded that

species of *Bacillus* are the predominant microflora in fish sauces. This reflects the salt resistant nature of these spore forming bacteria, since they were found throughout the fermentation period. Fungi isolated from certain samples were found only in the finished product. This was probably due to contamination during the aging process.

Fujii *et al.* (1980) reported that the total viable cell counts in fish sauce (patis) and fish paste (bagoong), produced in the Philippines, were 4.5×10^3 and 6.5×10^3 cells/ml, respectively. The dominant flora were *Bacillus*, *Micrococcus* and *Moraxella*, which accounted for 40, 25 and 17.5 %, respectively, of the total bacterial flora isolated. Some of these bacteria can grow well in media containing more than 20% NaCl.

In fish sauce, shotturu, the dominant microbial flora were *Vibrio* and *Bacillus* when a medium containing 2.5% NaCl was used. In medium containing 20% NaCl, *Halobacterium*, *Bacillus* and an unidentified coccus were dominant (Fujii and Sakai, 1984a). During fermentation, it was found that the dominant microbial flora on 2.5% NaCl medium was the genus *Micrococcus* and on 20% NaCl medium were *Micrococcus* and *Corynebacterium* (Fujii and Sakai, 1984c). Causative microorganisms of spoiled fish sauce were found to be of the genus *Halobacterium* and *Streptococcus* (Fujii and Sakai, 1984b)

Ooshiro *et al.* (1982) isolated halophilic strains of *Bacillus* from Burmese and Chinese fish sauce and observed that there was no growth in synthetic media containing 3 M NaCl when the pH of the media was below 5.0. These bacteria

showed positive results on lipase and gelatinase tests. Ok *et al.* (1982a) studied protease formation by a moderately halophilic *Bacillus* isolated from fish sauce. Two strains of a moderately halophilic *Bacillus* were isolated from Burmese and Chinese fish sauce and named B_3 and C_1. Both bacteria were capable of growth in a medium containing 4 M NaCl. Maximal protease formation for B_3 and C_1 were found in media with 4M and 1 M NaCl, respectively. The pH and temperature optima for enzyme production in B_3 were pH 7 and 44°C which were similar to the fermentation conditions in South East Asia.

1.7. Biochemical changes during the fermentation of fish sauce

Whitaker (1978) reviewed the biochemical changes occurring during fermentation of high-protein food. The hydrolysis of proteins and decomposition of amino acids (Fig 1-2) may involve endogenous enzymes, microbial enzymes or exogenous enzymes.

In fish sauce, the degradation of fish proteins into soluble proteins, peptides and amino acids is apparently due primarily to endogenous enzymes of the fish, especially digestive enzymes in the entrail (Ham and Clague, 1950; Uyenco *et al.*, 1953; Orejana, 1978; Beddows *et al.*, 1979; McIver *et al.*, 1982). During fermentation the number of bacteria decreases in the presence of high salt. However, bacteria were believed to play an important role in the development of flavor (Amano, 1962; Kasemsarn, 1963; Saisithi, 1967; Dougan and Howard, 1975; Beuchat, 1983).

During fermentation of fish where the brine is allowed to drain off, it is

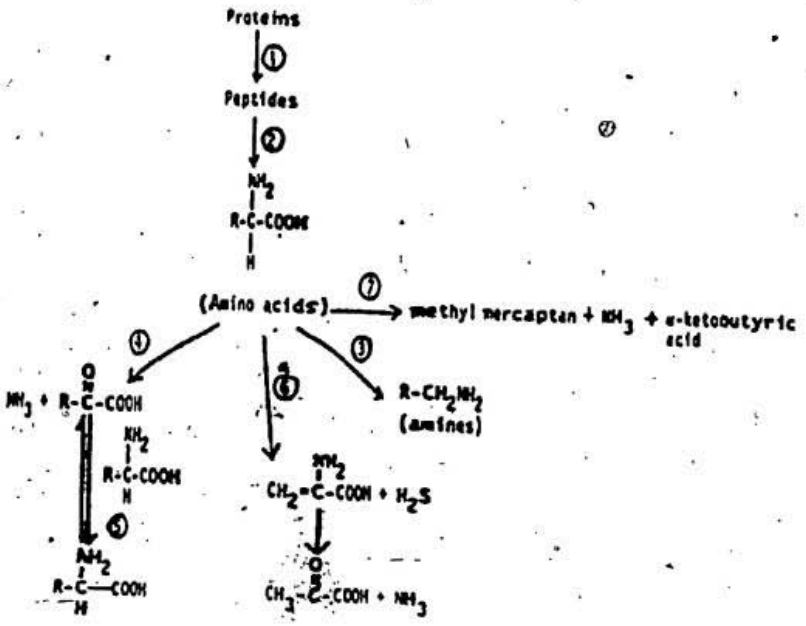


Figure 1-2: Hydrolysis of proteins and decomposition of amino acids.

Enzymes involved are:
(1) primarily endoproteases; (2) primarily exoproteases including carboxypeptidases, aminopeptidases, and di- and tri-peptidases; (3) decarboxylases; (4) deaminases; (5) transaminases; (6) cysteinase (substrate is cysteine); (7) probably through *B*-elimination of methionine by enzyme similar to cysteinase.
Source : Whitaker, 1978.

found that the average nitrogen loss is about 30% (Amano, 1962), however, in fish sauce the loss seems to be smaller because the process requires less drainage.

Uyenco *et al.* (1953) used long jawed anchovy as raw material for fish sauce and fish paste fermentation and suggested that the optimum fermenting time should be only 4 months, since the nitrogen content of the sauce reached a maximum around the period of 35-55 days. By the end of the second month, a large proportion of amino acids was produced, after that the ammoniacal nitrogen increased. The total nitrogen increased up to the fourth month and remained constant, while the ammoniacal nitrogen continued to increase from the deamination reaction of amino acids.

Ammonia in fish sauce may arise from two types of reactions (Amano, 1962), one is from the activity of the enzymes in fish, the other is bacterial reactions.

Early studies on Thai fish sauce by Kasemsarn (1963) and Saisithi (1967) showed that the pH of the sauce was slightly changed while the salt concentration remained close to 30%. The total soluble nitrogen in the sauce increased during the process, as well as total acid, such as lactic acid and volatile acids. Ammoniacal nitrogen was found to increase during the first six months and remained constant until the end of the process. Saisithi (1967) also reported that more volatile base was produced than volatile acid on a milliequivalent basis. Tongthai and Okada (1981; 1982) indicated 3 stages of the Thai fish sauce fermentation from which free amino acids were released :

Early or rapid stage (0-3 weeks), during which soluble nitrogen and free amino acids increase rapidly due to exopeptidases originating from the fish.

Middle or gradual stage (3-20 weeks), when endopeptidases are predominant and free amino acids increase gradually.

Late stage (20-50 weeks), during which time halophilic bacteria become active and there is no release of free amino acids.

Orejana (1978) observed that most of the changes in protein nitrogen took place during the first two months of fermentation. The amino nitrogen, soluble protein and peptide nitrogen increased significantly during this period while ammonia and other volatile bases increased throughout the fermentation with significant increases at the later stage. Lipolysis also occurred during the fermentation as observed from the increase in free fatty acids. The lipid breakdown products (carbonyl compounds and peroxides) may be involved in browning reactions. Flavor compounds such as nucleotides, amino acids, volatile and non-volatile acids were formed during fermentation. Orejana and Liston (1979) found that the main part of amino nitrogen in fish sauce was peptides rather than amino acids. The molecular weight range of these peptides formed during the first 40 days fell between 700-1500 daltons. They concluded that during the first 40 days endopeptidases of the trypsin type were most active. During the period of 70-140 days, the small molecular weight fraction increased significantly indicating a higher activity of exopeptidase including cathepsins.

1.8. Amino acids and peptides as a taste contributor

Amino acids have been well recognized as a taste contributor in foods. Monosodium glutamate (MSG) is the essential component of Japanese food seasoners (Kirimura *et al.*, 1969). The taste of cheese is influenced by amino acids formed during ripening. It was reported that sulfur containing amino acids play an important role in Cheddar cheese flavor (Manning, 1978). In Swiss cheese, only a combination of amino acids together with selected free fatty acids and selected volatiles gave reconstituted Swiss cheese flavor (Langler *et al.*, 1967). The tastes of miso and soy sauce were reported to be due to the amino acids which were released from protein during fermentation process (Kirimura *et al.*, 1969). Glutamic acid and its salts are very important in soy sauce flavoring (Steinkraus, 1983). According to Kiesvaara (1975) the Scandinavian barrel-salted herring and semi-preserves were judged ripe when the free amino acid content of the product had risen to a specified level.

Amino acids were characterized as being sweet, salty, sour, bitter and MSG-like (Kirimura *et al.*, 1969). The sweet amino acids were H-Pro (threshold value 50 mg/dl), Ala (60), Gly (130), Ser (150), Thr (260) and Pro (300). Sour taste was found in Asp (3), Glu (5) and His-HCl (5). MSG-like were sodium glutamate (30), Asp (100) and Glu (3). Bitter amino acids were His (20), Arg-HCl (30), Met (30), Val (40), Arg (50), Ile (90), Phe (90), Trp (90) and Leu (190). Solms (1969) reported on the taste of pure amino acids at pH 6.0. Amino acids with flat or no taste were D-Ala, D and L-Arg, D and L-Asp, D-Glu, L-His, D and L-Ile, D and L-Lys, D and L-Pro, D and L-Ser, D and L-Thr, D and L-Val. The sweet amino

acids were D-Trp > D-His > D-Phe > D-Tyr > D-Leu > L-Ala > Gly. Amino acids with bitter taste were L-Trp > L-Phe > L-Tyr > L-Leu. The sulfur containing amino acids, D and L-Cys, D and L-Met had sulfurous taste. L-Glu was found to have a unique taste-potentiating property. The characteristic taste modifying property of MSG is called "umami" in Japanese and it plays a predominant role in the flavor of foods, such as meat, poultry, fish and other seafoods (Yamaguchi, 1979).

Kirimura *et al.* (1969) indicated that amino acids may affect the taste of foodstuff in various ways e.g., some amino acids contribute to the inherent tastes of foodstuffs themselves; some specific pattern of amino acid mixture can intensify the taste of foodstuff and increase the mouthfulness without losing their inherent taste, and the buffer action of amino acids can also contribute to the taste of foodstuffs.

Kirimura *et al.* (1969) stated that the taste of peptides was weak compared to the taste of amino acids. The taste of peptides are not simply related to the amino acids; e.g., the peptide L-Gly-L-Trp is not bitter although it contains L-Trp (Solms, 1969). However, the taste of peptides from the hydrolysis of protein were found to be according to the terminal amino acids.

Bitterness of peptides can be predicted from their amino acids composition (Ney, 1971). The method is based on the value Q which is calculated from the solubility data of the individual amino acid. Peptides with $Q < 1300$ are not bitter, peptides with $Q > 1400$ show a bitter taste.

The distribution of peptides and their effect on taste has been studied. Some peptides containing glutamic acid are responsible for the brothy taste of the fish protein hydrolysate. The acidic peptides of molecular weight less than 1000 are responsible for a short time taste effect whereas fraction with a molecular weight higher than 1000 gives rise to a rather long time taste effect (Fujimaki *et al.*, 1973).

It was concluded that peptides contribute to both the complexity and favor balance of the taste of foodstuffs (Kirimura *et al.*, 1969).

Yamasaki and Maekawa (1978) isolated a peptide fraction from beef gravy using gel filtration with Sephadex G-25, chromatography on an ion exchange resin (Dowex 50 x 4) and paper electrophoresis. The amino acid sequence of the delicious taste peptide was determined by Edman degradation and carboxypeptidase A. The primary structure of the peptide was proposed as H-Lys-Gly-Glu-Ser-Leu-Ala-OH.

1.8.1. Taste of fish and shellfish

The taste of fish and shellfish originates from water-soluble low molecular weight components (Konosu and Yamaguchi, 1982). These extractive components are more abundant in the muscle of molluscs and crustacean than they are in the muscle of fish. Jones (1961) reported that part of the flavor of the fish is derived from sugar, sugar phosphate, amino acids and peptides, nucleotides and derivatives, organic acids, fats and degradation products of fat and nitrogen bases.

The content of free amino acids in crustacean muscle is higher than in fish muscle. The major free amino acids are taurine, proline, glycine, alanine and arginine. Shrimp has a very high content of glycine. Hujita *et al.* (1972) stated that glycine may contribute to the sweetness of shrimp. The free amino acid content of mollusk muscle appears to be in between fish and crustacean. Konosu (1979) used an omission test to determine compounds contributing to the taste of crab. Umami and sweetness decreased when glycine was omitted. Glutamic acid contributed greatly to umami. Alanine served to produce part of the sweetness and arginine was important for the overall taste as well as the crab-like taste. Na^+, K^+ and Cl^- were also important for the crab taste. Hashimoto (1965) reported on a synthetic flavor formulated based on the composition of the extract of uni (sea urchin gonad). It was found that omission of amino acids significantly influenced the taste. The most influential amino acids were glycine, alanine, valine, glutamic acid and methionine. Absence of glycine resulted in a decrease in sweetness and an increase in bitterness. According to Kiesvaara (1975) aspartic acid, glutamic acid, and methionine are amino acids responsible partly for the meaty flavor in fish semi-preserves. Although it is evident that several peptides are present in extracts of fish and shellfish, only a limited number of peptides such as carnosine, anserine, balenine and glutathione have been identified (Konosu and Yamaguchi, 1982).

1.8.2. Flavor compounds in fish sauce

All the nitrogen compounds in fish sauce were found to be dialyzable (van Veen, 1953), indicating that the protein was completely broken down. Kasemsarn (1963) suggested that the flavor of fish sauce is due to microbial reaction on proteins, or protein degradation products. Most of the bacterial species do not attack proteins but utilize more readily available protein-degradation products. Thus, it was concluded that the typical flavor of fish sauce was developed during the later stage of fermentation.

Howard and Dougan (1974) investigated the flavoring constituents of fish sauce. They reported that carbonyl compounds such as acetone, acetaldehyde, butan-2-one and n-valeraldehyde were found in low concentration and they appeared to make no contribution to the odor. Esters and sulphur compounds did not play important role in odor either. The volatile acids found were formic, acetic, propionic, iso-butyric, n-butyric and iso-valeric acids, these acids gave a distinct, sharp, cheesy odor when restored to their original concentration in water. Saisithi *et al.* (1966) also identified formic, acetic, propionic and iso-butyric in Thai fish sauce, and concluded that volatile fatty acids played an important part in the aroma of fish sauce.

Dougan and Howard (1975) stated that the aroma of fish sauce comprises three distinct notes. Cheesy aroma derives from lower fatty acids, the most important of which is n-butyric acid. Ammoniacal odor derives from ammonia and amines. Meaty aroma is complicated and can be produced by oxidation of

precursors present in the mature sauce by atmospheric oxygen. It was suggested that fatty acids in fish sauce are more likely to have been formed by the hydrolysis of fat than by any other mechanism (Dougan and Howard, 1975). Orejana and Liston (1979) assumed that free fatty acid levels would indicate lipase activity, since salt does not interfere with lipase activity (van Klaveren and Legendre, 1965). However, Beddows *et al.* (1980) investigated the origin and the mechanism of formation of the volatile fatty acids in budu (Malaysian fish sauce) and concluded that fatty acids did not appear to be derived from the breakdown of the fish lipid. Using (U-^{14}C)protein hydrolysate, it was shown that amino acids are the precursors of the n-butanoic and n-pentanoic acid and also contribute to the formation of other acids. Ooshiro *et al.* (1981) confirmed that volatile organic acids are important components for the development of fish sauce aroma. McIver *et al.* (1982) fractionated the solvent extract of fish sauce to acidic, neutral and basic fractions. The acidic fraction was composed of acetic (29% of fraction), propionic (14), iso-butyric acid (3), 4-hydroxyvaleric acid lactone (12), n-butyric (17), iso-valeric (6), levulinic (10), phenylacetic acid and 3-phenylpropionic acid (3). The relative and absolute amounts of the short chain volatile fatty acids have been found to be variable depending on the type of sauce (budu, nampla or patis) as well as the quality of the product. Fish sauce with less volatile fatty acids was described as less cheesy and more ammoniacal. A mixture of these volatile fatty acids in the same relative amount found in fish sauce gave a sharp, cheesy aroma similar to that of the acidic isolate.

Fish sauces made from flounder and trout were found to have isovaleric acid

at the highest percentage composition followed by acetic acid and isobutyric acids (Chayovan *et al.*, 1983a). Total volatile fatty acids (C-2 to C-4) in trout sauce (fatty fish, 9.2% fat) were about 3 times higher than in flounder sauce (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 the volatile fatty acids. It was concluded that the flavor of fish sauce could be due to the overall effects of both volatile and nonvolatile fatty acids along with other biochemical reactions that generally occurred in fermentation. It is possible that the flavor of fish sauce is also influenced by some breakdown oxidation products of long-chain polyunsaturated fatty acids which are found in fish lipids.

Sanceda *et al.* (1983) fractionated the steam volatile distillate of Philippine fish sauce into 4 fractions, neutral, acidic, basic and phenol. They concluded that the acidic fraction appeared to play a major role in the aroma. The major components in the acidic fraction were n-butyric and propionic acid. n-Butyric acid was found to be the most abundant, accounting for about 50% of the total acids in patis (Sanceda *et al.*, 1984). The five major acids in the acidic fraction were n-butyric, propionic, iso-butyric, valeric and acetic acids which accounted for 98% of the total acids. Fujii *et al.* (1980) found only acetic acid (2.03 mg/ml), propionic and isobutyric acid (0.06 mg), butyric acid (0.15 mg) and isovaleric (0.04 mg) in the patis sample. Isocaproic, n-valeric and n-caproic acid were not found.

The meaty aroma of fish sauce was found in the neutral fraction of the solvent extracted fish sauce (McIver *et al.*, 1982). The major compounds which

accounted for more than 10% of total components were lactones (γ-butyrolactone, γ-caprolactone and 4-hydroxyvaleric acid lactone), 3-(methylthiol) propanol and 2,3-butanediol.

The basic fraction contained mainly ammonia and trimethylamine. Dimethylamine and 2,3-butanediol were found in small amounts (McIver *et al.*, 1982). It was suggested that the volatile bases also contributed to the odours of fish sauce, but their effects depend upon both their concentration and the pH value of the sauce which was usually between 5.6 and 6.1 (Howard and Dougan, 1974).

1.9. Use of fermentation aids in fish sauce production

The fermentation time for fish sauce production requires a period as long as 6-12 months because of the very high concentration of salt. A lot of work has been undertaken to shorten the fermenting time of fish sauce.

Kasemsarn (1963) inoculated pure bacterial cultures isolated from fish sauce to the acid hydrolysate of fish muscle and reported that at least one organism produced a typical aroma and flavor after incubation for 2 weeks, thus it was concluded that fish sauce could be produced from the acid hydrolysate of fish muscle incubated with pure culture. Saisithi (1967) later described this organism which was *Pediococcus halophilus*.

Commercial proteolytic enzymes such as bioprase, pronase, papain, bromelain and ficin have been used to shorten fermentation time. The use of

bioprase and pronase was reported by Murayama *et al.* (1962). The fermentation time was shortened to 70 days and a fine quality fish sauce was produced. Guevara *et al.* (1972) used papain in patis (Philippine fish sauce) fermentation and claimed that the fermentation time was reduced to 4-7 days without any destruction in characteristic flavor. Papain was used in the production of fish sauce using *Stolephorus* sp. as raw material (Beddows and Ardeshir, 1979a) and after 21 days at 33°C the supernatant liquor had no particularly strong or unpleasant flavor. When bromelain (0.80%), ficin (2.50%) and papain (2.75%) were compared, bromelain was found to give a better result. Ooshiro *et al.* (1981) used papain, bromelain and trypsin to aid the fermentation of fish sauce. Papain was found to give a better result with sardine at a concentration of 0.3% enzyme based on the fish weight; however, the final enzyme treated product lacked the typical aroma of fish sauce, although the taste and color were satisfactory.

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

Trypsin, chymotrypsin, pronase, fungal protease or squid hepatopancreas enzyme extract were used in fermentation of capelin (Raksakulthai *et al.*, 1986).

The initial rate of fermentation was higher with added enzymes but after 13 months the fungal protease, pronase and trypsin supplemented sauces were somewhat low in free amino acid content. Squid hepatopancreas accelerated the rate of fermentation and resulted in a final product with a higher content of free amino acids. Sensory evaluation showed that an excellent quality fish sauce can be made from capelin supplemented with squid hepatopancreas.

The use of acid in fish sauce manufacture was studied (Beddows and Ardeshir, 1979b). Hydrochloric acid was used and the optimal conditions were reported to be either pH 2.0 and 10% salt (w/w) or pH 3.0 and 15% salt (w/w). The end product was found to have very little aroma or taste but the soluble nitrogen content was high. It was suggested that the product produced with acidification be mixed with traditional product. Gildberg *et al.* (1984) used acid to lower the pH of fish-salt mixture to pH 4.0 and reduced the salt content to as low as 5%. After the initial phase of rapid autolysis, the samples were neutralized and salt was added to the normal concentration (25% w/w). This process was reported to be able to produce acceptable fish sauce after 2 months, whereas the traditional fermenting time is 6 months or longer. Although the sauce had a lower level of volatile base and acid, it had a better balanced composition of essential amino acids than a first grade commercial fish sauce.

Three strains of halophilic bacteria were isolated from Chinese and Burmese fish sauce (Ooshiro *et al.*, 1982). Two strains were identified as *Bacillus* B_3 and C_1. Both bacteria were able to grow in a medium containing 4 M NaCl and

produced proteolytic enzymes (Ok *et al.*, 1982a). Use of these halophilic bacteria to aid the fish sauce fermentation was found to have a remarkable effect, the fermenting time was shortened to 3 months. Free amino acid profiles in control and bacteria added samples were similar (Ok *et al.*, 1982b).

Huang *et al.* (1980) reported, on using high temperature and low salt in fish sauce fermentation, that brine of 20-24°Be and temperatures of 50-55°C could be used successfully to shorten the fermentation time giving higher yields of amino acids than low temperature and high salt fermentation. The flavor of the product depends on the degree of salting of the raw materials. Higher salt content and longer time gives the fish sauce a better flavor.

Fish sauce which is prepared by accelerated fermentation normally has inferior consumer acceptability due to differences in appearance, taste and aroma from the traditional product. Howard and Dougan (1974) concluded that the fish sauce produced by rapid proteolysis was unlikely to give products with the traditional flavor unless the formation of fatty acids was also accelerated.

1.10. Amines in fish sauce

Amines are basic nitrogenous compounds usually formed by decarboxylation of amino acids by bacterial enzymes. Putrescine, spermidine and spermine probably occur universally in animals and plants and they are found in most bacteria (Smith, 1980). Putrescine and spermidine are important in the regulation of nucleic acid function and protein synthesis. Some of the amines are allergenic or biologically active. Tyramine and 2-phenylethylamine are known to cause

"cheese reaction"- the increase in blood pressure which cause severe headache and may induce a brain haemorrhage or heart failure (Smith, 1980). Histamine is the causative agent of scromboid food poisoning (Eitenmiller *et al.*, 1982). Moreover, in the presence of nitrite salts, amines may form carcinogenic nitrosamines (Warthesen *et al.*, 1975). Normally the exogenous amines absorbed from food will be rapidly detoxified by amine oxidases or by conjugation, but in patients treated with monoamine oxidase inhibitory drugs there will be high risk of toxicity. There are reports of nightmare, illness and even death from "overconsumption" of fish sauce (Steinkraus, 1983). Toxic symptoms may be caused by the presence of toxic biological amines. Steinkraus (1983) also suggested that it was possible for anaerobic bacteria including *Clostridium botulinum* to develop in improperly handled fish sauce fermentation. However, under normal circumstances it is not likely that *C. botulinum* will grow in fish sauce because of its high salt content (27%). Crisan and Sands (1975) reported on microflora of four fermented fish sauce samples that obligate anaerobic bacteria were not found in any sample.

1.11. Standard of fish sauce quality

According to the standard of nam pla, local Thai fish sauce (Anon., 1983), the requirements for fish sauce are shown in Table 1-5.

Sensory evaluation of fish sauce is based on color, aroma and flavor. The score for color is 10 and the desired hue is reddish-brown. The scores for aroma and flavor are 50 and 40, respectively. Total sensory score of the first grade and

Table 1-5: Local Thai fish sauce standard

Characteristics	Requirements for fish sauce Grade 1	Grade 2
Specific gravity at 27°C	> 1.2	> 1.2
pH	5 - 6	5 - 6
Sodium chloride (g/L)	> 230	> 230
Total nitrogen (g/L)	> 20	> 15
Glutamic acid N/ Total N	0.4-0.6	0.4-0.6
Amino acid nitrogen (g/L)	> 10	> 7.5

Source : Anon., 1983.

second grade fish sauce must be higher than 80 and 70, respectively. The standard also requires fish sauce to be clear, without sediment except salt crystal. No preservatives or sweeteners other than sugar are allowed to be used. Similarly only caramel is allowed to be used for improving color.

1.12. Problem approach and hypothesis

In Newfoundland the consumption of fresh capelin is normally limited to the local area where the fish are landed. Studies on quality of frozen stored capelin showed that the lean capelin can be well kept in frozen storage. Quick frozen capelin can be stored for at least one year (Jangaard, 1974a). The non-spawning capelin, with higher fat content, has the same keeping quality as that of spawning capelin (Shaw and Botta, 1977). Quality of long term frozen stored capelin was more affected by preprocessing treatment than by storage time at -23°C. The sensory evaluation of frozen capelin showed that the fish was acceptable after 21 months storage (Botta *et al.*, 1983). Besides the export of dried or frozen female capelin, the utilization of capelin in Newfoundland is still underexploited. Angel (1971) observed the extensive waste of male capelin which had been dumped into the sea or were left on the beach to rot. In 1981, the price of capelin for reduction to meal was 3.3 cents per kilogram which was much the same as the price in 1979-80. The price of 100% female capelin was 59.5 cents per kilogram (kg), while 20-35% female was priced only at 7.2 cents/kg. These prices were paid as per agreement between the capelin processor and Newfoundland Fishermen's Union (Anon., 1982a). In 1985, the price of 96-100% female capelin paid to fishermen was 22 cents/kg and the price for 36-45% female was only 3.75 cents/kg.

Capelin fish sauce was prepared from male capelin supplemented with squid hepatopancreas (Raksakulthai *et al.*, 1986). The yield of the fish sauce was approximately 600 ml/kg fish. Considering the cost of production was less than 50 cents/l compared to commercial products in the local market which cost

$3.33-4.67/L, the use of male capelin as raw material for fish sauce production seems to be promising. Preference tests by sensory evaluation showed capelin sauce had higher acceptability than the commercial product from the Philippines. Because of the growing population of Canadians and Americans of South-East Asian descent, there would appear to be a sizable market for this product in North America.

It is hypothesized that the initial autolysis as well as the ripening of fish sauce is catalyzed by proteolytic enzymes which are endogenous to capelin, squid hepatopancreas, and proteolytic enzymes of microbial origin. It is further hypothesized that the characteristic flavor of fish sauce is related to the amino acids and peptides formed as a result of protein hydrolysis.

The objectives of this study were

1. To develop a method to utilize male capelin by producing a food condiment with acceptable flavor and aroma.
2. To determine optimal conditions for the fermentation and the importance of aging or ripening on fish sauce quality.
3. To understand the chemical change of protein during fermentation.
4. To investigate the contribution of endogenous and bacterial enzymes to protein degradation in fish sauce preparation.
5. To understand the contribution of amino acids and peptides to the flavor of fish sauce.

Chapter 2

Materials and Methods

2.1. Biological Specimen

2.1.1. Capelin

Inshore capelin were harvested in Outer Cove, Newfoundland by handnet, in June 1983 and July 1984, and washed with tap water before grinding with a Hobart 7 horsepower, 6 mm plate, meat grinder.

2.1.2. Red feed capelin

Frozen capelin suspected to contain red feed, (rejected by the Japanese buyers) were obtained from Fogo Island. After receiving the fish, they were kept at -20°C for 4 months before use.

2.1.3. Squid hepatopancreas

Frozen Atlantic short-finned squid were purchased from Fishery Products Ltd., St. John's, Newfoundland. The hepatopancreas (SHP) was removed and mixed directly with the fish-salt mixture or was cooked in a water bath at 100°C for 30 min, cooled and added to the fish-salt mixture.

2.2. Chemicals and supplies

Commercial fish sauce, Rufina fish sauce made in the Philippines, was purchased from a local market, and was used as a reference in sensory evaluation.

Sifto coarse salt, used for pickling, was purchased from a local market.

Garamycin reagent solution (Gentamicin sulfate, USP), 50 mg/ml was purchased from Schering Corporation, (Kenilworth, N.J.).

Formaldehyde (38% v/v), HCl, Nessler's reagent and 4-chloromercuribenzoic acid (PCMB) were purchased from BDH Chemicals, (Toronto).

Lithium citrate sample dilution buffer 0.2 M, pH 2.2 and 0.15 M sodium citrate buffer pH 2.2 were purchased from Pierce Chemical Company, (Rockford, Illinois).

Microbiological media, Trypticase Soy Agar was purchased from BBL. Becton, Dickinson and Co., Canada. Bacto peptone was purchased from Difco Laboratories, (Detroit, Michigan).

All other chemicals were purchased from Sigma Chemical Company, (St. Louis, Missouri).

2.3. Preparation of fish sauce

2.3.1. Contribution of fish/squid hepatopancreas enzymes to fish sauce fermentation

2.3.1.1. Contribution of squid hepatopancreas enzymes

Minced capelin was mixed with salt at the ratio of fish to salt, 4:1 (w/w). The mixture was left for 16 h at 4°C. Samples of fish-salt mixture (1 kg) were packed in glass (1.5 L) jars. Duplicate 1 kg samples were supplemented with 25 g of either cooked or raw squid hepatopancreas. In one set of duplicate (1 kg) samples supplemented with raw squid hepatopancreas, the pH was adjusted to 4.5 using 6 N HCl. All fermenting jars were sealed and stored at ambient temperature (20-25°C). Another batch of fish-salt mixture was prepared in the same way using frozen capelin (3 months at -20°C) as raw material. Different concentrations of squid hepatopancreas (5 and 10% w/w of the fish-salt mixture) were also used to determine the optimum concentration of SHP in fish sauce fermentation.

2.3.1.2. Contribution of fish digestive enzymes

One lot of fish sauce was prepared from frozen female capelin which was suspected to contain red feed. The fish was thawed at 4°C for 16 h and separated into 2 parts. One part was ground, and mixed with salt (25 % salt w/w). The second part was headed, gutted and washed with tap water before grinding and then mixed with salt (25 % salt w/w). The fish-salt mixtures were left at 4°C for 16 h. Duplicate samples (1 kg) of each mixture were packed in 1.5 L glass jars, sealed and stored at ambient temperature (20-25°C).

2.3.2 Optimal conditions for fish sauce fermentation

2.3.2.1 Effect of salt concentration

Duplicate (1 kg) minced samples were mixed with salt to obtain 15, 20, 25 and 30 % salt based on fish weight. The samples were packed in jars, which were sealed and stored at ambient temperature.

2.3.2.2 Effect of temperature

Duplicate samples of fish-salt mixture (500 g): (i) 25 % salt w/w (A-37), (ii) 30% salt w/w (G-37) and (iii) SHP supplemented (B-37) were packed in glass jars, sealed and incubated at 37°C for 40 weeks. The brines formed were sampled at different time intervals for analysis of pH and degree of protein hydrolysis and compared to the samples incubated at ambient temperature (20-25°C).

2.3.2.3 Effect of pH

Samples of fish-salt mixture, 25 % salt w/w (300 g) were adjusted to pH 3, 4, 5, 6, 7 and 8 using 6 N HCl or 50 % (w/w) NaOH. These samples were stored in sealed glass jars at 37°C for 40 weeks and the brines formed were analyzed for pH and degree of protein hydrolysis at different fermenting times.

2.3.3. Contribution of bacterial enzymes to fish sauce fermentation

2.3.3.1 Effect of delayed salting

Minced capelin (500 g each) was held at room temperature for 6, 18, and 24 h before mixing with salt at the ratio of 4:1 (w/w). The salted mixtures were left for 12 h at 4°C prior to packing in 1 L glass jars, sealed and stored at ambient temperature.

2.3.3.2 Effect of antibiotics

To study the contribution of microbial enzymes to the proteolysis in fish sauce, salted mince (25 % salt w/w) was mixed with Garamycin reagent solution (gentamicin sulfate USP, 50 mg/ml) at the concentration of 2 ml/1 kg of fish-salt mixture. Another set of samples of minced capelin was held at room temperature for 24 h before mixing with salt (25 % salt w/w). The control without antibiotic (CO), the treatment with antibiotic (AN), and the delayed salting sample (DS) were stored in sealed glass jars at ambient temperature (20-25°C). Total bacterial counts were carried out between 1 - 40 days. Degree of protein hydrolysis, pH and soluble protein were also analyzed.

2.3.4 Recovery of fish sauce

The liquid formed during fermentation was sampled for chemical analysis at different time intervals. After 6 months, the liquid was recovered by filtration of the brined mince through Whatman No. 1 filter paper and stored in sealed 500 ml glass jars at ambient temperature. Sensory evaluation of fish sauce was normally conducted 6 months after filtration. The identification codes of samples of fish sauce prepared during the course of this study are shown in Table 2-1.

Table 2-1: Identification code for fish sauce samples

Sample code	Treatment
A, FA[1], C-FS	Control, fish:salt 4:1 (w/w)
B, FB[1], SQ-FS	2.5 % SHP supplement to control
C, FC[1]	2.5 % Heat treated SHP supplement to control
D, FD[1]	2.5 % SHP supplement to control, pH of the mixtures were adjusted to 4.5
E-6	Mince was held at room temperature for 6 h prior to salting
E-18	Mince was held at room temperature for 18 h prior to salting
E-24, DS	Mince was held at room temperature for 24 h prior to salting
H[2]	Fish : salt 6.6:1 (w/w)
K	Fish : salt 5:1 (w/w)
L	Fish : salt 4:1 (w/w)
G, M	Fish : salt 3.3:1 (w/w)
J_{3-8}	Fish : salt 4:1, pH of the mixtures were adjusted according to the subscript number
RRF	Round red feed capelin was used as raw material; fish : salt 4:1
GRF	Gutted red feed capelin was used as raw material; fish : salt 4:1
CO	Control, fish:salt 4:1
AN	Antibiotic treated fish-salt mixture (4:1 w/w)

[1] Capelin was frozen for 3 months at -20°C before use as raw material.
[2] Sample was spoiled after 2 weeks and was discarded.

2.3.5. Preparation of fish sauce for partial characterization of residual enzymes

To characterize the activity of enzymes retained after the fermentation, fish sauces were prepared and concentrated by a Millipore ultrafiltration unit.

A large batch of fish sauce was prepared from previously frozen capelin (3 months at -20°C). The fish was thawed for 16 h at 4°C prior to mincing. One lot of mince (8 kg) was mixed with 2 kg salt (C-FS), another 8 kg lot was similarly salted and supplemented with 200 g squid hepatopancreas (SQ-FS). The mixtures were stored in 20 L plastic containers with covers at ambient temperature for 6 months before filtration through double layered cheesecloth and finally through Whatman No. 1 filter paper. The liquid was frozen at -70°C until used for Millipore ultrafiltration.

2.4. Analytical Methods

2.4.1. pH

pH of fish sauce was measured directly using a Metrohm 632 pH meter.

2.4.2. Degree of protein hydrolysis

Degree of protein hydrolysis (DH) was measured by formol titration (Beddows *et al.*, 1976), using a Metrohm pH titrator (Brinkmann Instruments, Rexdale, Ont.). Sample (1 ml) was mixed with 40 ml of distilled water and titrated to pH 7.0 with 0.1 N NaOH then 10 ml of formalin solution (38 % v/v) was added to the neutralized sample. The titration was continued to pH 8.5 with

0.1 N NaOH. The degree of hydrolysis was expressed as mg formol nitrogen/ml fish sauce.

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

2.4.3. Browning development in fish sauce

Brown color of fish sauce was estimated by $A_{400\ nm}$ using a Beckman DU-8 spectrophotometer. Color of the fish sauce was also measured using a Hunter-Gardner XL 20 reflectance colorimeter. A white standard plate was used to standardize the colorimeter prior to obtaining L, a, b values of fish sauce samples. The total color difference (E) between a white standard plate and each sample was computed from the equation :

$$E = \{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2\}^{0.5}$$

2.4.4. Amino acid analyses

2.4.4.1. Free amino acids

Diluted fish sauce (1 part of fish sauce : 99 parts of water) was deproteinized with 12 % (w/v) sulfosalicylic acid and diluted with 0.2 M lithium citrate sample dilution buffer pH 2.2 to appropriate dilution and analyzed in the Beckman Amino Acid Analyzer Model 121 MB as described in the Beckman bulletin 121 M-TB-013.

2.4.4.2. Total amino acids

For analyses of total amino acids, diluted fish sauce (1:99) was hydrolyzed with 6 N HCl for 24 h at 110°C, appropriately diluted, and analyzed in the Beckman Amino Acid Analyzer Model 121 MB.

2.4.5. Soluble protein

Soluble protein in fish sauce was estimated by the Biuret Method (Cooper, 1977) using bovine serum albumin (BSA) as a standard. (Appendix A).

2.4.6. Total nitrogen

Total nitrogen in fish sauce was determined by the Micro-Kjeldahl method (Lang, 1958), using $(NH_4)_2SO_4$ as a standard (Appendix B).

2.4.7. Salt concentration

Sodium chloride content was determined according to AOAC (1980), by a volumetric method (Appendix C).

2.4.8. Polyamines

Fish sauce was diluted 5-fold with water before deproteinization with 12% sulfosalicylic acid and diluted to appropriate dilution with 0.15 M sodium citrate buffer (pH 2,2). The sample was applied to a Beckman Model 121 MB amino acid analyzer using the method described by Hall *et al.* (1978), with some modification. Beckman W-2 resin was packed in 0.2 M sodium hydroxide containing 0.01% EDTA in a glass column of 0.6 cm diameter. The resin was washed with buffer B (0.12 M trisodium citrate, pH 5.4, 3.65 M sodium chloride and 0.01% caprylic acid, v/v) for 30 min to ensure that the resin displayed maximum shrinkage. The

height of the column was set at 7.3 cm. Polyamines were eluted from the column at 55°C with buffer A (0.12 M trisodium citrate, pH 5.4, 2.0 M sodium chloride and 0.01% caprylic acid, v/v) for 10 min after the sample was injected followed by buffer B for 20 min. Flow rate of buffer was 35 ml/h and that of ninhydrin was 17.5 ml/h. Absorbance was monitored at 570 and 440 nm. The column was regenerated with 0.2 M sodium hydroxide for 4 min and equilibrated with buffer A for 14 min after each sample. Peak areas and concentration were calculated using HP 3390A computing integrator.

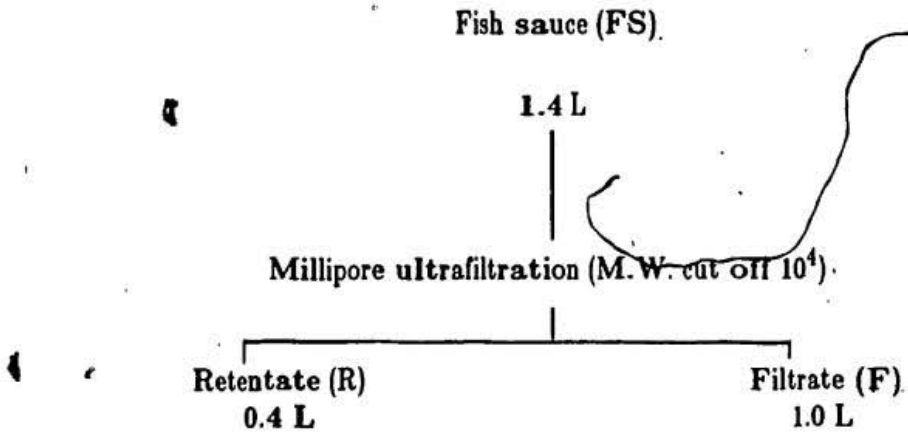
2.4.9. Separation of high molecular weight substances

A Millipore Ultrafiltration, Pellicon Cassette system with polysulfone filter of 10000 M.W. nominal exclusion limit was used to fractionate fish sauce.

Samples of fish sauce (1.4 L), control (C-FS) and squid hepatopancreas supplemented fish sauce (SQ-FS), were run through the system until 1 L of the filtrate was collected. The retentate (R) and filtrate (F) were frozen at -20°C before being used for further analyses (Figure 2-1).

2.4.10. Dialysis of fish sauce retentate

Fish sauce retentate (30 ml) was dialyzed against 4 L of distilled water or 4 L of 0.9 % saline using dialysis tubes of 6000 M.W. cut off for 72 h at 4°C with external solution changing every 24 h.



R and F were analyzed for:

1. Protease activity
2. Soluble protein
3. Amino acid composition
4. NaCl content
5. Formol N
6. Total N
7. Sensory properties and chemical analyses after aging

Figure 2-1: Flow diagram of ultrafiltration of fish sauce

2.4.11. Assay of protease activity.

2.4.11.1. Hide powder azure substrate

The assay mixture contained 5 mg hide powder azure, 1.8 ml of 0.2 M sodium acetate-HCl buffer (pH 6.0) and 0.2 ml of the enzyme solution (filtrate, retentate or fish sauce). After incubation at 30°C at different time intervals of 1-5 h, the mixtures were centrifuged at 1360 x g for 15 min in a Dynac centrifuge, (Clay Adam, New Jersey). The absorbance of the supernatant was read at 570 nm against a blank which contained only 1.8 ml acetate buffer and 0.2 ml enzyme solution.

The linearity of enzyme volume [E] vs reaction rate was carried out by incubation of hide powder azure (5 mg), 1.5 ml of 0.2 M sodium acetate-HCl buffer (pH 6.0), 0.1 - 0.5 ml of enzyme solution and 0-0.4 ml of 25% salt solution to give a final assay volume of 2 ml. After 5 h incubation at 30°C, the mixtures were centrifuged, and the absorbance of the supernatant was read at 570 nm against each blank of the same concentration of buffer and enzyme solution.

The activity of enzyme was reported as $\Delta A_{570\ nm}$/h-ml fish sauce.

2.4.11.2. Azocasein substrate

The assay mixture contained 1.0 ml of 1.5 % azocasein, 0.9 ml of 0.2 M sodium acetate-HCl buffer (pH 6.0) and 0.1 ml of enzyme solution (F, R and FS). After incubation at 30°C, at different time intervals the reaction was stopped by the addition of 0.5 ml of 20 % trichloroacetic acid (TCA). Thirty minutes after adding TCA, the mixtures were filtered through Whatman No.1 filter paper. The absorbance of the filtrate was read at 366 nm against the reading at zero time.

The enzyme concentration [E] vs reaction rate was conducted by incubating 1.0 ml of 1.5% azocasein in 0.2 M acetate-HCl buffer (pH 6.0), 0.025 - 0.3 ml of the enzyme solution, 0-0.275 ml of 25% salt solution, and 0.7 ml of distilled water to give a final assay volume of 2.0 ml. After 5 h incubation, the reaction was stopped by the addition of 0.5 ml 20 % TCA, incubated for 30 min and filtered through Whatman No.1 filter paper. The absorbance of the filtrate was read at 366 nm against a blank of the same [E] at zero time. For subsequent experiments an [E] was chosen in the linear range of [E] vs reaction rate.

The activity was reported as $\Delta A_{366\ nm}$/h-ml fish sauce.

2.4.12. Partial characterization of enzymes

2.4.12.1. Effect of pH on protease activity of fish sauce retentate

Azocasein solution, 1.5% (1.0 ml), 0.9 ml of buffer (pH 2.2 to 8.0) prepared from 0.1 M citric acid - 0.2 M Na_2HPO_4 (McIlvaine, 1921) or 0.05 M Tris-HCl buffer (pH 8.0-9.0), and 0.1 ml of the enzyme solution (fish sauce retentate) were incubated at 30°C for 5 h. The reaction was stopped by addition of 0.5 ml 20% TCA and incubated for 30 min before filtration through Whatman No. 1 filter paper. Absorbance of the filtrate was read at 366 nm against the pH 6 reaction mixture at zero time. The effect of high salt concentration on pH optimum of protease activity was carried out using azocasein substrate and McIlvaine buffer containing 4 M NaCl.

In one analysis, 1.0 ml of 2% azocasein solution was incubated with 1.0 ml McIlvaine buffer (pH 2.2-8.0), and 1.0 ml of the enzyme solution. The assay

mixture was incubated at 30°C for 16 h before the addition of 0.5 ml of 20% TCA and let stand for 30 min prior to filtration through Whatman No. 1 filter paper. The absorbance of the filtrate was read at 366 nm against a blank of pH 6 reaction mixture at zero time.

2.4.12.2. Effect of NaCl on protease activity of fish sauce retentate

The assay mixture, containing 1.0 ml of 1.5% azocasein , 1.0 ml of 0.2 M sodium acetate-HCl buffer (pH 5 or pH 6), and NaCl was added to give the final salt concentration of 1 (5.8%), 2 (11.7%), 3 (17.5%) and 4 M (23.4%) in the complete reaction mixtures, and 1.0 ml of dialyzed (M.W. cut off 6000, against distilled water for 72 h at 4°C with external solution changed every 24 h) fish sauce retentate, prepared with SHP, were incubated at 30°C for 16 h. The reaction was stopped by the addition of 0.5 ml 20% TCA, incubated for 30 min and filtered through Whatman No.1 filter paper. The absorbance of the filtrate was read at 366 nm against blank at zero time without addition of NaCl.

2.4.12.3. Effect of inhibitors on protease activity of fish sauce

Fish sauce (0.1 ml) was incubated with 0.1 or 0.2 ml of the following inhibitors: iodoacetate (1-2 mM in the final assay mixture), PCMB (1-2 mM), $HgCl_2$ (0.15-0.3 mM), EDTA (1-4 mM), SBTI (0.025 or 0.25 mg) in deionized water, PMSF (1-2 mM) in 10% 2-isopropanol. Distilled water was added to make the total volume of 0.3 ml in each tube. After incubation for 30 min, 1.0 ml of 1.5% azocasein and 0.7 ml of 0.2 M sodium acetate-HCl buffer (pH 6.0) were added. The reaction mixtures were incubated at 30°C for 5 h, then stopped by adding 0.5 ml 20% TCA and left to stand for 30 min before filtration through

Whatman No.1 filter paper. The absorbance of the filtrate was read against the reaction at zero time without inhibitor at 366 nm. The effect of inhibitors in the presence of 4 M NaCl in the final assay mixture was also conducted. After incubation of fish sauce with inhibitors, 1.0 ml of 1.5% azocasein solution and 0.7 ml of 0.2 M sodium acetate-HCl buffer containing 4 M NaCl were added. The reaction mixtures were incubated at 30°C for 5 h.

2.4.12.4. Cathepsin C activity (Dipeptidyl aminopeptidase I)

Hydrolase activity

The hydrolase activity of fish sauce and fish sauce retentate was determined using glycyl-L-arginine-4-methoxy-β-naphthylamide (Gly-Arg-MNA) as substrate (Hameed and Haard, 1985). Enzyme solution (0.1 ml) was incubated with 1.68 ml of distilled water, 0.12 ml of 10 mM Gly-Arg-MNA in dimethyl sulfoxide (DMSO), 0.5 ml of 0.2 M sodium acetate buffer (pH 6.0), and 0.6 ml of 125 mM mercaptoethanolamine-HCl. The absorbance was read at 340 nm, at 1 min intervals using a Kinetic Compuset Module attached to the DU-8 spectrophotometer at 30 ± 0.3°C. The retentate was replaced by water for the blank reading. The amount of 2-naphthylamine formed was determined from its molar extinction coefficient (1780 M) at 340 nm (Lee *et al.* 1971). The activity was expressed as nmoles 2-naphthylamine formed/min-ml fish sauce.

Transferase activity

The transferase activity of fish sauce and fish sauce retentate was determined as described by Mycek (1970), using glycyl-L-phenylalaninamide

(Gly-Phe-NH_2) as substrate. The mixture of 0.1 ml of 250 mM Gly-Phe-NH_2, 0.1 ml of 125 mM mercaptoethanolamine, 0.1 ml of 2 M hydroxylamine and 0.1 ml of distilled water was brought to a temperature of 30°C for 5 min, then 0.1 ml of the enzyme solution was added to the mixture. The reaction was stopped at 10 min intervals by adding 0.5 ml of 20% TCA and 0.5 ml of 5 % $FeCl_3$ in 0.1 N HCl. The mixture was diluted to 2 ml with distilled water and centrifuged at 15600 x g for 5 min. The absorbance of the supernatant was read at 510 nm against a blank reading at zero time. A standard curve was prepared using phenylalanine hydroxamate. The amount of dipeptide hydroxamate formed during the enzyme reaction was estimated from the standard curve. The transferase activity was expressed as the formation of 1 nmole dipeptide hydroxamate in 1 min/ml fish sauce under the condition of the assay.

Effect of NaCl on hydrolase activity

Fish sauce retentate, dialyzed against deionized water for 72 h at 4°C, M.W. cut off 6000, was incubated with hydrolase assay mixture containing glycyl-L-phenylalanine-β-naphthylamide (Gly-Phe-NA) substrate instead of Gly-Arg-MNA and mercaptoethanolamine-HCl was replaced by mercaptoethanol and 10-40 mM NaCl. The absorbance at 340 nm was read at 1 min intervals using a Kinetic Compuset Module attached to the DU-8 spectrophotometer against a blank of zero percent NaCl, and water was used to replace the retentate.

Fish sauce retentate was also incubated with hydrolase assay mixture containing Gly-Arg-MNA, mercaptoethanolamine-HCl and 5, 10, 15 and 20%

NaCl. Activities with and without NaCl were compared and calculated as % activity of the control.

Inhibition of hydrolase activity

Fish sauce retentate (0.1 ml) was incubated with hydrolase assay buffer containing the inhibitors: mercuric chloride (0.1, 0.2 mM), p-chloromercuribenzoic acid (PCMB, 1, 1.5, 2 mM), iodoacetic acid (1, 2 mM) and soybean trypsin inhibitor (SBTI 0.25, 0.5 mg). After 30 min incubation at 30°C, Gly-Arg-MNA substrate was added. The absorbance at 340 nm was immediately read using the Kinetic Compuset Module attached to the DU-8 spectrophotometer. Percent inhibition was determined by dividing the $\Delta A_{340\ nm}$/min of the enzyme preincubated with inhibitor by the $\Delta A_{340\ nm}$/min of the same amount of enzyme preincubated without inhibitor.

Aminopeptidase activity

Aminopeptidase activity was determined as described by Pfleiderer (1970), using L-leucine-p-nitroanilide (Leu-p-NA) as a substrate. The assay mixture contained 1.7 ml of 0.06 M potassium phosphate buffer (pH 7.0), or 1.7 ml of 0.2 M sodium acetate - HCl buffer (pH 6.0), 0.2 ml of 16.6 mM Leu-p-NA and 0.1 ml enzyme solution (fish sauce, retentate or filtrate). The total test volume was 2 ml. The absorbance at 405 nm was measured using a Kinetic Compuset Module attached to the DU-8 spectrophotometer. The activity was expressed as nmoles of nitroanilide (molar extinction coefficient at 405 nm = 9620) formed in 1 min/ml fish sauce.

The effect of NaCl on the aminopeptidase of fish sauce retentate was determined using 0.06 M potassium phosphate buffer (pH 7.0) containing 5, 10, 15, 20 and 25% NaCl. The activity was expressed as % of that in the absence of added NaCl.

2.4.13. Molecular size distribution of fish sauce protein and peptides

2.4.13.1. Gel filtration

Gel filtration (Bio-Gel P-2, Bio-Rad Laboratories (Canada) Ltd.) was used to analyze molecular size distribution of peptides resulting from proteolysis in fish sauce fermentation. A chromatography column, 80 x 1.5 cm (Pharmacia Fine Chemicals) was packed with deaerated Bio-Gel P-2 suspension. Fish sauce sample (0.25 ml) was applied to the column and eluted with 0.05 M HCl containing 0.1 N NaCl at a flow rate of 20 ml/h. Fractions obtained were collected by an automatic fraction collector (LKB-Redi Rac 2112) and the absorbance at 214 nm was read and recorded by an Absorbance Detector Model 441 (Waters Associates) and Servogor 120 Recorder (BBC-Georz Metrawatt), respectively. Standard compounds of known molecular weight were run to obtain the relationship between retention time and molecular weights.

2.4.13.2. HPLC

High performance liquid chromatography (HPLC) was also used to estimate the molecular size of peptides in fish sauce. A Bio-Sil TSK-125 column, 300 x 7.5 mm (Bio-Rad Laboratories Ltd.) was used with 0.1 M Na_2SO_4, 0.02 M NaH_2PO_4, (pH 6.8) buffer at a flow rate of 1.0 ml/min. Fish sauce (10 μl) sample (diluted 1:1 or 1:2 with buffer) was injected using a Model U6K Universal Liquid

Chromatograph Injector (Waters Associates). Absorbance at 214 nm was read and recorded by Absorbance Detector Model 441 (Waters Associates) and Servogor 120 Recorder (BBC-Georz Metrawath). The retention time was recorded using a Waters QA-1 Data System.

2.4.14. Total Viable Bacterial Count

Trypticase soy agar (TSA) and Trypticase soy agar with 10 % NaCl (TSA + NaCl) were used for total viable counts of fresh capelin, 24 h delayed salting fish and fish - salt mixtures (control, delayed salting and antibiotic treated mixtures). 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 the boiling temperature. After boiling for 1 min, the solutions were distributed in 150 ml amounts in 250 ml Erlenmeyer flasks, sterilized in an autoclave at 121°C for 15 min and kept at 4°C until used. Serial decimal dilution of samples were prepared according to the pour plate method using 0.1 % peptone solution with 10% NaCl. Duplicates of each dilution were incubated aerobically at 37°C for 48-72 hours after which the number of colonies formed were counted. The results were reported according to Gilliland *et al.* (1976).

2.4.15. Sensory Evaluation

Fish sauces were evaluated for preference by a panel of 5 Vietnamese - Canadians using a nine point hedonic scale and by ranking as described by Larmond (1977). Coded samples were presented to panelists in partitioned booths with standardized lighting. The judges evaluated each sample twice.

A triangle test was used to determine whether selected samples could be distinguished based on the flavor. The panelists were asked to identify the odd sample and state the degree of difference between the samples.

The results were analyzed for statistical significance by the methods described by Larmond (1977). Questionnaires for hedonic scale, ranking and triangle tests are shown in Appendix D-1 to D-3.

2.4.16. Ripening of fish sauce

Unaged fish sauce (kept frozen at -20°C) and aged fish sauce (left at ambient temperature for 4 and 6 months) were compared for browning intensity, amino acid composition and sensory evaluation.

To investigate the importance of residual enzymes in the ripening process, ultrafiltration of fish sauce was done prior to aging the filtrate. Fish sauces (control, C-FS and squid hepatopancreas supplemented, SQ-FS) were filtered through a Millipore ultrafiltration (M.W. 10^4 cut off) system until 1 L of filtrate was obtained from 1.4 L fish sauce. A portion of each retentate (R) obtained was heated in a 100°C water bath for 10 min. One part of the retentate or heated

retentate (HR) was added back to the filtrate (6 parts), mixed, covered and left at ambient temperature. After 4 months, the mixtures of the filtrate (F) and the retentate (R) were analyzed for free amino acids, total amino acids, and sensory acceptability.

2.4.17. Use of fish sauce as a flavoring agent in kamaboko

Frozen surimi was obtained from the Terra Nova Fishery Co. Ltd., Clarenville, Newfoundland. The surimi was left at 5°C until half-thawed. Salt or squid hepatopancreas supplemented fish sauce were used as flavoring agents. To inactivate residual enzymes in fish sauce, it was boiled for 5 min and cooled prior to addition to the surimi. Surimi (1 kg) was mixed with either salt (25 g), fish sauce (100 ml) or heat-treated fish sauce (100 ml), 50 g of potato starch and 100 g of ice in a Stephan UM 12 Food Mixer for 3 min with a stop at 1 min intervals. The jacketed chopping bowl was kept cool by circulation of cold water. The dough was packed into a 4.5 cm diameter glass tube and steamed for 20 min in a Rival automatic steamer or shaped into a ball of 2.5 cm and deep fat fried until golden brown. The steamed fish cake was cooled in ice water, sliced into 3 mm thick discs and subjected to a fold test (Appendix E). Both steamed and fried fish balls were subjected to sensory evaluation by eight panelists (7 N. American and 1 Ghanaian), using a triangle test and hedonic preference [illegible]re as described by Larmond (1977).

Chapter 3

Results and Discussion

3.1. Contribution of fish/squid hepatopancreas enzymes to fish sauce fermentation

3.1.1. Squid hepatopancreas enzymes

Squid hepatopancreas has been reported to aid capelin fish sauce fermentation (Raksakulthai *et al.*, 1986). The SHP-supplemented sauce was of an exceptional high quality. To test the thesis that enzymes of SHP were responsible for their role as a fermentation aid, the SHP enzymes were inactivated by cooking the SHP at 100°C for 30 min prior to the addition to the fish-salt mixture. The proteolytic activity of cooked and uncooked SHP was determined using hide powder azure substrate following the method described in 2.4.11.1. The result showed that heating the SHP at 100°C for 30 min inactivated 99.9% of its proteolytic activity. The activity ($\Delta A_{570\ nm}$/h-g) of SHP with hide powder azure substrate was 3.170 prior to heating and 0.003 after heating.

3.1.1.1. Kinetics of pH change

During fermentation, the pH of all samples dropped gradually during the first 4 weeks, slightly increased during the next 2 weeks, and then remained more or less constant for the final 18 weeks (Fig 3-1). After aging for 6 months the pH of all samples were found to be below pH 6. The pH of the SHP-supplemented mixture (B) was 0.2 unit lower than the control (A). An initial decline in pH followed by an increase in pH during fermentation has been reported by others. The initial acidification has been attributed to proteolysis and the formation of volatile and non-volatile acids (Saisithi, 1967; Orejana, 1978). The increase in pH after one month has been attributed to the formation of ammonia and amines such as di- or trimethylamine (Orejana and Liston, 1979). The increase in total volatile bases was reported until the ninth month of fermentation (Saisithi *et al.*, 1966) then decreased toward the twelfth month.

3.1.1.2. Rate of autolysis

After one month, it was observed that the texture of the SHP-supplemented sample was more liquefied than the control. The SHP-supplemented sample was orange color with an aroma reminiscent of squid hepatopancreas. The heat-treated-SHP-supplemented salted mince (C) appeared to have a finer texture than the control but was less liquefied than sample prepared with unheated SHP. The control and heat-treated-SHP-supplemented samples differed from the SHP-supplemented sample in that they were grey color and had fish aroma.

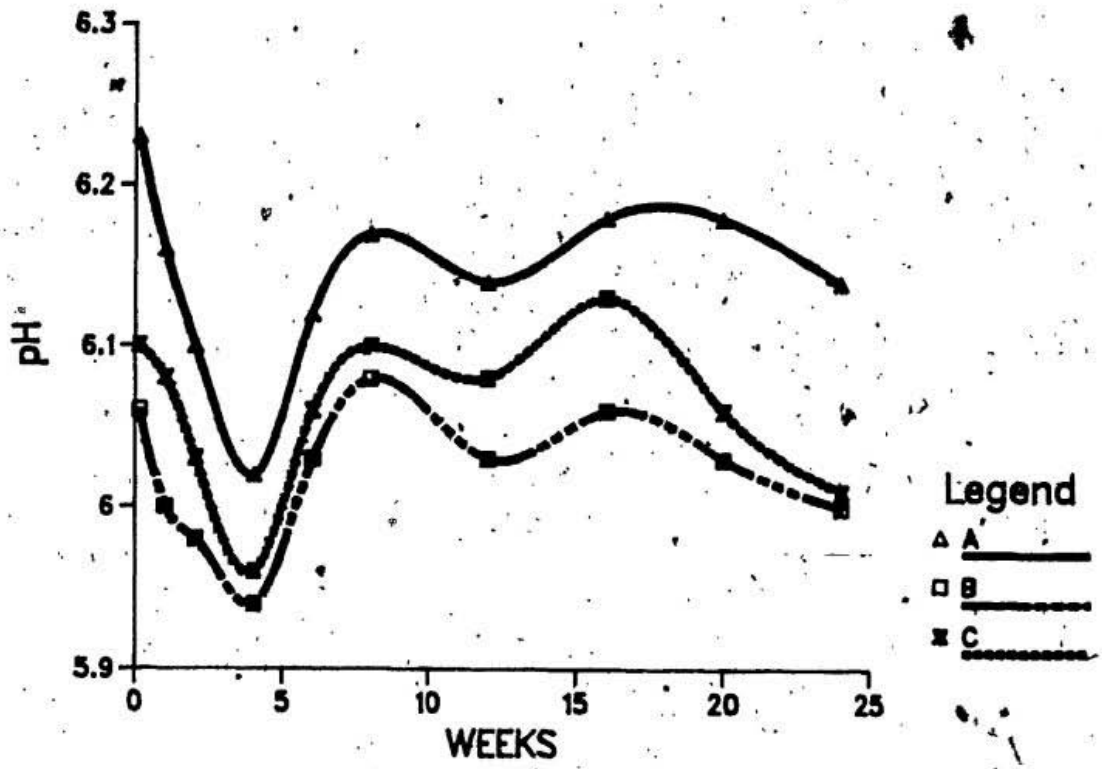


Figure 3-1: Changes in pH during fish sauce fermentation

Values plotted are averages of duplicate batches.
A = Control, B = SHP-supplemented, C = Heat-treated-SHP-supplemented

3.1.1.3. Rate of protein hydrolysis

The rates of protein hydrolysis of the fish sauce samples are shown in Fig 3-2. Analysis of variance of the rate of protein hydrolysis (DH), expressed as mg formol N/ml, during the fermentation period indicated that DH of SHP-supplemented sample (B) was much higher than in control (A), ($P<0.01$). The rate of protein hydrolysis was not significantly affected by supplementation with heat-treated SHP ($P<0.05$). This finding supports the thesis that SHP aided the fish sauce fermentation by the virtue of its proteolytic enzyme activity.

3.1.1.4. Free amino acid formation

The changes in non-amino acid ninhydrin positive components and free amino acids during fermentation are shown in Figs 3-3 and 3-4. According to some researchers, the level of amino acid nitrogen in fish sauce decreased when fermentation time was prolonged (Uyenco *et al.*, 1953; Orejána, 1978; Tongthai and Okada, 1981); however, in the fermentation of capelin fish sauce under the conditions employed, free amino acids increased throughout the period of fermentation (6 months) and aging or ripening (6 months), although the increase was slow after the fourth week. The increase in free amino acids, although at a slower rate, might be due to the remaining active enzymes in the fish-salt mixture.

The major components of non-amino acid ninhydrin reactive substances in fish sauce are summarized in Table 3-1.

At an early stage of fermentation, non-amino acid ninhydrin positive

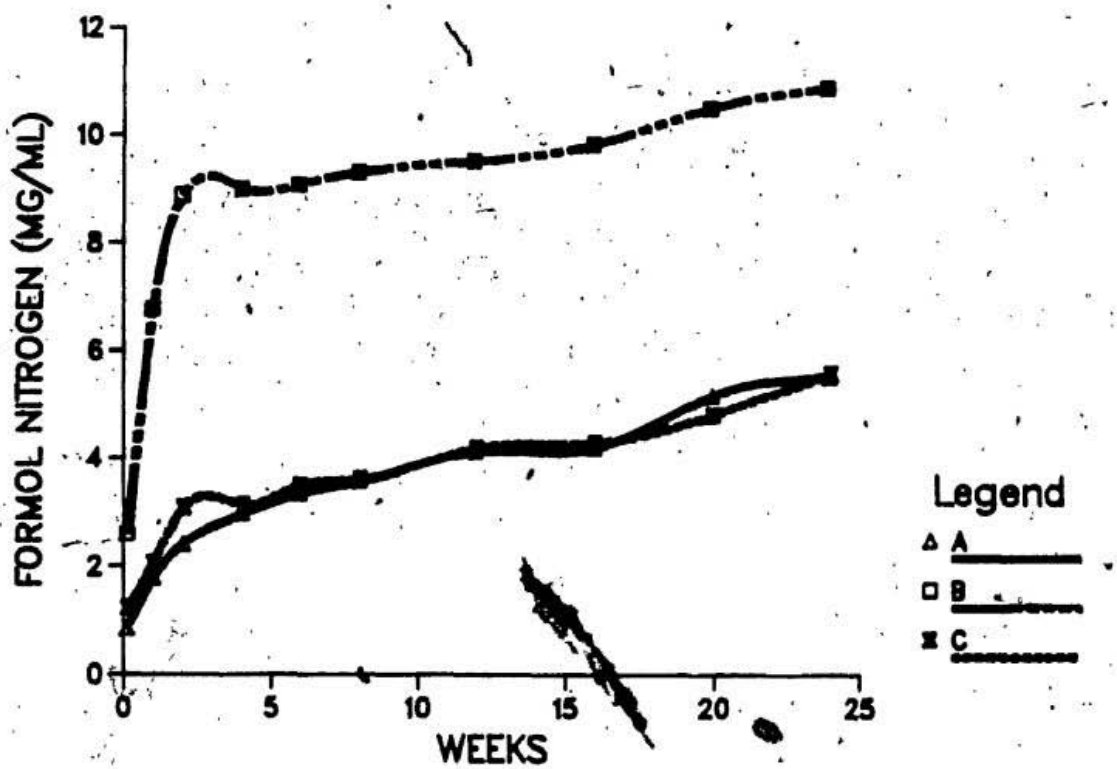


Figure 3-2: Changes in protein hydrolysis during fish sauce fermentation

Values plotted are averages of duplicate determinations for two lots of fish sauce

Protein hydrolysis calculated as mg formol nitrogen/ml fish sauce.

A = Control, B = SHP-supplemented, C = Heat-treated-SHP-supplemented

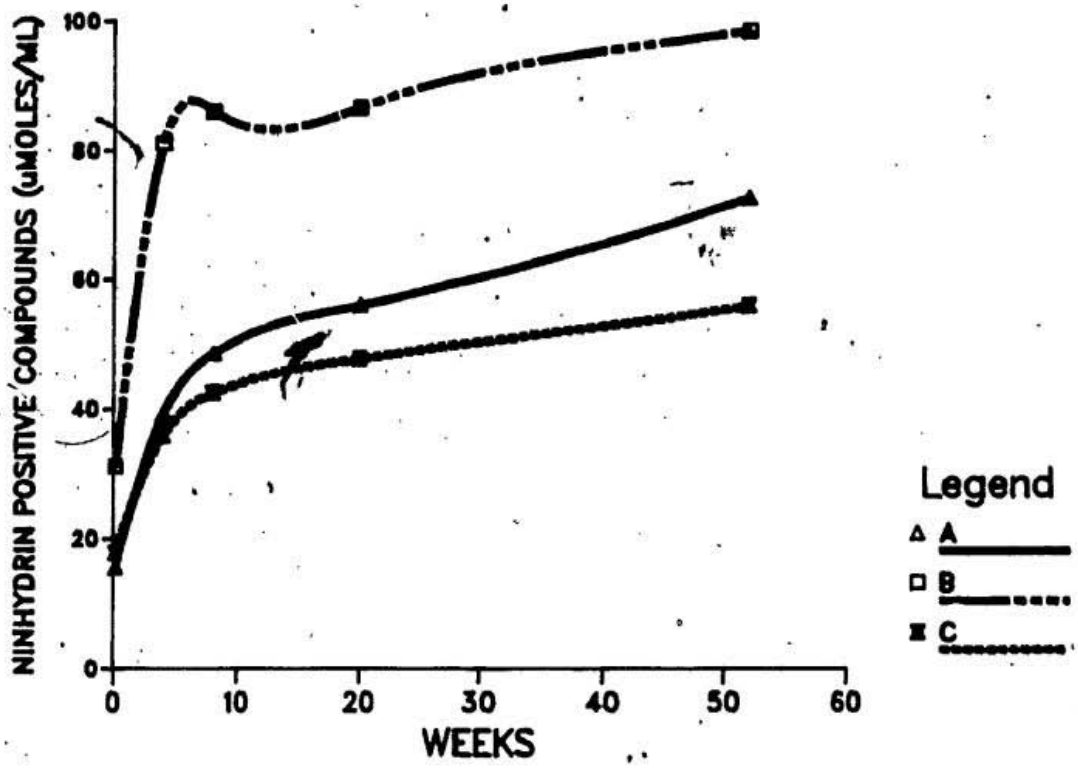


Figure 3-3: Changes in non-amino acid ninhydrin positive compounds during fish sauce fermentation

Values plotted are averages of duplicate batches
A = Control, B = SHP-supplemented, C = Heat-treated-SHP-supplemented

Compounds analyzed were glycerophosphoethanolamine, phosphoethanolamine, urea, methionine sulfoxide*, asparagine, glutamine*, sarcosine, α-amino adipic acid**, citruline*, α-amino-n-butyric acid*, homocitruline, cystathionine, γ-glutamyl-ε-lysine, β-aminoisobutyric acid, homocystine, γ-aminobutyric acid*, ethanolamine, ammonia*, ornithine*, anserine*, carnosine, glutathione, glucosamine, galactosamine.

Compounds with * were present in all samples.
Compounds with ** was present in sample B.

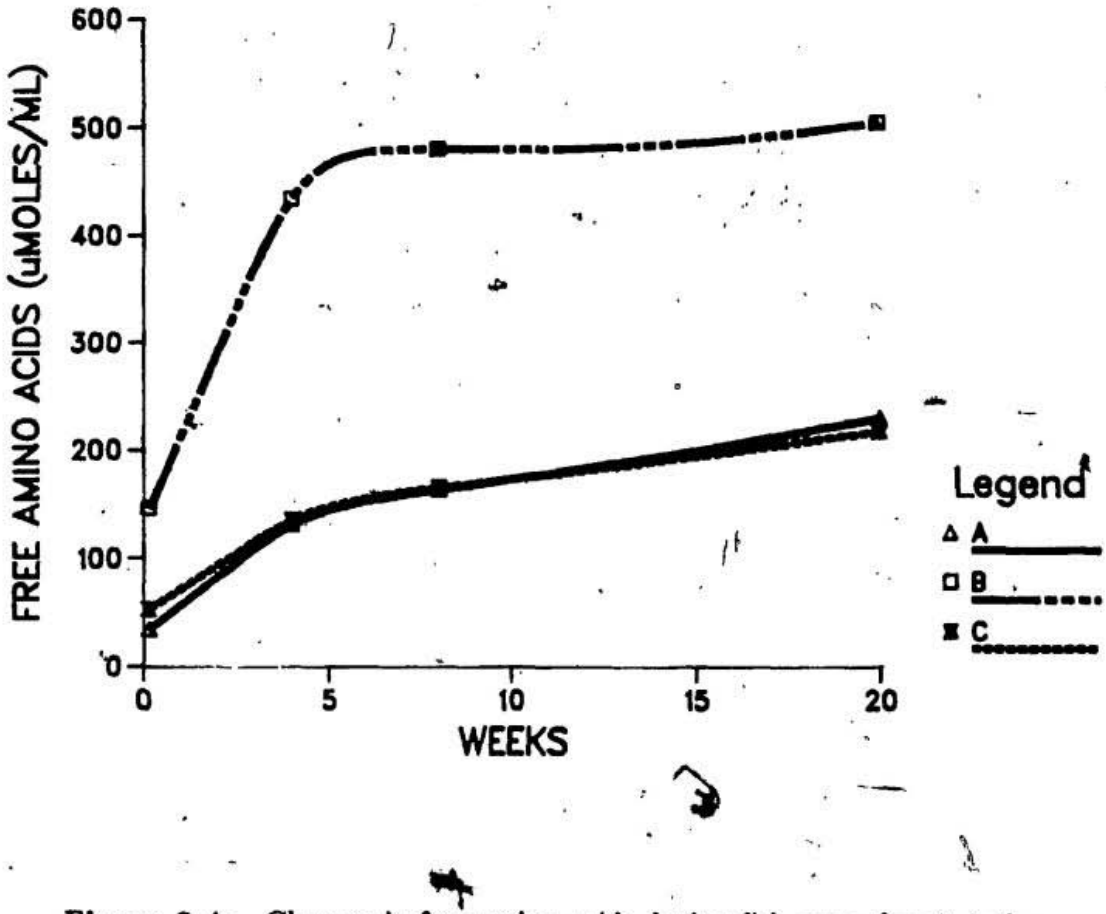


Figure 3-4: Changes in free amino acids during fish sauce fermentation

Values plotted are averages of duplicate batches
A = Control, B = SHP-supplemented, C = Heat-treated-SHP-supplemented

Table 3-1: Non-amino acid ninhydrin positive substances in fish sauce

Sample[1]	Ninhydrin positive components[2] (μmoles/ml)	Major components (mole%)
Control	72.64	NH_3(72.8) citruline (13.6) ethanolamine (9.1)
+ SHP	98.28	NH_3(77.3) citruline (12.9) ethanolamine (3.7)
+ Heat treated SHP	55.88	NH_3(77.3) citruline (12.6) ethanolamine (4.6)

[1] Samples were fermented for 6 months, aged for 6 months.

[2] Data are for duplicate analyses.
Compounds analyzed were glycerophosphoethanolamine, phosphoethanolamine, urea, methionine sulfoxide*, asparagine, glutamine*, sarcosine, α-amino adipic acid**, citruline*, α-amino-n-butyric acid*, homocitruline cystathionine, γ-glutamyl-ε-lysine, β-aminoisobutyric acid homocystine, γ-aminobutyric acid*, ethanolamine, ammonia*, ornithine*, anserine*, carnosine, glutathione, glucosamine, galactosamine.

Compounds with * were present in all samples.
Compound with ** was present in sample B.

compounds in fish sauce increased at a fast rate. After 8 weeks, the increased rate declined. After 1 year, the non-amino acid fractions of control, SHP-supplemented and heat-treated-SHP-supplemented samples were, respectively, 23, 16 and 20% of the total ninhydrin positive compounds in fish sauce samples. Ammonia was predominant in this fraction and accounted for 73-77% of the total non-amino acid ninhydrin positive compounds. Ammonia and other volatile bases were reported to contribute to the ammonia-like aroma (Dougan and Howard, 1975). Citruline and ethanolamine were the other major components and accounted for 23, 17 and 17% of the total non amino acid fraction in samples A, B and C, respectively.

The free amino acid content of the fermentation brine increased rapidly from the start to the fourth week. From the fourth week to the end of fermentation, it increased gradually (Fig 3-4). Statistical analysis indicated that the increase of free amino acids in SHP-supplemented sauce was much higher than the control as well as the heat-treated-SHP-supplemented samples ($P<0.01$). The increase in free amino acids in control and heat-treated-SHP-supplemented samples was not significantly different ($P<0.05$). After one year, the free amino acid content of SHP-supplemented sauce (B) was 2.5-fold higher than the control (A). The heat-treated-SHP-supplemented sauce (C) and the control had approximately the same content of free amino acid. The amino acid compositions of the fish sauces, after fermentation for 6 months and storage at ambient temperature for 6 months, are shown in Fig 3-5 and Appendix Table A-1. Comparison of amino acid compositions between control and SHP-supplemented

sauce, and control and heat-treated-SHP-supplemented sauce are shown in Appendix Figs A-1 and A-2, respectively. The major free amino acids in the fish sauce accounted for 62-64% of the total free amino acids. The distribution of the major free amino acids is summarized in Table 3-2. Acidic amino acid fraction was found at highest % composition (21 mole%) in the sample prepared with SHP, compared to 16 and 17 mole% in control and heat-treated-SHP-supplemented samples.

3.1.1.5. Quantitative changes of free amino acids during fermentation of fish sauce

Free amino acid compositions during fermentation of fish sauces, as mole percent, are shown in Tables 3-3 and 3-4. The concentrations of free amino acids, as μmoles/ml fish sauce are shown in Appendix Tables A-2 and A-3. Among the major free amino acid components of the control sample, alanine was readily found at a high ratio in the first-day brine and the ratio of alanine to the total free amino acids remained similar throughout the fermentation. The mole percentages of aspartic acid, leucine and lysine increased greatly while those of glutamic acid and glycine only slightly increased. The % composition of histidine and arginine slightly decreased. Taurine was found at the highest % composition in the first-day brine (37 mole%). However, at the beginning, the concentration of taurine in the brine increased slightly, then slightly decreased after 4 weeks of fermentation. In SHP-supplemented sample, the ratio of alanine, glutamic acid, glycine, leucine and lysine to the total free amino acids appeared to be the same throughout the whole period of fermentation. A great increase was found in %

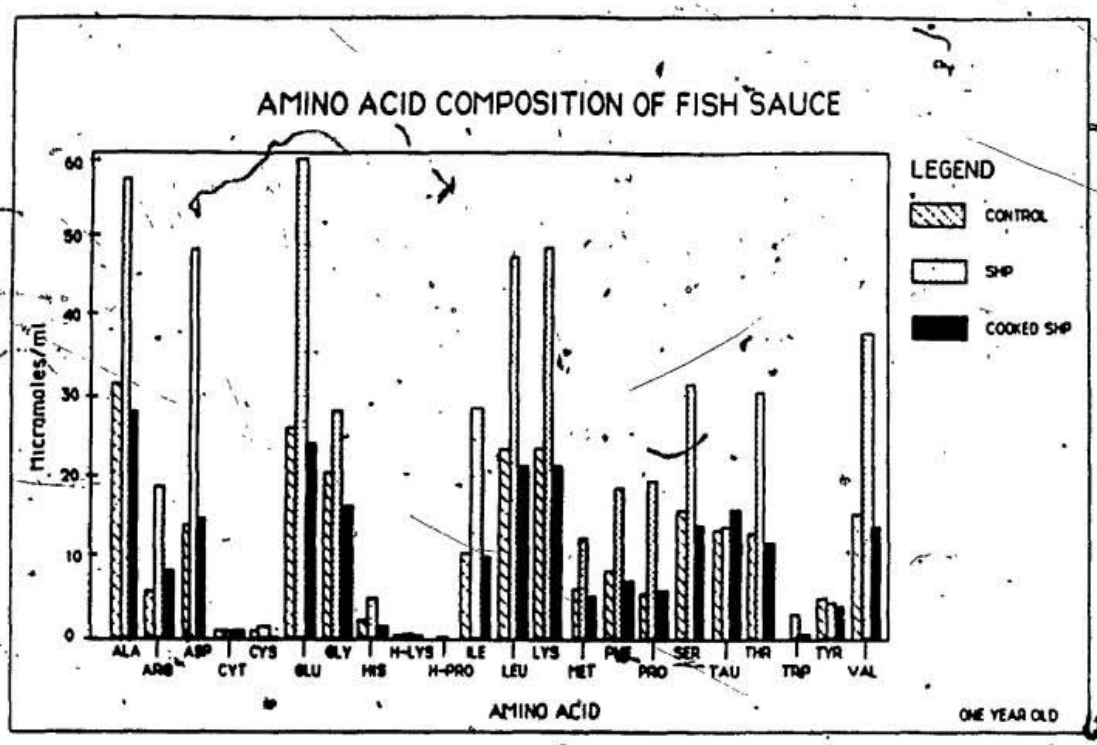


Figure 3-5: Free amino acid concentrations of fish sauce

Values plotted are averages of duplicate batches.
Samples were fermented for 6 months, aged for 6 months.

Table 3-2: Major free amino acids in fish sauce samples

Free amino acid	mole%[1] Sample[2] A	B	C
Ala	13.0	11.0	12.4
Asp	5.8	9.4	6.5
Glu	10.7	11.6	10.9
Gly	8.5	5.5	7.3
Leu	9.6	9.2	9.5
Lys	9.7	9.4	9.6
Val	6.4	7.3	6.2

[1] Values are averages of duplicate batches.

[2] Samples were fermented for 6 months, aged for 6 months.
A=Control; B=SHP-supplemented; C=Heat-treated-SHP-supplemented.

Table 3-3: Free amino acid composition of control fish sauce during fermentation

Amino acid	Mole%[1] Time (weeks) 1 d	4 wk	8 wk	20 wk	52 wk
Ala	12.2	12.9	12.3	12.6	13.0
Arg	2.9	3.4	2.9	2.8	2.3
Asp	1.8	4.6	5.5	6.2	5.8
Cyst acid	0.2	0.2	0.4	0.3	0.3
Cys	0	0.2	0	0	0.3
Glu	8.0	9.3	9.0	10.6	10.7
Gly	6.8	8.0	8.6	8.4	8.5
His	1.3	1.2	1.1	0.4	0.8
HLys	0	0.1	0.1	0.1	0.1
HPro	0	0	0.2	0.1	0
Ile	2.1	3.5	3.9	3.9	4.5
Leu	4.1	9.3	8.4	9.9	9.6
Lys	4.8	8.3	8.9	9.5	9.7
Met	1.6	3.6	3.3	3.6	2.6
Phe	1.6	2.6	3.0	3.2	3.4
Pro	2.2	1.4	1.8	1.4	2.2
Ser	4.3	6.4	6.7	6.7	6.6
Tau	37.0	12.3	8.7	6.7	5.8
Thr	3.2	4.2	4.5	4.7	5.4
Trp	0.5	0.3	0.2	0.1	0
Tyr	1.2	2.0	2.1	2.1	2.0
Val	4.2	6.0	6.4	6.6	6.4

[1] Values are averages of duplicate batches.

Table 3-4: Free amino acid composition of SHP-supplemented fish sauce during fermentation

Amino acid	Mole%[1] Time (weeks) 1 d	4 wk	8 wk	20 wk	52 wk
Ala	12.3	10.8	10.8	10.9	11.0
Arg	4.5	3.4	3.8	3.4	2.6
Asp	1.1	10.8	9.6	10.1	9.4
Cyst acid	0.2	0.1	0.1	0.1	0.1
Cys	0.2	0.6	0.7	0.3	0.3
Glu	11.5	11.2	11.4	11.5	11.6
Gly	4.7	4.7	5.1	5.5	5.5
His	1.6	1.2	1.2	0.8	0.9
HLys	0	0	0.2	0.1	0.1
HPro	0	0	0.3	0.2	0.2
Ile	4.6	5.4	5.3	5.3	5.5
Leu	10.6	11.0	10.6	10.6	9.2
Lys	8.4	8.8	9.1	9.2	9.4
Met	3.4	3.6	3.5	3.4	2.4
Phe	3.5	3.6	3.7	3.6	3.6
Pro	1.7	1.8	2.4	2.9	3.8
Ser	6.3	5.4	5.6	5.9	6.1
Tau	9.7	4.1	3.0	2.9	2.7
Thr	4.9	4.9	5.1	5.3	6.0
Trp	0.5	0.4	0.3	0.2	0.6
Tyr	2.7	1.0	0.9	0.6	0.8
Val	7.5	7.0	7.3	7.2	7.3

[1] Values are averages of duplicate batches.

composition of aspartic acid and proline. Taurine was found to decrease from 9.7 to 2.7 mole%, but the concentration of taurine increased slightly during 1-4 weeks of fermentation then slightly dropped after the fourth week. The mole % of arginine, histidine and methionine decreased slightly.

Lee *et al.* (1981; 1982a) suggested that the taste compounds in fermented sardine and anchovy were mainly free amino acids and nucleotides. Chung and Lee (1976) concluded that the most important taste compounds of fermented shrimp were free amino acids such as lysine, proline, alanine, glycine, serine, glutamic acid and leucine. The taste-active amino acids in fermented squid were reported to be glutamic acid, alanine, leucine, serine, lysine, arginine and proline (Lee *et al.*, 1982b). All of these amino acids were abundant in the fish sauce especially the SHP-supplemented sauce. Chayovan *et al.* (1983b) stated that it was most likely that free amino acids contributed to the taste of fish sauce.

3.1.1.6. Total amino acids

The concentrations of acid hydrolyzate amino acids in fish sauce are shown in Appendix Table A-1. The major amino acid residues in fish sauce are shown in Table 3-5. The major amino acid residues from acid hydrolyzates of all samples were alanine, aspartic acid, glutamic acid, glycine, leucine and lysine but the amino acid residues that accounted for the peptide/protein fractions were aspartic acid, glutamic acid, glycine, proline and serine. These results indicate that in the fermentation process, the residual peptides in fish sauce are rich in aspartic acid, glutamic acid, glycine, proline and serine.

Table 3-5: Major peptide/protein amino acids in fish sauce

Amino acid	mole%[1] Sample[2] A	B	C
Ala	6.5	5.6	8.0
Asp	14.2	11.1	11.8
Glu	21.5	18.6	18.9
Gly	17.9	25.1	17.7
Lys	5.9	4.9	6.7
Pro	9.3	8.3	7.1
Ser	6.2	6.1	6.3

[1] Values are averages of duplicate batches. Peptide/protein amino acid was determined by subtracting free amino acids from total hydrolyzate amino acids.

[2] Samples were fermented for 6 months, aged for 6 months. A=Control; B=SHP-supplemented; C=Heat-treated-SHP-supplemented.

Acidic oligopeptides having flavor potentiation in fish protein hydrolyzate were analyzed by Noguchi *et al.* (1975). The peptides containing high molecular ratios of glutamic acid residue were found to have a flavor activity qualitatively resembling that of monosodium glutamate (MSG). These peptides were Gly-Asp-Glu, Glu-Asp, Asp-Glu-Ser, Thr-Glu, Glu-Gly-Ser, Glu-Ser, Glu-Glu, Ser-Glu-Glu, Glu-Gln-Glu. Since the amino acid residues of fish sauce contained a very high proportion of glutamic acid, peptides containing glutamic acid and/or the high level of free glutamate may contribute to the typical or desired taste of the squid hepatopancreas-supplemented sauce.

For fish sauce which was fermented for 6 months and aged for 6 months, the free amino acids in the control, the SHP-supplemented and the heat-treated-SHP-supplemented were 63, 76 and 54% of the total amino acid residues, respectively.

3.1.1.7. Color of fish sauce

Color is one of the criteria for judging the quality of fish sauce, however, color is a subjective quality which is dependent upon individual preference. The standard for Thai local fish sauce (Anon., 1983) requires a reddish-brown hue for the full sensory score of 10.

The brine became darker as the fermentation period was prolonged. Color of samples prepared with SHP measured by absorbance at 400 nm appeared to be darker than control sauce (Table 3-6). The brown color of the finished products, as judged by the panelists, were SHP-supplemented > heat-treated-SHP-supplemented > control. The absorbance at 400 nm showed that SHP-

supplemented > heat-treated-SHP-supplemented > control for 16-week old products and this was similar to L value and the total color difference (E) for 12-month old sauces (Table 3-7).

Saisithi (1967) concluded that the brown color of fish sauce was caused by non-enzymatic Maillard type reactions involving ribose and amino acids. From model systems of amino acids and ribose, it was found that lysine, 1-methyl histidine, taurine and β-alanine are actively involved in the non-enzymatic browning reaction, but Orejana (1978) suggested that carbonyl compounds derived from lipids are more likely to contribute to browning in fish sauce than sugar. Oxypolymerization of the lipid components could be another possible mechanism of browning in fish sauce.

The SHP-supplemented sauce contained larger quantities of amino acid residues which are active precursors of Maillard browning. Hence the darker color of SHP-supplemented sauce could be reasonably explained on the basis of its amino acid content. Besides, the squid hepatopancreas was reported to contain 22% lipid with 3.7% unsaponified matter, and iodine value of 190 (Jangaard and Ackman, 1965). Oxidation products of lipid have been known to react in the browning reactions. Fish sauce prepared with heat-treated SHP, contained approximately the same concentration of free amino acid as control fish sauce, but having different lipid content, appeared to exhibit darker color than control sample. However, statistical analysis indicated that the absorbance at 400 nm of control and heat-treated-SHP-supplemented samples was not significantly

Table 3-6: Browning development in fish sauce prepared with and without SHP

Sample[2]	Absorbance (400 nm)[1] Time (Weeks)					
	2	4	6	8	12	16
A	0.50	0.67	0.83	1.03	1.27	1.43
B	1.11	1.74	2.11	2.39	2.89	3.00
C	0.78	1.01	1.11	1.30	1.63	1.65

[1] Values are averages of duplicate batches.
[2] A = Control; B = SHP-supplemented; C = Heat-treated-SHP-supplemented.

Table 3-7: Difference in tristimulus color of fish sauce prepared with and without SHP

Sample[1]	L[2]	a[2]	b[2]	E[3]
A	31.4	8.6	20.3	64.4
B	18.8	17.8	12.3	76.5
C	26.2	13.6	17.0	69.4

[1] Samples were fermented for 6 months, aged for 6 months.
A = Control; B = SHP-supplemented; C = Heat-treated-SHP-supplemented.
[2] Values are averages of duplicate batches.
[3] Values calculated from average values, compared to the white plate standard.

different, while that of the SHP-supplemented sample was significantly higher than that of the control and the heat-treated-SHP-supplemented samples ($P<0.01$).

3.1.1.8. Effect of acidification on SHP-supplemented fermentation

SHP was reported to be a rich source of acid proteases, for example, cathepsins B, D and E (LeBlanc and Gill, 1982). To test the contribution of these acid proteases to fish sauce fermentation, the fish-salt mixture supplemented with SHP was acidified to pH 4.5. The changes in pH during fermentation of samples supplemented with SHP at natural pH and at pH 4.5 are shown in Fig 3-6. The pH of salt-mince mixture initially adjusted to pH 4.5 showed a similar pattern of pH change as did the sample fermented at the natural pH of fermentation.

The rates of protein hydrolysis during fermentation of fish sauce supplemented with SHP at pH 4.5 and 6.0 are shown in Fig 3-7. Acidified fermentation mixtures (pH 4.5) containing SHP exhibited a lower rate of hydrolysis than did SHP-supplemented sample fermented at natural pH. This result differs from other studies (Beddows and Ardeshir, 1979b; Gildberg *et al.*, 1984) where endogenous proteolysis is normally accelerated by acidification. It appears that SHP is a rich source of salt tolerant enzymes which are more active at a pH around 6 than at pH 4.5 of the fermentation. The pH optimum for the hydrolase activity of cathepsin C from squid hepatopancreas is at pH around 6 (Hameed and Haard, 1985).

The free amino acids formed in the SHP-supplemented sauce fermented at

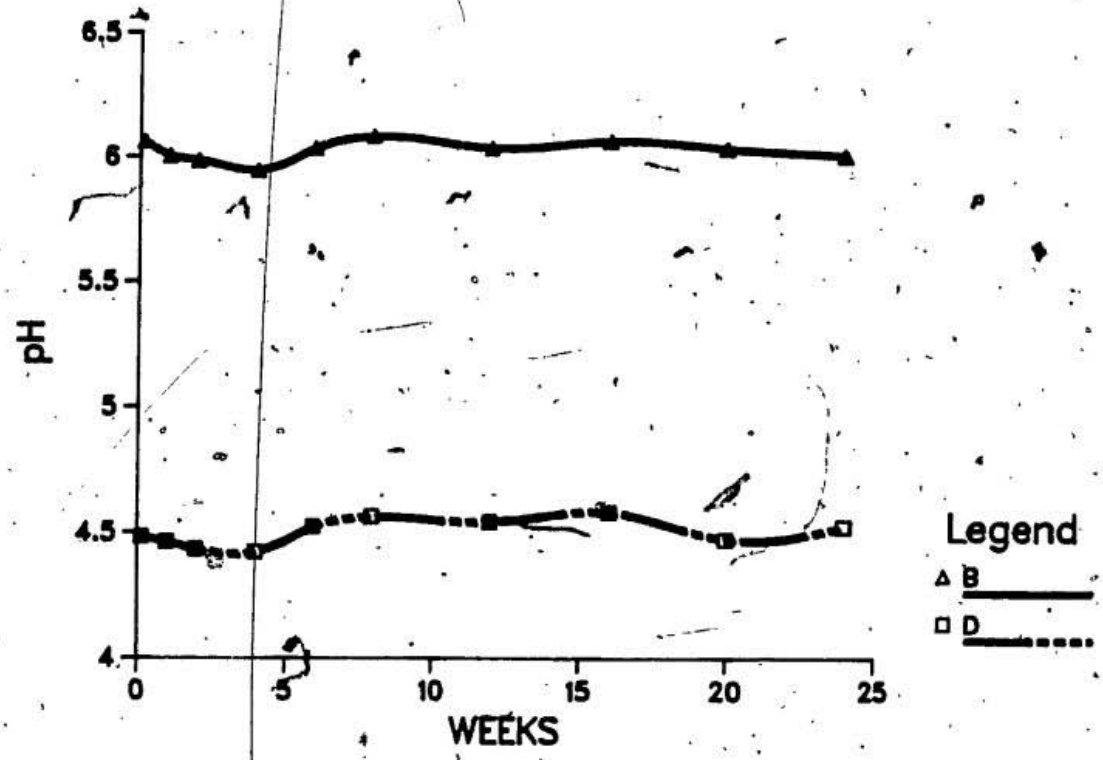


Figure 3-6: Changes in pH during fermentation of fish sauce

Values plotted are averages of duplicate batches
B = SHP-supplemented, D = SHP-supplemented (pH 4.5)

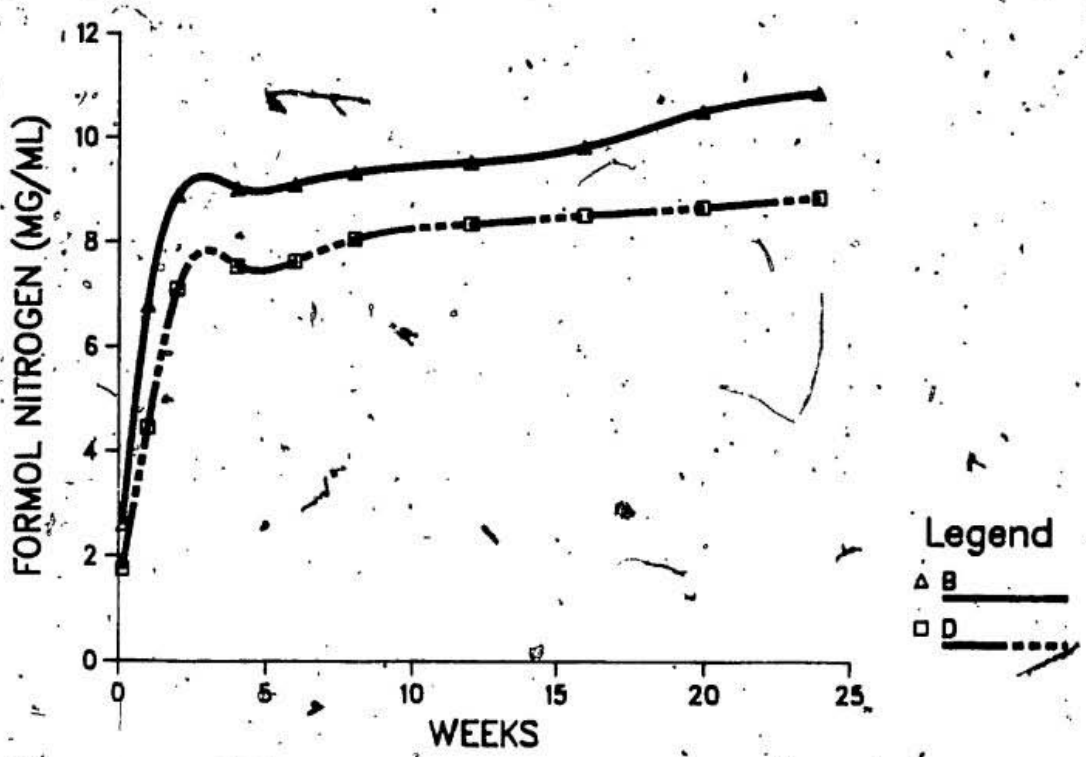


Figure 3-7: Changes in the protein hydrolysis during fermentation of fish sauce

Values plotted are averages of duplicate determinations for two lots of fish sauce.
B = SHP-supplemented (pH 6), D = SHP-supplemented (pH 4.5)

natural pH and at pH 4.5 are shown in Fig 3-8. The free amino acid compositions of 1-year old fish sauces fermented at natural pH and pH 4.5 are compared in Fig 3-9. A notable difference between these samples is the higher content of acidic residues, glutamic acid and aspartic acid, in sauce prepared with SHP at its natural pH. Also, only a trace of arginine was found in sample fermented at pH 4.5.

The change in brown color of an acidified fermentation mixture containing SHP is shown in Table 3-8. The tristimulus color values of the final product after 6 months aging are shown in Table 3-9. Although sample prepared with SHP, fermented at pH 4.5, contained more free amino acids, which are precursors of the Maillard reaction, than control and heat-treated-SHP-supplemented samples, it was lighter in color. Non-enzymatic Maillard browning reaction is generally favored at the more alkaline conditions, thus the lighter color of sauce at pH 4.5 may be due to the acidic environment.

3.1.1.9. Effect of concentration of squid hepatopancreas

The effects of concentration of SHP on the rate of protein hydrolysis and free amino acid formation during the fermentation of fish sauce are shown in Figs 3-10 and 3-11, respectively. The changes in pH, the increase in browning intensity during the fermentation and the differences in tristimulus color values of the finished products are shown in Tables 3-10, 3-11 and 3-12, respectively.

The rates of protein hydrolysis and free amino acid formation were higher at the higher concentration of SHP. Statistical analysis indicated that there was

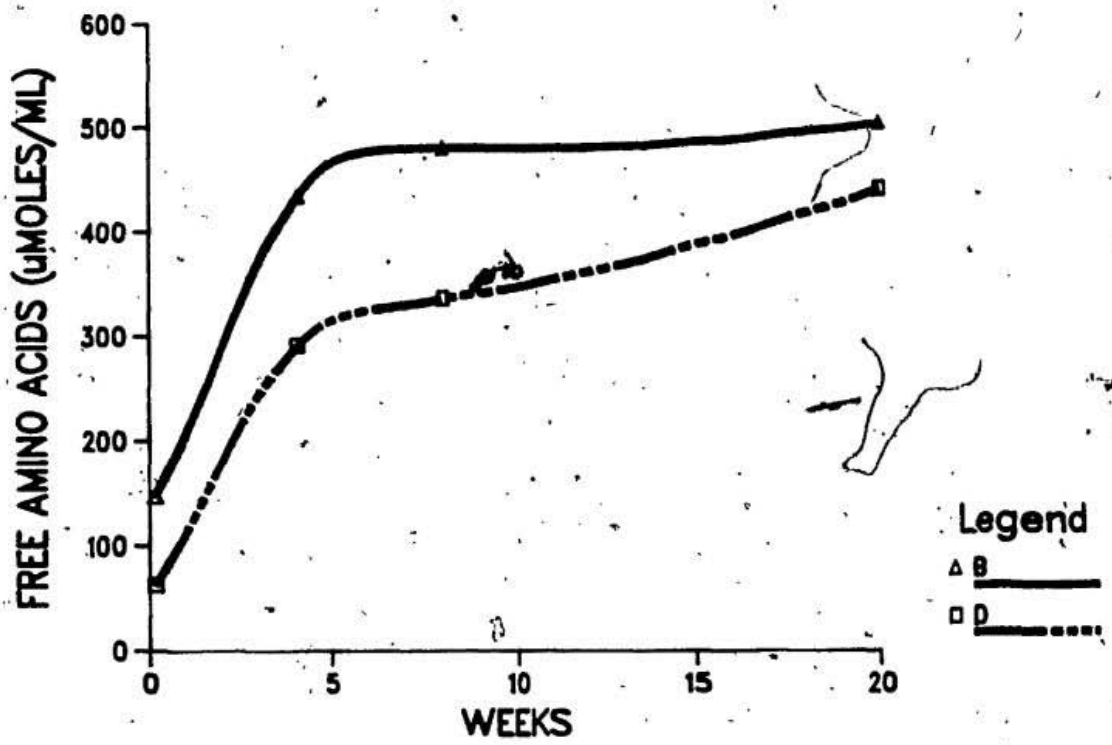


Figure 3-8: Free amino acid formation during fermentation of fish sauce

Values plotted are averages of duplicate batches.
B = SHP-supplemented (pH 6), D = SHP-supplemented (pH 4.5)

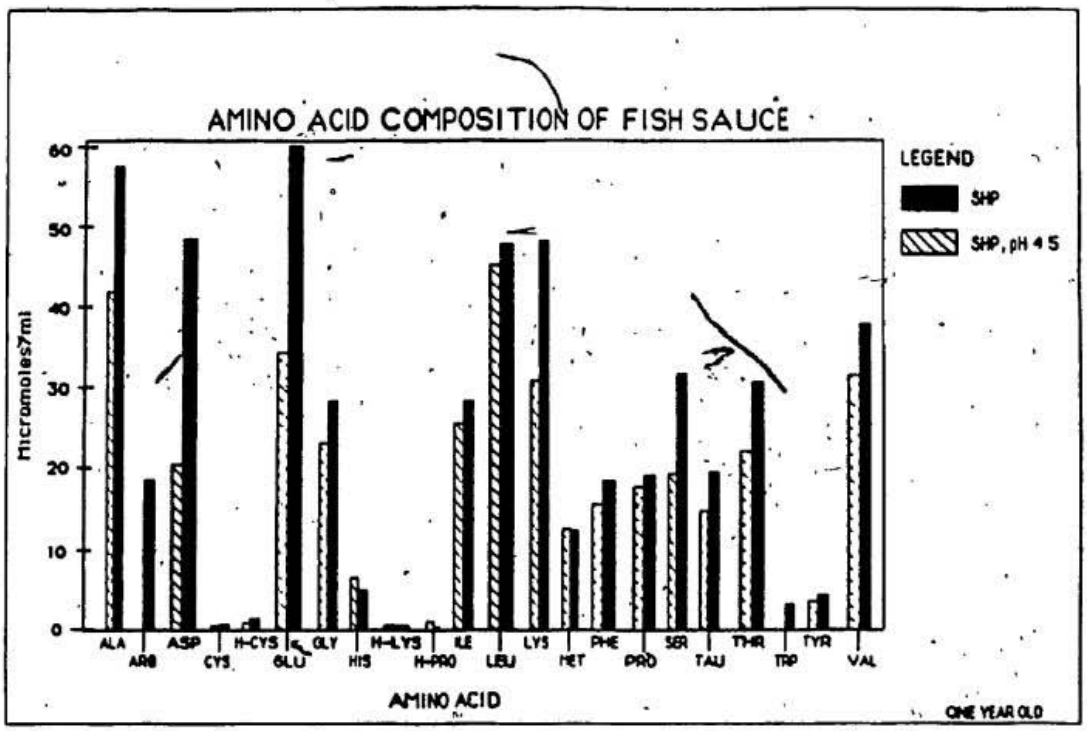


Figure 3-9: Free amino acid concentrations of SHP-supplemented fish sauce fermented at different pH

Values plotted are averages of duplicate batches
Samples were fermented for 6months and aged for 6 months

Table 3-8: Browning development in fish sauce prepared at natural pH and at pH 4.5

Sample[2]	Absorbance (400 nm)[1] Time (Weeks)					
	2	4	6	8	12	16
B	1.11	1.74	2.11	2.39	2.89	3.00
D	0.38	0.66	0.72	0.94	1.23	1.27

[1] Values are averages of duplicate batches.
[2] B = SHP-supplemented (pH 6); D = SHP-supplemented (pH 4.5).

Table 3-9: Tristimulus color of fish sauce prepared at natural pH and at pH 4.5

Sample[1]	L[2]	a[2]	b[2]	E[3]
B	18.8	17.8	12.3	76.5
D	30.5	12.2	20.4	65.9

[1] Samples were fermented for 6 months, aged for 6 months.
B = SHP-supplemented (pH 6); D = SHP-supplemented (pH 4.5).
[2] Values are averages of duplicate batches.
[3] Values calculated from average values.

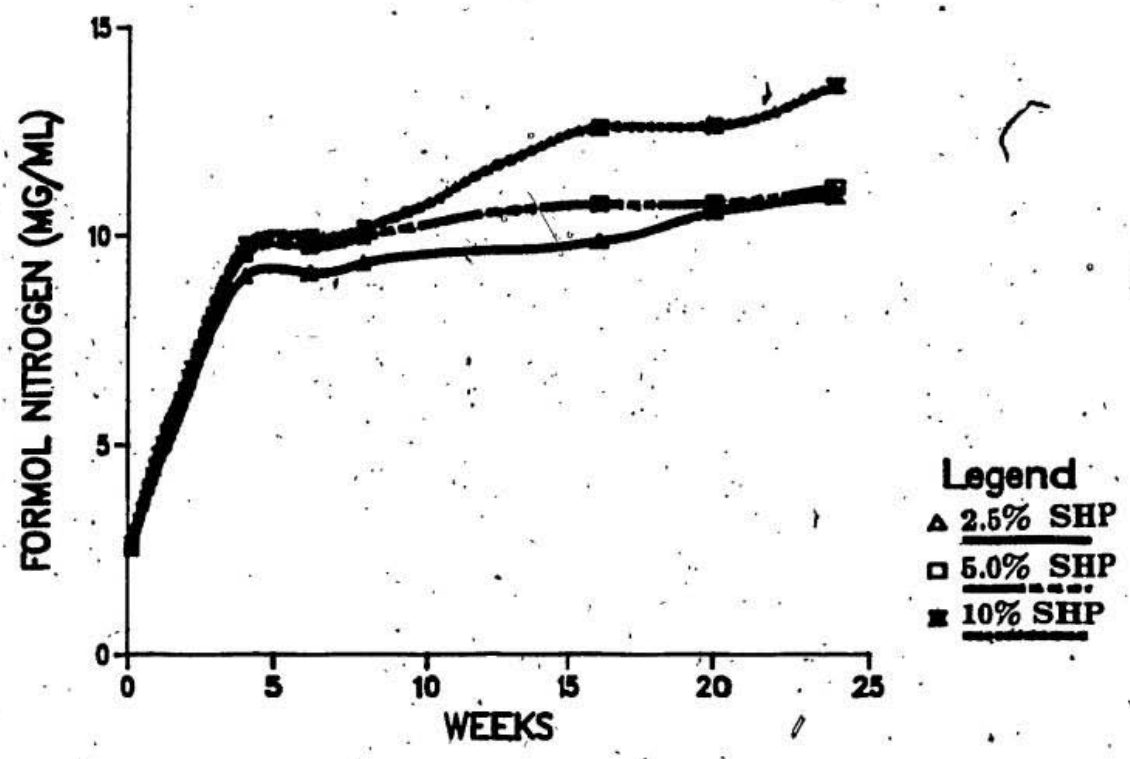


Figure 3-10: Effect of SHP concentration on protein hydrolysis during fish sauce fermentation

Values plotted are averages of duplicate determinations for two lots of fish sauce.

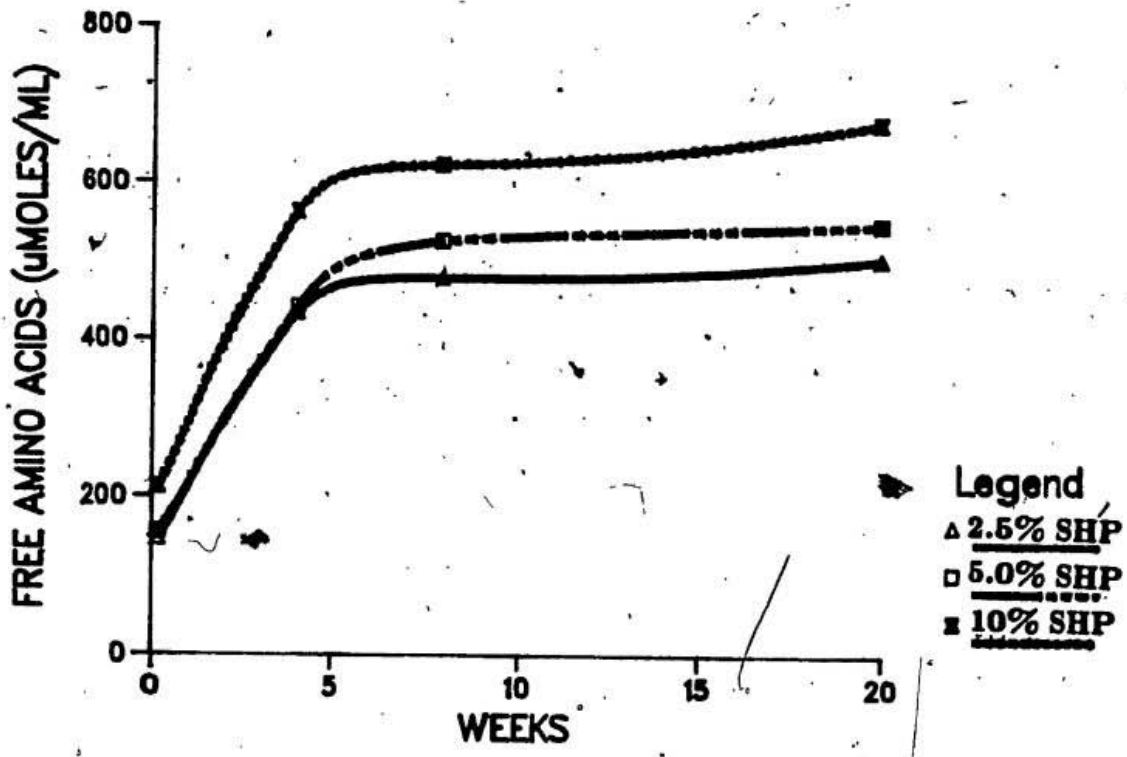


Figure 3-11: Effect of SHP concentration on free amino acid formation during fish sauce fermentation

Values plotted are averages of duplicate batches.

no significant difference in protein hydrolysis of the samples supplemented with 5 and 10% w/w of SHP. Protein hydrolysis in 2.5% SHP supplementation was lower than that of 5 and 10% SHP ($P<0.05$). All samples exhibited the same pattern of pH change. The absorbance at 400 nm was higher in samples with 5 and 10% SHP (Table 3-11). After 2 weeks of fermentation, the sample prepared with 10% SHP exhibited a very dark color. Statistical analysis indicated that the absorbance at 400 nm of sample supplemented with 10% SHP was significantly higher than that of 2.5 and 5% supplemented samples ($P<0.01$). The values for 2.5% SHP-supplemented samples were not significantly different from 5% SHP supplement. An informal sensory evaluation for fish sauce prepared with different concentrations of SHP was conducted, the result showed that higher concentration of SHP did not improve the sensory score. The sample with 10% SHP supplement appeared to have a strong flavor of the hepatopancreas. Therefore, it was concluded that there is no advantage to use more than 2.5% of SHP as a fermentation aid.

3.1.1.10. Sensory evaluation

The results of sensory analyses of control, SHP-supplemented, heat-treated-SHP-supplemented, SHP-supplemented (pH 4.5) and commercial fish sauce are summarized in Table 3-13. A triangle test between the control (A) and the squid hepatopancreas-supplemented (B) sauces revealed that the two samples were significantly different ($P<0.001$). All eight panelists could identify the odd sample and all stated that they preferred SHP-supplemented sauce to control sauce.

Table 3-10: Effect of SHP concentration on pH of fish sauce during fermentation

Sample	pH[1] Time (Weeks)					
	1d	4	8	12	16	20
2.5% SHP	6.06	5.94	6.08	6.03	6.06	6.03
5% SHP	6.07	5.96	6.04	6.04	5.94	5.94
10% SHP	5.93	5.90	6.04	5.93	6.00	5.92

[1] Values are averages of duplicate batches.

Table 3-11: Effect of SHP concentration on browning intensity of fish sauce during fermentation

Sample	Absorbance (400 nm)[1] Time (Weeks)				
	2	4	6	8	12
2.5% SHP	1.11	1.74	2.11	2.39	2.89
5% SHP	1.39	1.69	2.36	2.53	3.03
10% SHP	2.10	2.63	3.03	3.19	3.57

[1] Values are averages of duplicate batches.

Table 3-12: Effect of SHP concentration on tristimulus color of fish sauce (Hunter-Gardner Colorimeter)

Sample[1]	L[2]	a[2]	b[2]	E[3]
2.5% SHP	18.8	17.8	12.3	76.5
5% SHP	14.2	17.6	6.4	80.2
10% SHP	8.4	12.5	2.7	84.7

[1] Samples were fermented for 6 months, aged for 6 months.
[2] Values are averages of duplicate batches.
[3] Values calculated from average values.

Sensory evaluation using hedonic scale showed that SHP-supplemented sauce was preferred to control, heat-treated-SHP-supplemented and SHP-supplemented (pH 4.5) sauces ($P<0.05$). The result was confirmed by the ranking test where SHP-supplemented sauce was significantly ranked higher than control, heat-treated-SHP-supplemented, SHP-supplemented (pH 4.5) and commercial sauce ($P<0.05$).

Fish sauce prepared with SHP at pH 4.5 got the lowest preference score and the highest total rank sum when compared to control, SHP-supplemented, heat-treated-SHP-supplemented and commercial sauce (Table 3-13). The preference scores for control, heat-treated-SHP-supplemented and SHP-supplemented (pH 4.5) were not significantly different ($P<0.05$), but from the ranking, the sample

Table 3-13: Sensory evaluation score of capelin and commercial fish sauce

Sample[1]	Total rank sum[2]	Preference score[3]
SHP (B)	6 a	8.3 a
Commercial	11 b	5.8 b
Control (A)	18 b	5.3 b
Heat-treated SHP (C)	17 b	5.3 b
SHP, pH 4.5 (D)	23 c	4.8 b

[1] Laboratory samples were fermented for 6 months, aged for 6 months.

[2] Values followed by the same letter are not significantly different ($P<0.05$), $n = 5$.

[3] Values followed by the same letter are not significantly different ($P<0.05$), $n = 6$.

prepared with SHP (pH 4.5) was significantly inferior to the other samples ($P<0.05$).

To compare the flavor of SHP-supplemented fish sauce, when its amino acid content was adjusted to approximately the same concentration as control fish sauce, SHP-supplemented fish sauce was diluted 1 to 1 with 25% NaCl solution (Dil B), then subjected to triangle test, ranking and preference test comparing with control, SHP-supplemented and commercial fish sauce (Rufina).

The results of this sensory evaluation are summarized in Table 3-14. There were significant differences between samples B and Dil B as well as control and Dil B ($P<0.01$ and 0.05, respectively). The panelists stated that B was preferred to Dil B. From the triangle test, it was not clear whether Dil B or control samples was more preferable (3 preferred A, 3 preferred Dil B), but the result from ranking and preference test showed that Dil B had a lower rank sum and higher preference score than A, although it was not statistically significant. However, this result may indicate that amino acid content alone was not responsible for the flavor of fish sauce since Dil B was still preferred to control fish sauce when amino acid content of the two samples were approximately the same.

3.1.1.11. Composition of fish sauce

The salt concentration, pH, Biuret soluble protein, free amino acid nitrogen and total nitrogen concentration of the control (A), SHP-supplemented (B), heat-treated-SHP-supplemented (C) and SHP-supplemented, pH 4.5 (D) fish sauce samples are summarized in Table 3-15. The sodium chloride content of control,

Table 3-14: Sensory evaluation score of fish sauce diluted with brine

Sample[1]	Free a.a. µmole/ml	Triangle test[2] Correct	Incorrect	Total rank[3]	Preference score[3]
B : Dil B	-	7 a	2	-	-
A : Dil B	-	6 b	3	-	-
B	520.3	-	-	7 a	8.5 a
Dil B	260.2	-	-	15 b	6.7 ab
A	242.1	-	-	20 b	5.2 b
Commercial	414.7	-	-	18 b	4.8 b

[1] Laboratory samples were fermented for 6 months, aged for 1 year. A=Control; B=SHP-supplemented; Dil B=B diluted 1:1 with 25% NaCl solution; Commercial=Rufina fish sauce

[2] Values followed by a are significantly different ($P<0.01$), Values followed by b are significantly different ($P<0.05$), $n = 9$.

[3] Values in the same column followed by the same letter are not significantly different ($P<0.05$), $n = 9$.

Table 3-15: Analyses of capelin fish sauce prepared with and without SHP[1,5]

Sample[2]	NaCl[3] %	Total N[3] mg/ml	Formol N[3] mg/ml	Soluble[3] protein (mg/ml)	pH[4]
A	27.20 ± .35a	13.94 ± .42a	7.00 ± .35a	23.40 ± .16a	5.99
B	27.00 ± .18a	23.85 ± .59b	13.72 ± .23b	21.36 ± .20b	5.92
C	27.35 ± .46a	12.82 ± .50a	6.23 ± .15a	25.57 ± .24c	5.97
D	28.80 ± .87a	14.43 ± .44a	9.80 ± .41c	20.76 ± .12b	4.59

[1] Analyzed after 6 months fermentation and 6 months storage.
[2] A=Control; B=SHP-supplemented; C=Heat-treated-SHP-supplemented; D = SHP-supplemented (pH 4.5)
[3] Values are averages of duplicate determinations for two lots of fish sauce.
[4] Values are averages of duplicate batches.
[5] Values in the same column followed by the same letter are not significantly different ($P < 0.05$).

SHP-supplemented and heat-treated-SHP-supplemented sauces were similar at 27%, while the salt content of the acidified sample (D) was slightly higher at 29%. Sample prepared with SHP contained about 1.7-fold more total nitrogen and 2.0-fold more free amino acid nitrogen than the control. However, the sample supplemented with heat-treated SHP has lower total nitrogen and free amino acid nitrogen content than the control. The Biuret protein for control, SHP-supplemented, heat-treated-SHP-supplemented and SHP-supplemented (pH 4.5) sauce were, respectively, 27, 14, 32 and 23% of the total crude protein (total N x 6.25). This result indicates that the percentage of protein hydrolyzed, as well as the amount of total crude protein recovered in the sauce, is significantly higher in fish sauce prepared with enzymatically active SHP at the natural pH of fermentation. Also, acidification of SHP-supplemented capelin mince resulted in a greater free amino acid nitrogen (1.37-fold) than in total nitrogen (1.04-fold) over the control (Table 3-16). This observation may indicate that acidification depressed the proteolytic activity more so than the peptidase activity of SHP enzymes. The nitrogen distributions of sample A-D (control, SHP-supplemented, heat-treated SHP-supplemented and pH 4.5 SHP-supplemented) are shown in Table 3-16. The formol nitrogen in samples A, B, C and D were 50, 58, 48 and 68% of the total nitrogen, respectively. According to Rose (1918), as cited in Uyenco *et al.* (1953), formol nitrogen in fish sauce should be between 60 and 70% of the total nitrogen. Ammoniacal nitrogen in fish sauce should be less than 50% of the formol nitrogen. The ammoniacal nitrogen, (formol N - free amino acid N), in samples A, B, C and D accounted for 36, 35, 41 and 37% of the formol nitrogen, respectively. The free amino acid nitrogen, (calculated from free amino

Table 3-16: Nitrogen distribution in fish sauce sample[7]

Sample[1]	Total N[2] (mg/ml)	Formol N[3] (mg/ml)	Free Amino acid[4] N (mg/ml)	NH_3-N[5] (mg/ml)	Biuret N[6] (mg/ml)
A	13.94 ± .42a	7.00 ± .35a	4.48	2.52	3.74 ± .16a
B	23.85 ± .59b	13.72 ± .23b	8.94	4.78	3.42 ± .20b
C	12.82 ± .50a	6.23 ± .15a	3.68	2.55	4.09 ± .24c
D	14.43 ± .44a	9.80 ± .41c	6.14	3.66	3.32 ± .12b

[1] A=Control; B=SHP-supplemented; C=Heat-treated-SHP-supplemented; D=SHP-supplemented (pH 4.5)

[2] MicroKjeldahl method. Values are averages of duplicate determinations for two lots of fish sauce. Values following means are standard deviation.

[3] Included amino acid, primary and secondary amines. Values are averages of duplicate determinations for two lots of fish sauce. Values following means are standard deviation.

[4] From free amino acid analysis data. Values are averages of duplicate batches.

[5] Formol N - Amino acid N

[6] Biuret soluble protein/6.25. Values are averages of duplicate determinations for two lots of fish sauce.

[7] Values in the same column followed by the same letter are not significantly different ($P<0.05$).

acid analysis data), in capelin fish sauce was found to be 32, 37, 29 and 42% of the total nitrogen in samples A, B, C and D, respectively. According to the Standards for fish sauce in Thailand, amino acid nitrogen in first and second grade fish sauce must be > 10 and 7.5 mg/ml fish sauce, respectively. However, estimation of amino acid nitrogen by the method used in this standard, which is based on the difference between formaldehyde nitrogen and ammoniacal nitrogen, would give somewhat higher value than by direct estimation from free amino acid because of the contribution of peptide α-amino groups to formaldehyde nitrogen. From the amino acid analyses, the free amino acid nitrogen in samples A, B, C and D were 4.5, 8.9, 3.7 and 6.1 mg/ml, respectively.

3.1.2. Contribution of fish digestive enzymes

To study the contribution of fish digestive enzymes to fermentation of capelin, fish sauce was prepared from gutted fish (GRF) and from round fish (RRF). The raw material was suspected to contain red feed, which might cause belly burst. Red feed capelin is rejected by the Japanese buyers.

From Fig 3-12, analysis of variance indicated that the rate of protein hydrolysis of RRF was higher than that of GRF ($P<0.05$). The hydrolysis of protein in RRF was also compared with a sample prepared from frozen inshore capelin; caught in Outer Cove in 1983, these capelin were not suspected of containing red feed. A Student t-test indicated that the protein hydrolysis in RRF was significantly higher than that of the inshore samples ($P<0.10$), (Appendix Fig A-3). The soluble proteins (Biuret) in GRF brine appeared to be higher than that of RRF after 8 weeks (Table 3-17). This is probably due to the

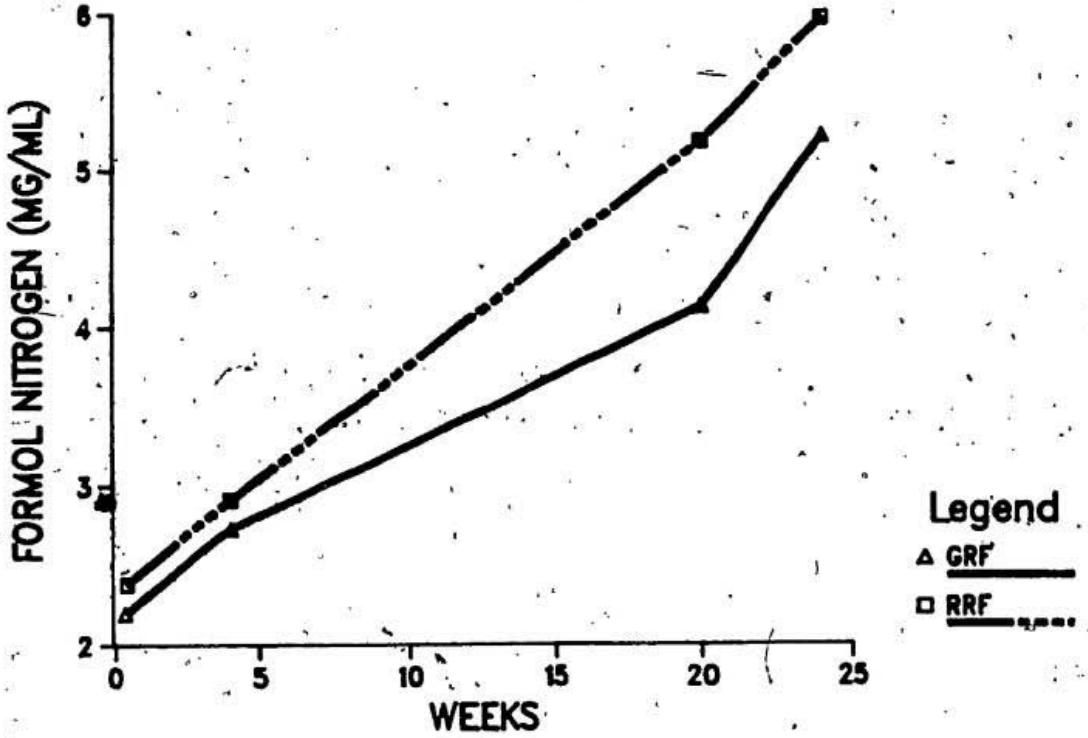


Figure 3-12: Contribution of fish digestive enzymes to protein hydrolysis during fish sauce fermentation

Values plotted are averages of duplicate determinations for two lots of fish sauce.
RRF = Round capelin, GRF = Gutted capelin

Table 3-17: Soluble protein (Biuret) in round and gutted red feed capelin.

Sample[2]	mg/ml[1] Time (Weeks)		
	8	20	24
RRF	28.77	23.30	21.72
GRF	32.37	20.36	18.58

[1] Values are averages of duplicate determinations for two lots of fish sauce.
[2] RRF=Round capelin; GRF=Gutted capelin

fact that GRF has less enzymes to hydrolyze the fish protein into free amino acids leaving the greater amounts of protein and peptides to react with Biuret reagent. However, after 20 weeks the soluble proteins in RRF appeared to be higher than in GRF (Table 3-17). This could be due to the fact that after 8 weeks the higher proteolysis in RRF resulted in the higher extractable proteins in the liquid. The Biuret soluble proteins appeared to decrease when fermentation time progressed. Biuret reagent reacts on the peptide bonds except for dipeptide. It is apparent that the increase in Biuret soluble protein and then the decrease are due to the hydrolysis of muscle proteins to polypeptides and later dipeptides and free amino acids.

Orejana and Liston (1979) reported that during the first 40 days of fish sauce fermentation endopeptidases were most active and after 70 days exopeptidases were more active. However, this conclusion was contrary to that of Tongthai and Okada (1981). They stated that during the first 3 weeks exopeptidases were predominant (indicated by the rapid increase of free amino acids) and during 3-20 weeks endopeptidases were predominant. According to Kirschke *et al* (1977), some exopeptidases are able to hydrolyze protein molecules but extensive rapid digestion will take place only after the reaction of endopeptidases on the protein. Thus, in early stages of fish sauce fermentation, it was more likely that endopeptidases were more active than exopeptidases.

In the fermentation of capelin fish sauce, digestive enzymes appeared to contribute somewhat to the hydrolysis of protein. However, when a triangle test

was conducted to distinguish GRF and RRF, the result indicated that there was no significant difference between the two samples. Only three out of eight panelists could distinguish between the two samples. Thus, it was apparent that enzymes associated with viscera was less important than muscle tissue enzymes, which play a major role in protein hydrolysis during fermentation of capelin fish sauce.

The contribution of digestive enzymes to fish sauce fermentation was studied by Uyenco *et al.* (1953). It was reported that the amino-nitrogen in sauce prepared from round fish was about 2-fold higher than that from gutted fish. They concluded that digestive enzymes were of importance in fish sauce fermentation. Lindgren and Pleje (1983) reporting on the fermentation of fish silage with lactic acid bacteria and found that the proteolytic activity is usually attributable to the enzymes associated with the gut such as pepsin at acidic pH. Fermentation of gutted and ungutted herring at pH 4.4 and 5 indicated that the gut contains proteases with activity at pH 4.4. However, protease activity of gutted and ungutted herring at pH 5 was found to be insignificantly different. The activity in gutted, as well as ungutted herring, was reported to be attributable to cathepsins. This group of tissue proteases is known to occur at a high level in fish (Siebert and Schmitt, 1965). A combination of cod viscera and cod frames liquefy at a faster rate than viscera or frames alone during preparation of fish silage at pH 4 (Haard *et al.*, 1985).

In this experiment the raw material employed might have affected protein

hydrolysis. Capelin used in this experiment was suspected to contain red feed. According to Hjelmeland and Raa (1980), belly burst was caused by the activity of proteolytic enzyme. However, rate of belly burst cannot be determined by the activity of proteolytic enzymes alone. The rate of belly burst was affected by the type of feed ingested, the condition of the connective tissue and the postmortem pH. The type of feed ingested influenced the rate of acid production in the stomach (Gildberg and Raa, 1980). It was found that the pH of the stomach content consisting mainly of red feed (Calanus) was as low as 3.2 while the stomach content of the fish that had ingested mainly a small ampheipod seafly (Hyperia) had neutral pH. It was reported that the autolysis of capelin was maximized at pH around 4 (Gildberg, 1982). The influence of red feed on pH of the stomach was consistent with the assumption that capelin containing red feed was susceptible to belly burst and apparently desirable in fish sauce fermentation.

3.2. Optimal conditions for fish sauce fermentation

3.2.1. Salt concentration

Salt has been widely used for food preservation. Salt, at high concentration, inhibits the growth of most microorganisms by lowering the water activity. However, halophiles require more than 12% of salt for growth. In fermentation of fish, salt is required to prevent putrefaction but when salt concentration is increased protein hydrolysis decreases.

3.2.1.1. Rate of protein hydrolysis

The effect of salt concentration in the fermentation brine on protein hydrolysis is shown in Fig 3-13. The lower the salt concentration, the higher the rate of protein hydrolysis. However, at 15% salt (w/w) the mixture of fish and salt started to spoil after two weeks of incubation at ambient temperature. Because of the offensive odor this sample was discarded and the data were not presented. Statistical analysis by ANOVA showed that the sample with 20% salt (w/w) had highest protein hydrolysis compared to the samples with 25 and 30% salt ($P<0.01$). The sample with 25% salt had a higher rate of protein hydrolysis than the 30% salt ($P<0.05$). Salt is found to suppress the activity of most enzymes especially pepsin (Gildberg *et al.*, 1984).

3.2.1.2. Free amino acid formation

The effect of salt concentration in the fermentation brine on free amino acid accumulation are shown in Fig 3-14. The changes in amino acid composition during fermentation of samples prepared with 20, 25 and 30% salt are shown in Appendix Tables A-4, A-5 and A-6, respectively. At 20% salt the rate of free amino acid formation was higher than at 25 or 30% salt ($P<0.01$). During fermentation, the % compositions of alanine, arginine, cysteic acid, cysteine, and hydroxy lysine were more or less constant. The mole% of aspartic acid and glutamic acid increased in all samples during fermentation. The mole% of glycine increased in samples prepared with 20 and 25% salt but remained the same in sample prepared with 30% salt. The mole% of leucine, and lysine increased in samples prepared with 30 > 25 > 20% salt. The mole% of methionine, phenylalanine, serine and threonine increased slightly in all samples. Taurine was

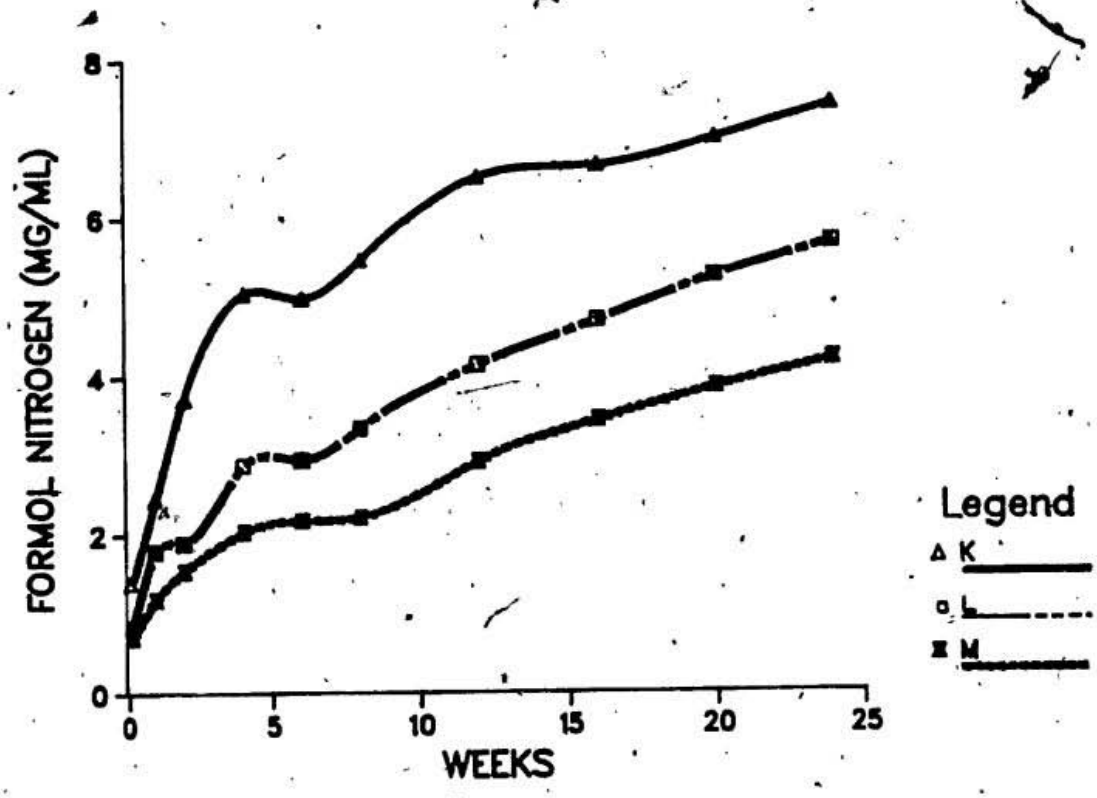


Figure 3-13: Effect of salt concentration on protein hydrolysis during fish sauce fermentation

Values plotted are averages of duplicate determinations for two lots of fish sauce.
K = 20% salt w/w, L = 25% salt w/w, M = 30% salt w/w

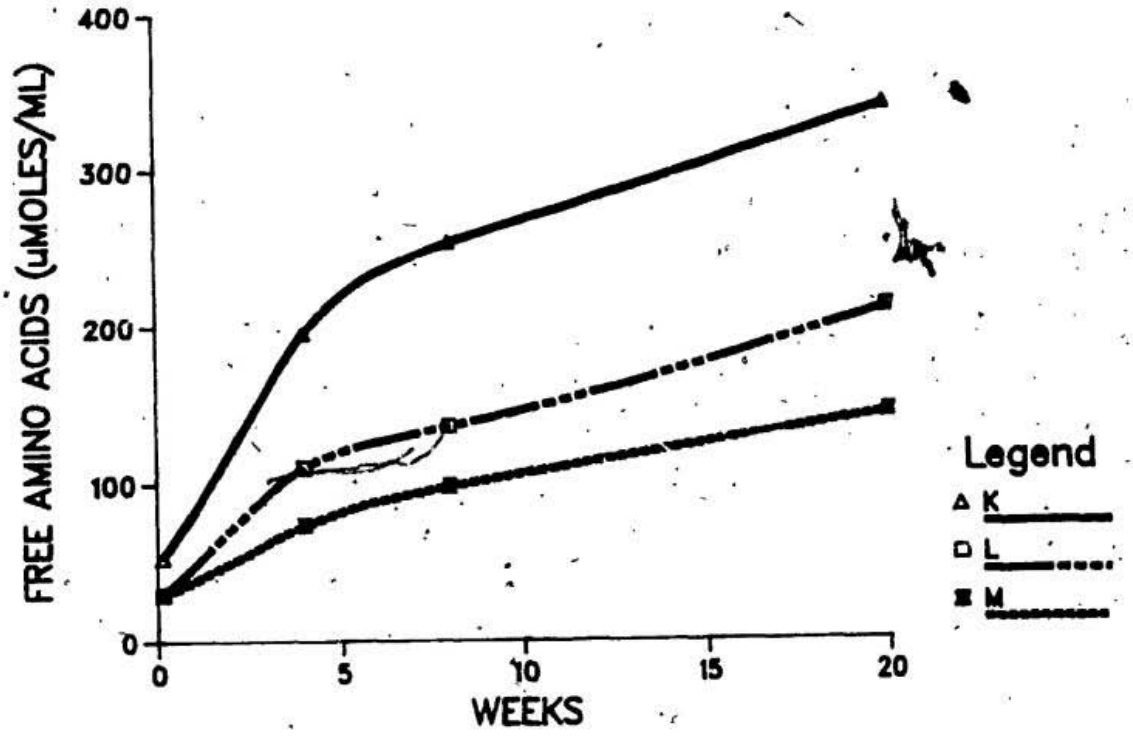


Figure 3-14: Effect of salt on free amino acid formation during fish sauce fermentation

Values plotted are averages of duplicate batches.
K = 20% salt, L = 25% salt, M = 30% salt

found at the highest concentration in the first-day brine of all samples. The % composition of histidine and proline slightly decreased in all samples. After 1 year, sample prepared with 20% salt (w/w) had 1.6-fold greater free amino acid content than the control (25% salt w/w). The free amino acid content in 1-year old samples prepared with 30% salt (w/w) was only about half of the control. Free amino acids in 1-year old sample prepared with 20, 25 and 30% salt were 68.6, 61.0 and 49.6% of the total hydrolyzate amino acids, respectively. The free amino acid content of fish sauces fermented at different salt concentrations after 6 months fermentation plus 6 months aging is shown in Fig 3-15 and the free and total amino acid contents are shown in Appendix Table A-7. The amino acid composition, as mole percent, is shown in Fig 3-16. Alanine was found at the highest ratio in all samples. Sample K contained higher mole% of glutamic and aspartic acids than did samples L and M. It appeared that enzymic hydrolysis of fish protein during fermentation releases these two amino acids. At lower concentration of salt in the fermentation mixture, the proteolytic activity was higher than at higher salt concentration, and thus resulted in higher free amino acid content. However, Rose (1918), as cited in Amano (1962), reported that in the case of too low salt fermentation amino acid nitrogen decreased and volatile nitrogen increased.

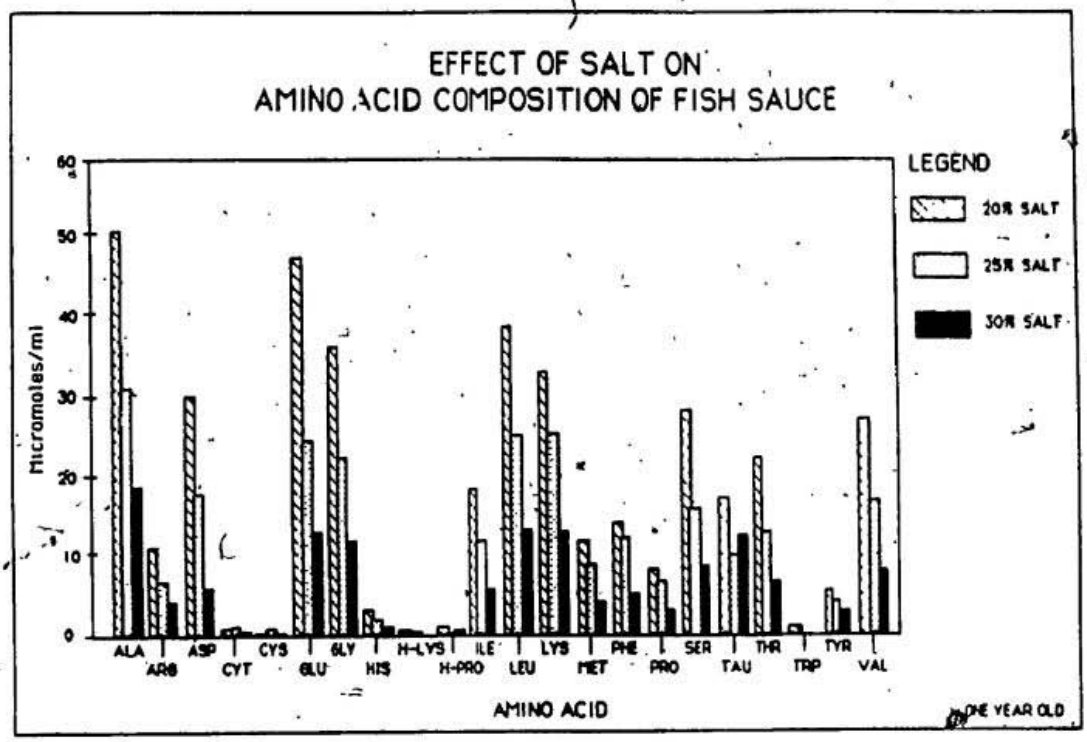


Figure 3-15: Free amino acid content of fish sauce fermented at different concentrations of NaCl

Values plotted are averages of duplicate batches.
Samples were fermented for 6 months, aged for 6 months.

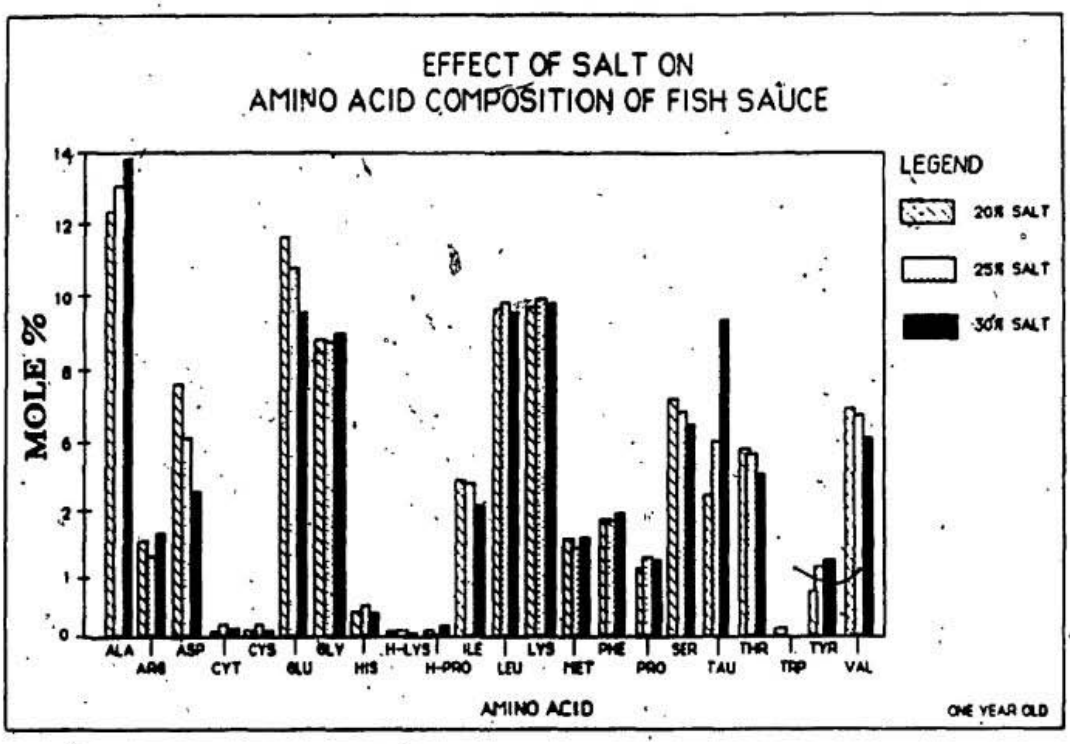


Figure 3-16: Free amino acid composition of fish sauce fermented at different concentrations of NaCl

Values plotted are averages of duplicate batches.
Samples were fermented for 6 months, aged for 6 months.

3.2.1.3. Color

The effect of salt concentration in the fermentation mixture on the browning intensity of fish sauce during fermentation are shown in Table 3-18. The Hunter-Gardner color indices of the finished products are shown in Table 3-19. The lower the salt concentration, the darker the color of fish sauce (higher absorbance at 400 nm and lower L value). Since the rate of free amino acid formation was influenced by salt concentration, it is possible that salt inhibits browning reaction indirectly by limiting formation of the precursors. The very active amino acids in browning reaction of squid muscle are lysine, proline, methionine and taurine (Haard and Arcilla, 1985).

3.2.1.4. Sensory evaluation

Preference scores and NaCl content of samples prepared with 20, 25 and 30% salt (w/w) after 6 months fermentation and 6 months aging are shown in Table 3-20. Sample prepared with 20 % salt (w/w) had the highest preference score ($P<0.05$) probably because of the less salty taste and higher total amino acid and glutamic acid content. However when this sample was kept for another year the color of the sample was changed from reddish-brown to dark brown and the aroma was offensive. The sample fermented with >25% salt had good shelflife, even after two years storage. Thus, a salt concentration below 25% is not recommended. The use of a low salt concentration in the fermentation of fish sauce must be accomplished by other means e.g., lower pH, higher fermentation temperature and use of preservatives. The sauce prepared by fermentation of fish at low salt concentration (300 g/kg fish) was reported to be more preferable than at high salt concentration of 400 g/kg fish (Ohayovan *et al*,, 1983a).

Table 3-18: Browning development in fish sauce fermented at different salt concentrations

Sample[2]	Absorbance (400 nm)[1] Time (Weeks)					
	2	4	6	8	12	16
K	0.71	0.92	1.45	1.62	1.82	1.92
L	0.57	0.61	0.75	1.00	1.18	1.26
M	0.37	0.45	0.51	0.63	0.89	0.90

[1] Values are averages of duplicate batches.
[2] K = 20% salt w/w, L = 25% salt w/w, M = 30% salt w/w.

Table 3-19: Effect of NaCl concentration of fish sauce on tristimulus color

Sample[1]	L[2]	a[2]	b[2]	E[3]
K (20% NaCl w/w)	24.0	13.6	5.3	69.7
L (25% NaCl w/w)	29.8	11.6	19.3	66.2
M (30% NaCl w/w)	37.6	8.0	23.4	59.6

[1] Fish sauce samples were fermented for 6 months and aged for 6 months.
[2] Values are averages of duplicate determinations.
[3] Values calculated from average values.

Table 3-20: Sensory evaluation of fish sauce prepared at different concentration of NaCl

Sample[1]	Preference score[2]	% NaCl[3]
20% salt	6.7a	22.9
25% salt	5.3b	27.2
30% salt	4.3b	30.3

[1] Fish sauce samples were fermented for 6 months and aged for 6 months. Values indicate percent by weight of salt added to fish prior to fermentation.

[2] Values followed by the same letter are not significantly different ($P<0.05$), $n = 6$.

[3] Values are averages of duplicate determinations for two lots of fish sauce.

3.2.2. Temperature

The effect of incubation temperature on protein hydrolysis in control, SHP-supplemented and 30% salt (w/w) samples is shown in Fig 3-17. Statistical analyses of these data by Student t-test indicated that the fish-salt mixtures (25% salt w/w) incubated at 37°C (A-37) had lower protein hydrolysis than the same batch of sample incubated at ambient temperature ($P<0.05$), but the protein hydrolysis of the sample with 30% salt w/w (G-37) and the sample supplemented with SHP (B-37) were not significantly different from the sample incubated at ambient temperature.

Fermentation of fish sauce at higher temperatures has been reported to hasten the rate of protein hydrolysis and the accumulation of free amino acids. Orejana (1978) reported that the level of free amino acids observed after 14 months at room temperature (23°C) can be reached in only 1 month at 37°C. Ham and Clague (1950) also recommended the use of a higher fermentation temperature, but temperatures higher than 40°C was not recommended because an adverse burnt flavor occurred in the product (Orejana *et al.*, 1984).

The effect of temperature on fish sauce fermentation was studied (Miyazawa *et al.*, 1979) using anchovy as raw material. After 150 days, the percentages of the liquefied protein were 25.3, 17.6, 6.3 and 2.3 of the total protein at 50, 30, 10 and 2°C respectively. Free amino acid content in fish sauce prepared at 50°C was about 45% higher than the one at 30°C. Ooshiro *et al.* (1981) compared the fish sauce fermentation at room temperature (24°C), 37 and 50°C and reported that at

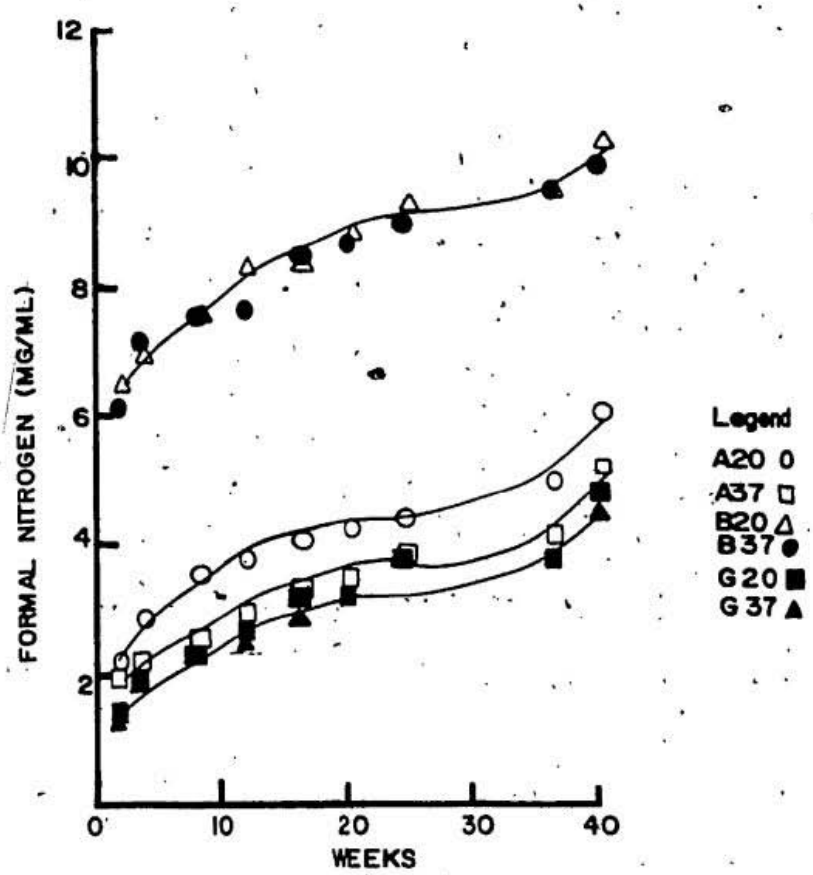


Figure 3-17: Effect of temperature on protein hydrolysis in fish sauce

Values plotted are averages of duplicate determinations for two lots of fish sauce.
A20 = Control at ambient temperature, A37 = Control at 37°C
B20 = SHP-supplemented at ambient temperature,
B37 = SHP-supplemented at 37°C
G20 = 30% salt at ambient temperature, G37 = 30% salt at 37°C.

the beginning of the fermentation at 37°C, the concentration of the amino nitrogen and soluble nitrogen were higher than the sample at room temperature but after 153 days the values came to be very close. The concentration of volatile base nitrogen was higher at 24°C than at 37°C. The fish sauce fermented at 50°C had the lowest amino nitrogen and the highest volatile nitrogen. They concluded that at 50°C the activity of the enzymes was depressed and the nitrogen contents were concentrated because of the moisture loss.

According to Gildberg (1982) and Haard *et al.* (1982), the digestive proteases from the cold water species are relatively more active at low temperature than those from warm water species of fish. Also digestive and intracellular enzymes from cold water fish are inactivated at lower temperature than homologous enzymes from temperate or warm water temperature (Simpson and Haard, 1986). Proteases from warm water fish have a higher temperature optimum than those from cold water. Activity of capelin and Indian oil sardine alkaline protease, at pH 9.0, have temperature optima at 43 and 54°C, respectively. Moreover, both purified and crude preparation of capelin were found to retain relatively high activity at low temperature (Gildberg, 1982). The temperature optimum of cathepsin C from squid hepatopancreas was found to be at 40°C (Hameed and Haard, 1985).

The fermentation of capelin fish sauce prepared with 25% salt (w/w) and without SHP was found to occur at a faster rate at ambient temperature than at 37°C, this was apparently due to the instability of part of the enzymes from

capelin at 37°C. However, when salt was increased to 30% (w/w), the activity of enzymes involved with protein hydrolysis was suppressed and there was no significant difference between fermentation at temperatures of 20 or 37°C. It is possible that part of the enzymes in the fermentation which are sensitive to temperature increase at 25% NaCl are inactivated by 30% NaCl at both temperatures.

3.2.3. pH

The effect of initial pH of the fermentation mixtures (capelin:salt 4:1 w/w) on protein hydrolysis during fermentation is shown in Fig 3-18. The highest rate of protein hydrolysis was found in pH 6.0 adjusted sample. During fermentation, the pH of all samples changed from the initial adjusted pH values of 3 to 8. The pH of the finished products after fermentation for 40 weeks were 3.5, 4.0, 4.8, 5.6, 6.3 and 7.6, respectively (Appendix Table A-8). A previous study on autolysis of capelin in the absence of added salt showed that acid proteases are more active than neutral or alkaline proteases (Gildberg, 1982). Activity of capelin digestive proteases on hemoglobin was also reported to be maximal at pH 4 and minimal at pH 5-6; but on glycoprotein extracted from the skin of capelin the optimal pH was at pH 7.0 (Gildberg, 1982). Two trypsin type enzymes were isolated from the gut of capelin (Hjelmeland and Raa, 1982). The activity of these two enzymes at pH 6.0 was only 15% of the optimum activity at pH 8-9, but Gildberg and Raa (1979) found that digestive enzymes of capelin degraded protein in a native skin only at pH below 6.0; not at neutral or alkaline pH. Capelin pepsin was also reported to be able to hydrolyze muscle protein at a significant rate even at pH 6.0 (Gildberg,

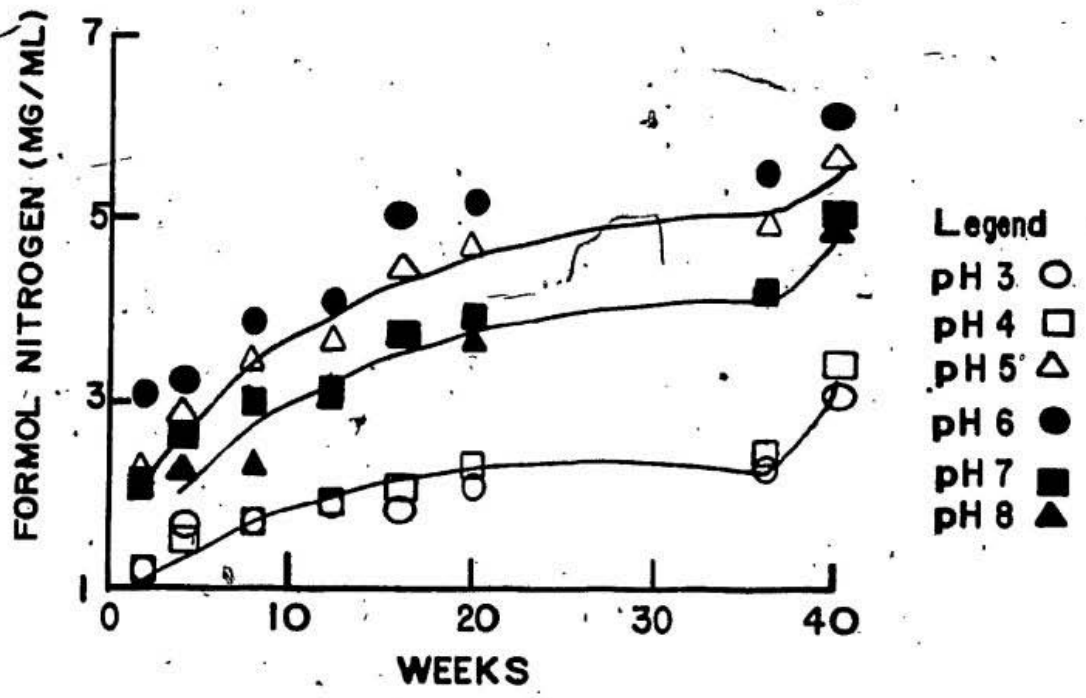


Figure 3-18: Effect of pH on protein hydrolysis during fish sauce fermentation

Values plotted are averages of duplicate determinations for two lots of fish sauce.

1982). Although activity of pepsin is higher at acidic pH, the activity of pepsin is inhibited by salt (Gildberg *et al.*, 1984), thus at pH below 5 the activity of pepsin in the fish-salt mixture is depressed. At initial pH of 3 and 4 or 7 and 8, the protein hydrolysis was not significantly different from each other ($P<0.05$). Protein hydrolysis was lowest at pH 3.0, followed by pH 4.0. Rates of protein hydrolysis in salted capelin at different initial pH are shown in Fig 3-19. From these results, it appeared that the optimum rate of protein hydrolysis was at initial pH of 5.0-6.0. Thus, the pH optimum for fermentation of fish sauce appeared to be at the natural pH of fermentation.

3.3. Contribution of bacterial enzymes to fish sauce fermentation

3.3.1. Delayed-salting

The principle of delayed-salting is that it gives a chance for autolysis and bacterial growth to develop prior to addition of salt. After autolysis has taken place, the microorganisms, both natural flora and contaminants, use the autolytic products as nutrients. Although microorganisms may be subsequently killed by the addition of salt, their enzymes may remain active in the brined fish.

Protein hydrolysis during fermentation of capelin held at ambient temperature for 0 to 24 h prior to addition of salt is shown in Fig 3-20. The ANOVA of the data indicated that there was no significant difference between the 6-18 h delayed-salting samples and control ($P<0.05$). However, delayed-salting for 24 h increased the rate of protein hydrolysis significantly ($P<0.05$).

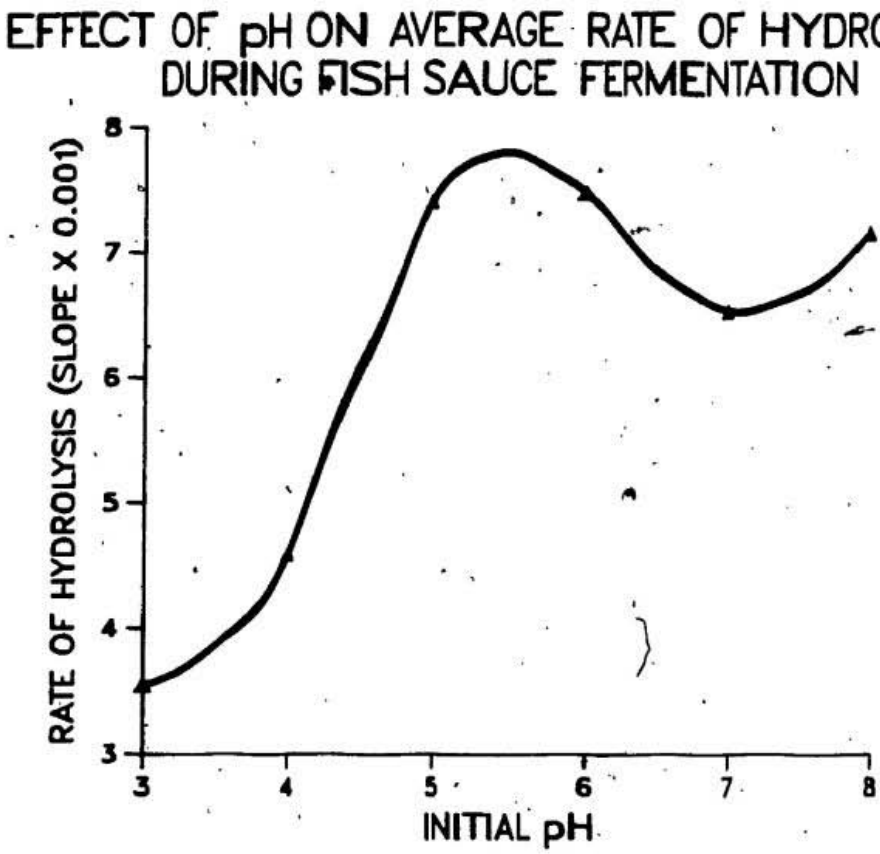


Figure 3-19: Rate of protein hydrolysis at different pH in fish sauce fermentation

Rate calculated from slope of plots shown in Fig 3-18

Coefficient of determination (r^2) for linear regression of data at pH 3 to pH 8 were 0.9306, 0.9381, 0.9299, 0.9437, 0.9596 and 0.9775, respectively

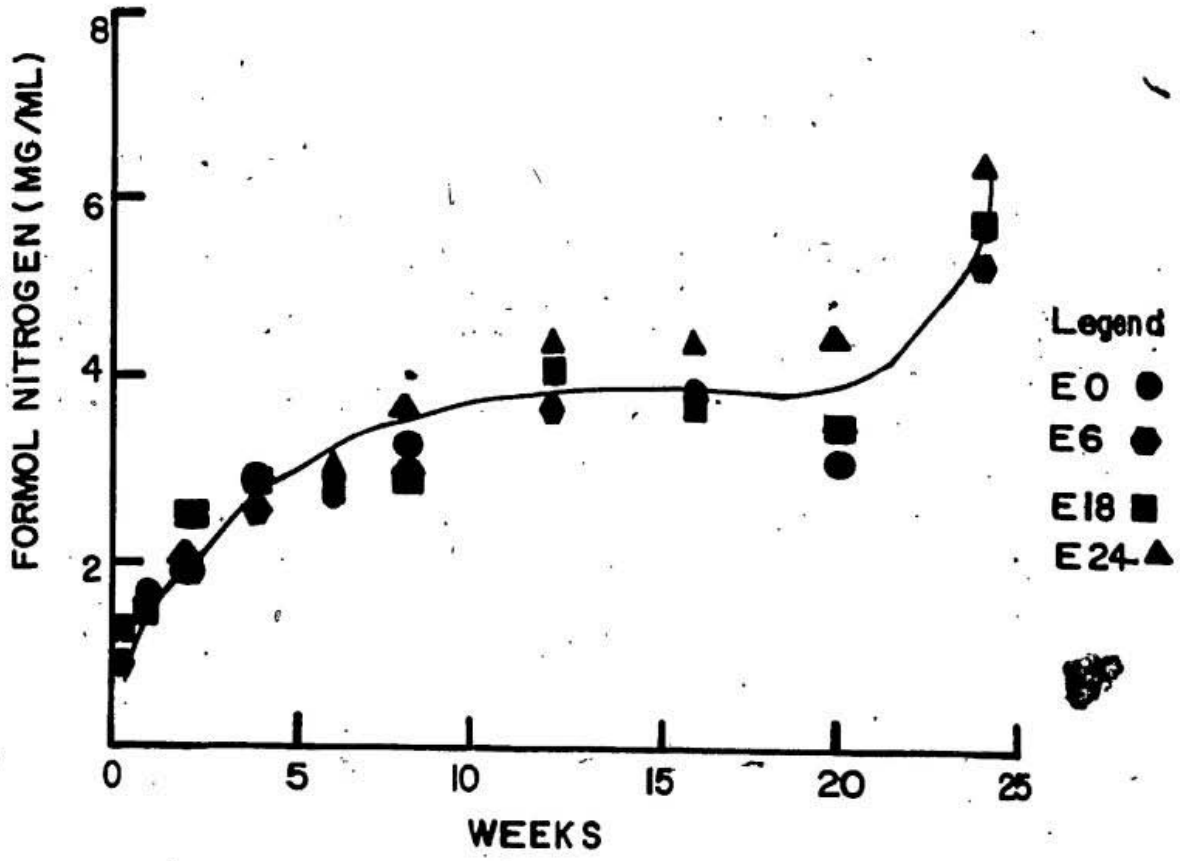


Figure 3-20: Effect of delayed-salting on protein hydrolysis during fish sauce fermentation

Values plotted are averages of duplicate determinations for two lots of fish sauce. Number following sample code indicates hours of delayed salting.

Total colony forming units in 24 h delayed-salting sample (DS) were very high, in both TSA and TSA +10% NaCl, compared to the fresh capelin (Table 3-21), which indicated the growth of salt tolerant bacteria. Capelin held 24 h at ambient temperature did not have an offensive odor. The reason for the insignificant difference in protein hydrolysis of the 6-18 h delayed-salting and the control may be due to the existence of a lag phase before the microorganisms started to produce significant levels of enzymes. It also may indicate that bacterial proteases are mostly inactivated by 25% salt and are therefore not important in the fish sauce fermentation. According to Liston (1965), number of the microorganisms must reach 10^7 before microbial enzymes become significant in the spoilage of fish. The total viable counts in capelin held 24 h at ambient temperature dropped from $>6.5 \times 10^7$ to 5.2×10^3 after the fish was mixed with salt for 1 day. After 1 week, no viable microorganisms were detected (Table 3-22).

Studies on the microbiology of fish sauce have shown that in the warm and damp climate of South East Asia, where the raw material for fish sauce production might be left without refrigeration for a period up to 24 h before salting, the total viable bacterial counts can be very high. Ham and Clague (1950) reported the initial bacterial count of fresh anchovies used for fish sauce production in the Philippines to be 6.5×10^6/g, compared to the total viable counts of the fresh capelin and the capelin held for 24 h at ambient temperature which were 5.3×10^2 and $>6.5 \times 10^7$ CFU/g, respectively. Beddows *et al.* (1976) reported that delayed addition of salt aided proteolysis in budu (Malaysian fish sauce) fermentation. The protein conversion to soluble protein increased from

Table 3-21: Total viable counts in capelin[1] (CFU/g)

	TSA	TSA + 10% NaCl
Fresh capelin	5.3×10^2	1.0×10^2
24 h holding at ambient temperature (20-25°C)	$> 6.5 \times 10^7$ Est	$> 6.5 \times 10^7$ Est

[1] Interpretation was according to Gilliland *et al.* (1976).
Est = Estimated number.

Table 3-22: Total viable counts in fish sauce[1] (CFU/ml)

	CO[2]		AN[3]		DS[4]	
Time (d)	TSA	TSA + NaCl 10%	TSA	TSA + NaCl 10%	TSA	TSA + NaCl 10%
1	5.5×10^2	< 10 Est	< 10 Est	< 10 Est	5.2×10^3	1.7×10^3
7	2.8×10^2 Est	< 10 Est	< 10 Est	< 10 Est	< 10 Est	< 10 Est
14	3.3×10	< 10 Est	< 10 Est	< 10 Est	< 10 Est	< 10 Est
21	< 10 Est	< 10 Est	< 10 Est	< 10 Est	< 10 Est	< 10 Est
40	< 10 Est	< 10 Est	< 10 Est	< 10 Est	< 10 Est	< 10 Est

[1] Interpretation was according to Gilliland *et al.* (1976).
Est = Estimated number.
[2] Control fish-salt mixture (25% salt w/w)
[3] Gentamicin sulfate USP (100 mg) was added to 1 kg of fish salt mixture (25% salt w/w).
[4] Capelin was held at ambient temperature 24 h prior to addition of salt (25% w/w).

41.7 in control to 76.5 and 89.7% when the raw material was held at ambient temperature for 7 and 20 h, respectively. It was suggested that the production of the volatile fatty acids, which were necessary for the development of aroma, was complex and probably depended upon a period of bacterial action prior to salting, since the fish used for commercial preparation usually would be left at ambient temperature (normally above 30°C in South-East Asia) for some hours. The change in free amino acid concentration during fermentation of 24 h delayed-salting sample is shown in Appendix Table A-9. The amino acid composition of the 24 h delayed-salting fish sauce, after fermentation for 6 months and aging for 6 months, is shown in Fig 3-21 and Appendix Table A-10. It was noted that this fish sauce had lighter color than control, but the fish-salt mixture itself developed a pink color with pleasant aroma. The tristimulus color values and the absorbance at 400 nm of the finished product are shown in Table 3-23. The sensory evaluation of the 24 h-delayed-salting sample was not improved much. The preference score was not significantly different from the control (Table 3-24). The data led to the conclusion that the contribution of microbial enzymes to the formation of fish sauce from capelin is not a major factor. This conclusion is contrary to previous reports describing the contribution of microorganisms to formation of fish sauce from other species of fish (Saisithi, 1967; Beddows *et al.*, 1976; Beuchat, 1983).

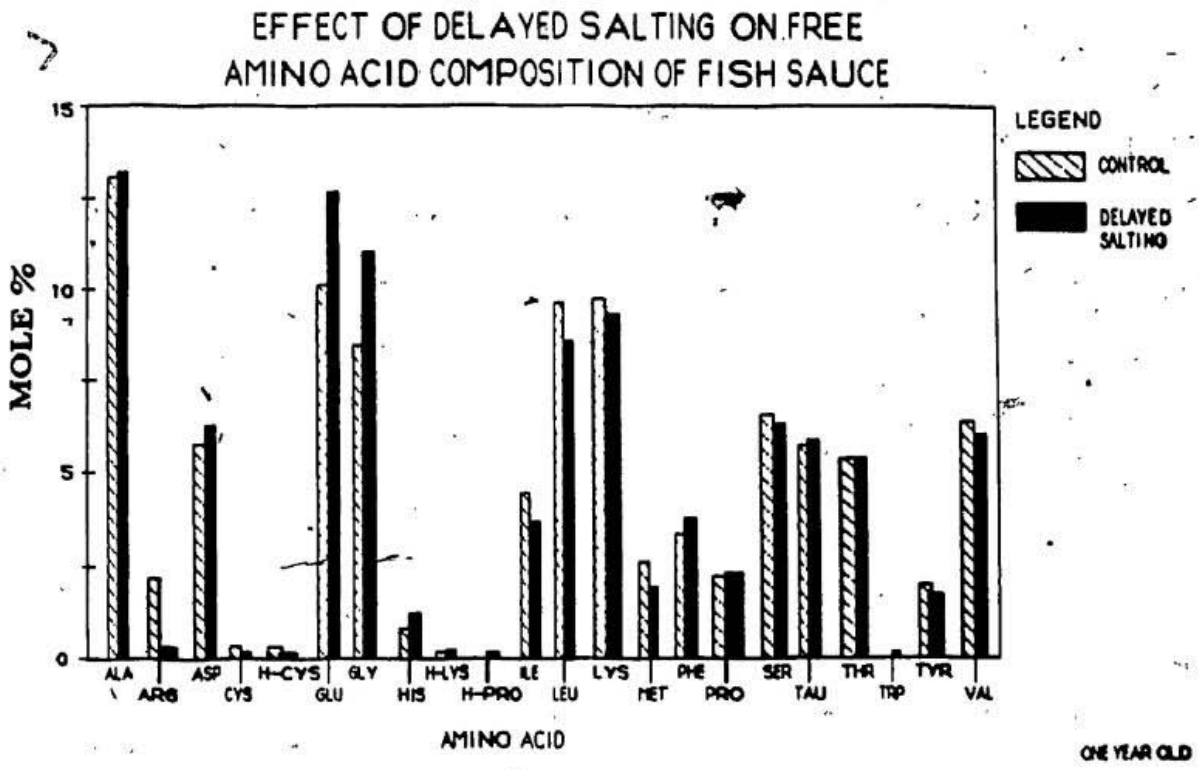


Figure 3-21: Free amino acid composition of fish sauce

Values plotted are averages of duplicate batches.
Samples were fermented for 6 months, aged for 6 months.

Table 3-23: Color indices of control and delayed-salting fish sauce

Sample[1]	L[2]	a[2]	b[2]	E[3]	$A_{400\ nm}$[2]
Control	31.4	8.6	20.3	64.4	1.11
24 h-delayed salting	35.2	10.3	22.3	61.8	0.77

[1] Samples were fermented for 6 months, aged for 6 months.
[2] Values are averages of duplicate batches.
[3] Values calculated from average values.

Table 3-24: Effect of delayed-salting on sensory evaluation of fish sauce

Sample[1]	Preference score[2]
24 h delayed-salting	6.3a
Control	5.3a

[1] Samples were fermented for 6 months and aged for 6 months.
[2] Values followed by the same letter are not significantly different ($P<0.05$), $n = 6$.

3.3.2. Antibiotic

To further study the contribution of viable microorganisms to proteolysis during fish sauce fermentation, an antibiotic of broad spectrum, gentamicin sulfate, was added at a concentration of 100 mg/kg fish-salt mixture. The use of antibiotic was effective against the growth of microorganisms (Table 3-22). There were no viable cells detected in antibiotic-treated-salted mince sample in both TSA and TSA + 10% NaCl. However, the total viable counts of the untreated sample were also low. This might be due to the fact that the raw material was very fresh and had low total viable counts (5.3×10^2 CFU/g), even before mixing with salt.

The total viable bacterial counts were reported to decrease during the course of fermentation of fish sauce (Ham and Clague, 1950; Guevara *et al.*, 1972; Orejana and Liston, 1979). The total viable bacterial count was found to be lower in a medium with 10% NaCl (5.3×10^2 in TSA, 1.0×10^2 in TSA + NaCl). The result was consistent with that reported by Orejana and Liston (1979) and indicated that bacteria in fish sauce are salt-tolerant bacteria rather than halophilic bacteria. Crisan and Sands (1975) isolated microorganisms from four types of fermented fish sauce and also reported that no true halophile was found in any of the fish sauce samples. However, Fujii and Sakai (1984a, b, c) found halophilic bacteria in Japanese fish sauce and suggested that halophilic bacteria were responsible for the spoilage of shotturu.

The rates of protein hydrolysis during fermentation of 24 h delayed-salting

and antibiotic-treated sample are shown in Fig 3-22. ANOVA of these data showed that there was no significant difference between the control and antibiotic treated samples. However, protein hydrolysis in the delayed-salted sample was significantly higher than in the control ($P<0.05$). The greater rate of protein hydrolysis in delayed-salting sample could be due to the activity of microbial enzymes produced before the addition of salt. The soluble proteins in all samples increased up to 10 weeks. After the twelfth week the drop in soluble protein content was probably due to the decreased rate of protein solubilization relative to its hydrolysis to amino acids (Table 3-25). Soluble protein content in antibiotic-treated and control samples was not significantly different ($P<0.05$), but delayed-salting sample had a higher content of soluble protein ($P<0.05$). The finding that antibiotic treatment did not significantly alter protein hydrolysis and protein solubilization in fish sauce indicates microorganisms do not make a significant contribution to the fermentation of fish sauce made from freshly harvested capelin.

3.4. Regression analyses

To determine the relationship between sensory preference score and chemical components in fish sauce, regression analysis was carried out using SPSSX programme for multiple regression on VAX system at Memorial University of Newfoundland. Linear regression analyses of preference score and free amino acid, total amino acid, aspartic acid, glutamic acid and glycine content are summarized in Table 3-26. The relationships between the preference score and the absorbance at 400 nm, L, a and b values are summarized in Table 3-27. The contributions of free amino acid content on color are summarized in Table 3-28.

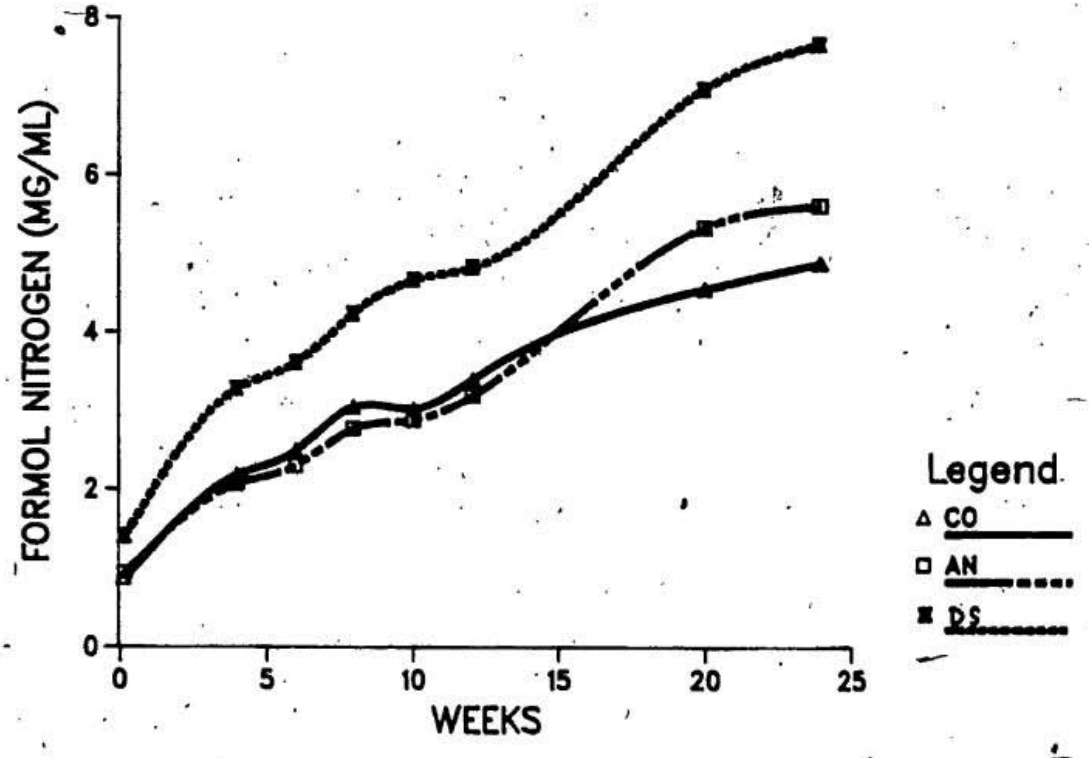


Figure 3-22: Effects of antibiotic and 24 h delayed salting on protein hydrolysis during fish sauce fermentation

Values plotted are averages of duplicate determinations
for two lots of fish sauce.
CO = control, AN = Antibiotic treated, DS = 24 h delayed salting

Table 3-25: Effects of delayed salting and antibiotic on Biuret soluble protein during fish sauce fermentation

	mg/ml[1]								
	1d	2	4	6	8	10	12	20	24
Control	14.25	19.10	20.40	22.65	24.53	23.15	23.27	21.00	21.73
Antibiotic	14.75	17:30	18.25	21.20	21.53	22.08	23.33	21.28	19.23
Delayed Salting	18.05	22.82	25.25	26.45	26.42	26.48	27.10	25.08	22.73

[1] Values are averages of duplicate determinations for two lots of fish sauce. Biuret soluble protein was measured as described in section 2.4.5 of Materials and Methods.

Table 3-26: Regression analysis of sensory preference score as a function of different amino acid fractions

Dependent variable (Y)[1]	Independent variable (X)[1]	Correlation coefficient (r)	Coefficient of determination (r^2)
Preference score	Total free amino acid (1)	0.851[2]	0.724
	Hydrolyzate amino acid (2)	0.775[2]	0.600
	Aspartic acid (3)	0.770[2]	0.592
	Glutamic acid (4)	0.829[2]	0.687
	Glycine (5)	0.812[2]	0.660
	Peptide amino acid (6)	0.031	0.001
	3, 4, 5, 6	0.958[2]	0.917[3]

[1] From regression analysis of data for 7 different samples of fish sauce.

[2] The relationship is significant ($P \leq 0.05$), $df = 5$, $r_{0.05} > 0.754$.

[3] $Y = 6.243 - 0.006(X_3) + 0.149(X_4) - 0.095(X_5) - 0.029(X_6)$.

Table 3-27: Regression analysis of sensory preference score as a function of objective measurement of color

Dependent variable (Y)[1]	Independent variable (X)[1]	Correlation coefficient (r)	Coefficient of determination (r^2)
Preference score	L (1)	-0.774[2]	0.600
	a (2)	0.799[2]	0.638
	b (3)	-0.652	0.425
	E (4)	-0.736	0.541
	1, 2, 3, 4	0.911[2]	0.830[3]

[1] From regression analysis of data for 7 different samples of fish sauce.
[2] The relationship is significant ($P<0.05$), df = 5, $r_{0.05} > 0.754$.
[3] $Y = 6.528 - 0.080(X_1) + 0.151(X_2) - 0.128(X_3) - 0.044(X_4)$.

Table 3-28: Regression analysis of objective color measurement as a function of amino acid content

Dependent variable (Y^1)	Independent variable (X^1)	Coefficient of correlation (r)	Coefficient of determination (r^2)
$A_{400\ nm}(Y_2)$	Total free amino acid (X_1)	0.764[4]	0.584
	Active amino acid[2] (X_2)	0.810[4]	0.656
	Very active amino acid[3] (X_3)	0.749	0.561
L (Y_2)	X_1	-0.794[4]	0.631
	X_2	-0.828[4]	0.686
	X_3	-0.794[4]	0.631
a (Y_3)	X_1	0.829[4]	0.687
	X_2	0.870[4]	0.756
	X_3	0.846[4]	0.715
b (Y_4)	X_1	-0.670	0.436
	X_2	-0.660	0.448
	X_3	-0.665	0.436
E (Y_5)	X_1	0.799[4]	0.638
	X_2	0.841[4]	0.708
	X_3	0.802[4]	0.644

[1] From regression analysis of data for 7 different sample of fish sauce.
[2] Ala, Arg, His, Lys, Met, Pro, Tau, Trp.
[3] Lys, Met, Tau.
[4] Significant correlation ($P<0.05$), $n = 7$, $df = 5$, $r_{0.05} > 0.754$.

The results indicated that there was a positive correlation between preference scores and free amino acid content of the seven fish sauce samples being analyzed ($r^2 = 0.724$). Glutamic acid concentration had a higher correlation with preference score than did glycine or aspartic acid concentration. Although, total peptide amino acid content (Hydrolyzate amino acids - free amino acids) alone did not show any correlation with preference score, multiple regression with peptide amino acids, glutamic acid, glycine and aspartic acid as independent variables showed a highly significant correlation ($r^2 = 0.917$).

Regression analysis to compare sensory evaluation score and amino acid content of enzyme-supplemented capelin fish sauce showed that total amino acid content, ratio of acidic to basic amino acid residues, hydrophobicity of free amino acids and non-amino acid content as the independent variables appear to account for most of the variations in the preference scores with a coefficient of determination (r^2) of 0.792 (Raksakulthai *et al.*, 1986).

The linear regression of preference score and color measurement showed a significant negative correlation between preference score and L value (lightness), thus the result indicated that the sauces preferred by panelist were darker. For redness, there was significant positive correlation between preference score and "a" (redness) value. The result indicated that the fish sauce preferred by panelists were reddish-brown in color. However, there was no significant correlation between "b" value (yellowness) or "E" (total color difference) and preference score.

There was a significant correlation between objective measurement of browning ($A_{400\ nm}$, L and a values) and free amino acid content, especially the concentration of active amino acids in the browning reactions, alanine, arginine, histidine, lysine, methionine, proline, taurine and tryptophan (Haard and Arcilla, 1985), which showed the highest correlation with the "a" (redness) value.

3.5. Amines in fish sauce

Since there are reports on toxicological problems and since some of the fish sauces in the experiment were prepared under the conditions that encourage the growth of microorganisms i.e. delayed-salting and lower salt concentration; it was of interest to determine the content of amines in these samples. The result of amine analysis is shown in Table 3-29.

The delayed-salting sauce had highest level of polyamines when compared to the other samples. Histamine was found at a very low level, (27.8 nmoles/ml or 3.0 μg/ml), compared to other reports on amines in foods. The other aromatic amines which are reported to be "toxic amines" were not found in any of the samples. The level of histidine which is a precursor of histamine did not decrease during the fermentation (Appendix Table A-5). The bacterial count in the delayed-salting capelin prior to addition of salt was very high (Table 3-20). This might be the reason for the higher content of amines in this sample. Histamine was reported at higher level in Cheddar cheese, 50-1300 μg/g (Voigt *et al.*, 1974). Futhermore, fish paste made from anchovy was reported to contain up to 404 μg/g histamine (Fardiaz and Markakis, 1979). Besides the delayed-salting sample,

Table 3-29: Amines in fish sauce prepared by different methods

Amines	nmole/ml[1]			
	Control (25% salt)	SHP	20% salt	Delayed-salting 24 h
Ethanolamine	6600.0	3600.0	5800.0	7140.0
Putrescine	194.0	232.6	254.3	3273.0
Cadavarine	7.2	16.9	26.5	2653.0
Spermidine	55.2	81.2	91.6	112.0
Spermine	29.2	71.0	27.8	52.0
Agmatine	0.0	0.0	0.0	526.0
Histamine	0.0	0.0	0.0	app 28[2]

[1] Values are averages of duplicate analyses. Samples were fermented for 6 months and kept in sealed jars at ambient temperature for 2 years.

[2] Approximate value due to limit of sensitivity for detection.

neither other fish sauce samples analyzed contained any detectable amount of histamine. The small amount found in delayed-salting sauce indicates that there is potential for biologically active amine formation in fish sauce.

3.6. Partial characterization of enzymes in fish sauce

Attempts to fractionate fish sauce by ultrafiltration with the Millipore Pellicon filtration unit fitted with a 1000 M.W. cut-off membrane were not successful. This would appear to be due to the high solid content of the fish sauce (>38%) and the limited operating pressure of the filtration system. When a 10,000 M.W. cut-off membrane was used, approximately 70% of the sauce was able to pass the filtration unit. The filtrate had a light color and mild odor. The retentate had a dark color with strong odor, characteristic of fish sauce. The retentate and filtrate fractions were assayed for proteolytic activity, NaCl, pH, soluble protein (Biuret), total nitrogen, formol nitrogen and amino acid content. Chemical analysis of fish sauce, retentate and filtrate are summarized in Table 3-30.

3.6.1. Assay of protease activity

The assay of protease activity using both hide powder azure and azocasein showed that there was no activity in filtrate, moderate activity in fish sauce and high activity in retentate. The data indicated that ultrafiltration of fish sauce was effective in removing residual proteolytic enzyme activity. The effects of retentate volume and time on the rate of proteolysis on hide powder azure are shown in Figs 3-23 and 3-24, respectively. The effects of retentate volume and time on hydrolysis of azocasein are shown in Figs 3-25 and 3-26, respectively. It

Table 3-30: Analyses of retentate and filtrate from Millipore ultrafiltration of fish sauces prepared with and without SHP

Sample[1]	Protease[2] activity	NaCl[3] %	pH[3]	Soluble[3] protein mg/ml (Biuret)	Total[3] N mg/ml	Formol[3] N mg/ml	Free[4] a.a. μmoles/ml	Total[4] a.a. μmoles/ml
C-FS	+	27.48	6.13	25.80	8.79	4.75	216.72	405.27
C-F	-	24.23	6.16	7.00	8.65	4.13	216.93	429.80
C-R	++	24.47	6.16	38.40	10.36	4.59	223.22	750.14
SQ-FS	+++	27.04	5.99	18.53	16.28	9.72	488.26	721.22
SQ-F	-	23.45	6.03	13.27	14.70	8.55	449.22	697.71
SQ-R	++++	22.94	6.03	21.20	16.28	8.81	457.50	856.07

[1] C=Control; SQ=SHP-supplemented; F=Filtrate; R=Retentate; FS=Fish sauce.

[2] - No activity
+ Relative activity, more + indicate greater activity

[3] Values are averages of 3 determinations.

[4] Values are averages of duplicate determinations. Total amino acids determined after the samples were hydrolysed with 6 N HCl at 110°C for 24 h.

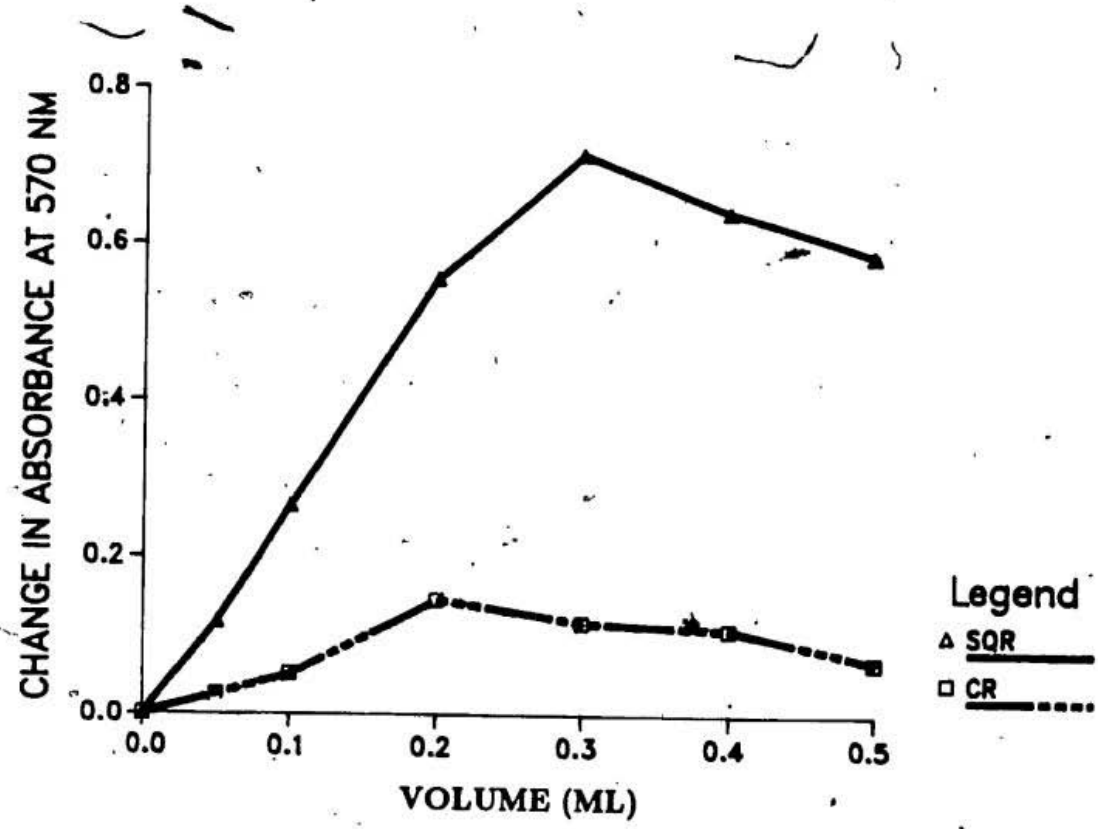


Figure 3-23: Effect of retentate volume on the rate of proteolysis on hide powder azure substrate

Values plotted are averages of duplicate analyses.
Fish sauce was concentrated 3.5-fold by a Millipore ultrafiltration unit with Pellicon cassette system M.W. cut off 10000.
Assay was at pH 6.0, incubation time 5 h at 30°C.
SQ-R=SHP-supplemented retentate; C-R=Control retentate.

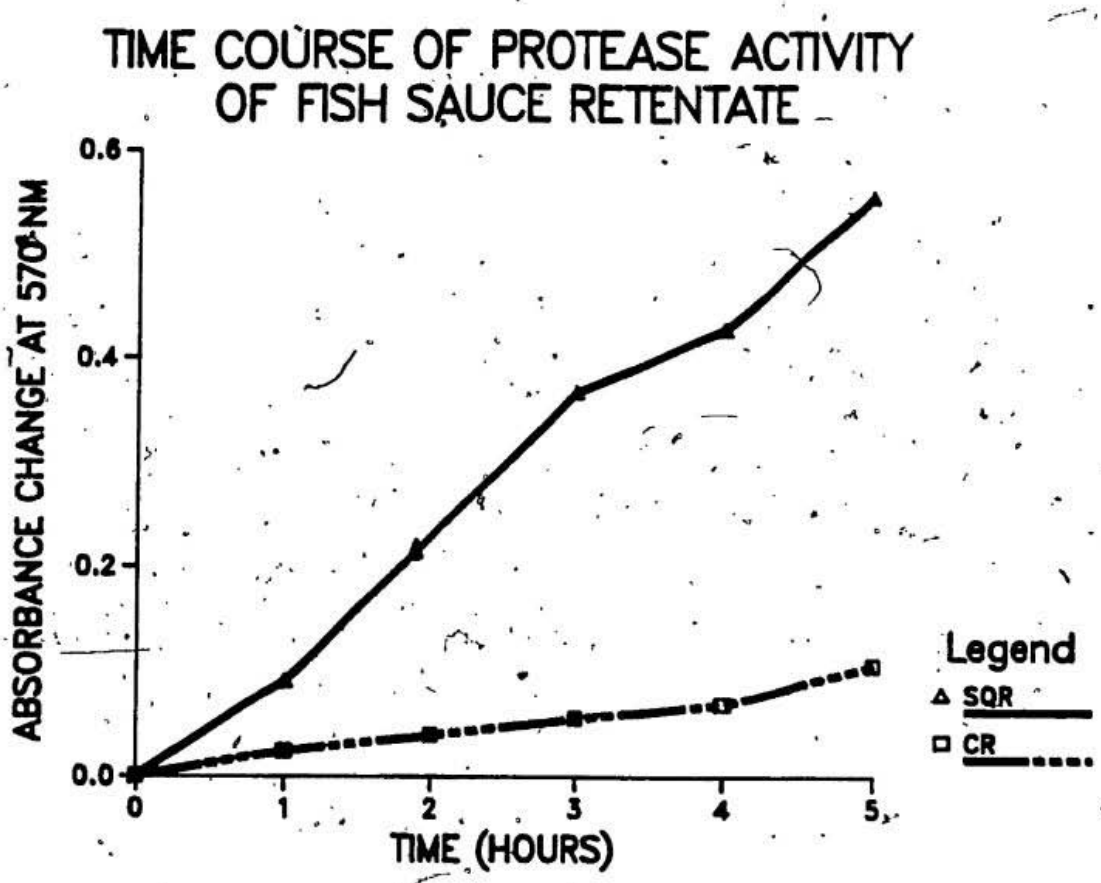


Figure 3-24: Effect of time on the rate of proteolysis on hide powder azure substrate

Values plotted are averages of duplicate analyses.
The assay mixture contained 5 mg hide powder azure, 0.2 ml of fish sauce retentate and 1.8 ml of buffer pH 6.0. Incubation time was 1-5 h at 30°C.

Slope for retentate prepared with SHP (SQ-R)=0.112, r^2=0.9957

Slope for retentate prepared without SHP (C-R)=0.020, r^2=0.9885

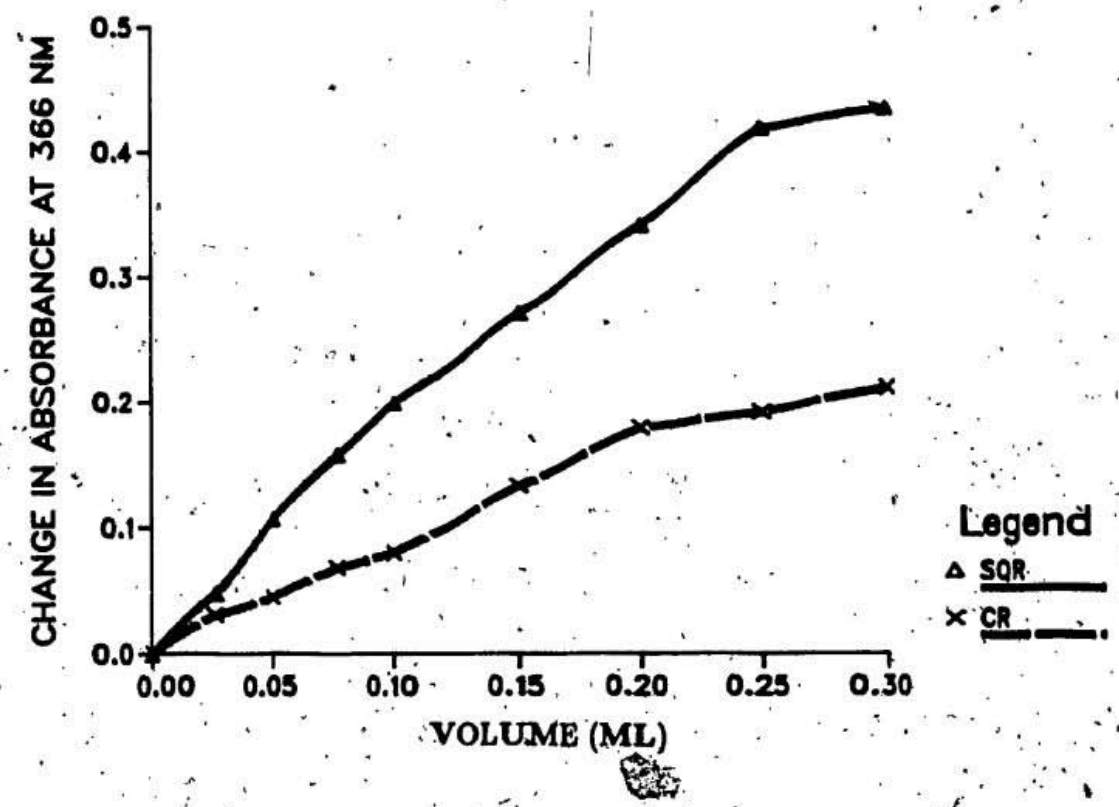


Figure 3-25: Effect of retentate volume on the rate of proteolysis on azocasein substrate

Values plotted are averages of duplicate analyses

Assay was at pH 6.0 on 0.75% azocasein, incubation time 6 h at 30°C.

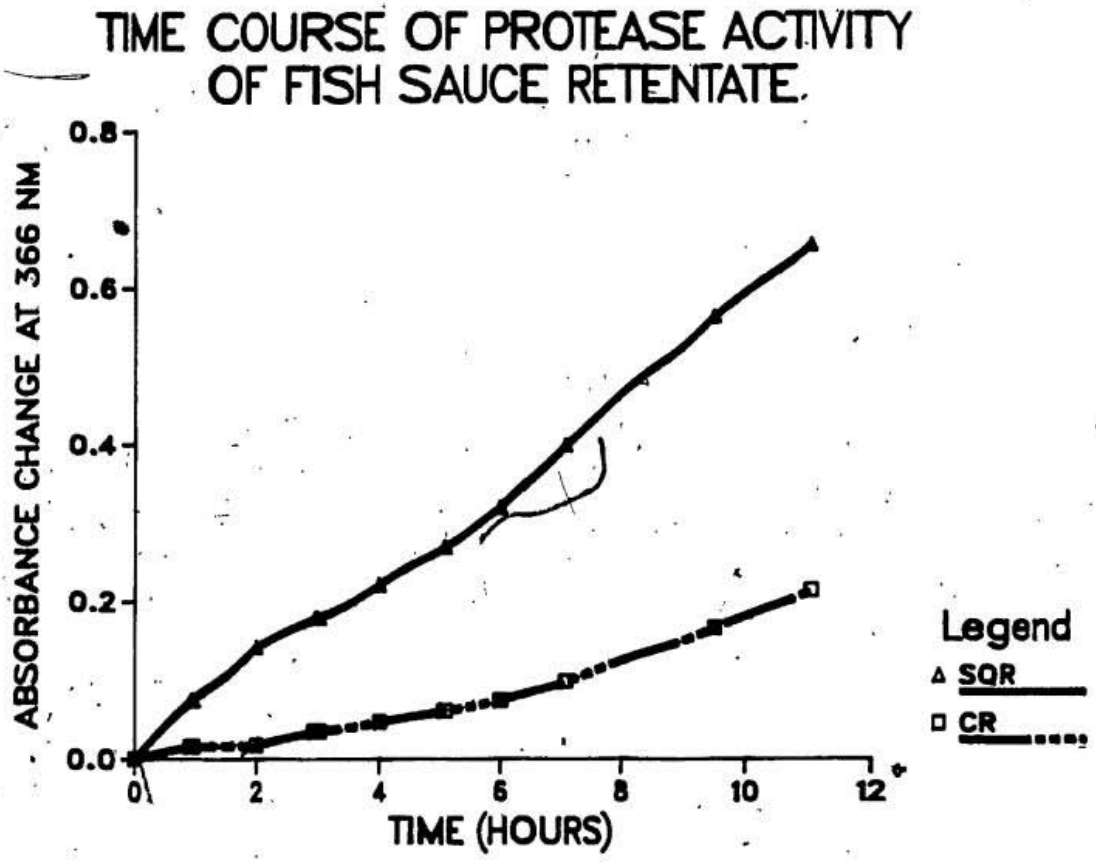


Figure 3-26: Effect of time on the rate of proteolysis on azocasein substrate

Values plotted are averages of duplicate analyses.
The assay mixture contained 0.1 ml of fish sauce retentate, 1.0 ml of 1.5% azocasein and 0.9 ml of sodium acetate-HCl buffer pH 6.0, incabation time 5 h at 30°C.

Slope for retentate prepared with SHP (SQ-R)=0.048, r^2=0.9987

Slope for retentate prepared without SHP (C-R)=0.016, r^2=0.9999

appeared from Fig 3-23 that, the activity of the proteolytic enzymes in fish sauce increased to a certain level when the volume of the fish sauce retentate was increased; after that the enzyme activity decreased indicating that there might have been some inhibitors present in the fish sauce. The decrease in the activity at higher volume of retentate was not caused by salt in the sample since the NaCl content of all samples were normalized at 25%. Orejana and Liston (1982) suggested that limited proteolysis in fish sauce fermentation was probably due to the presence of a naturally occurring trypsin inhibitor in the blood of fish and the end products of proteolysis i.e. amino acids and small peptides. The extract of internal organs of squid *Loligo vulgaris* contained protease inhibitor which inhibited proteolytic enzymes of different specificity (Tschesche and Riicker, 1973). Both aqueous and acidic extracts of the internal organs showed inhibitory activity against the proteolytic activity of trypsin and chymotrypsin. The inhibitory effect found in the SHP-supplemented retentate might be due to an endogenous inhibitor present in the squid hepatopancreas tissue.

3.6.2. Effect of pH on protease activity

The residual protease activity of control and SHP fish sauce retentate was found to be maximum at pH 4, when NaCl from fish sauce retentate accounted for approximately 0.2 M in the assay mixture (Fig 3-27). The presence of 4 M NaCl in the assay mixture shifted the pH optimum from pH 4 to pH 6 (Fig 3-28). The effect of salt on pH optimum was confirmed when higher concentration of retentate (1.0 ml) was used in the assay mixture (3 ml) which increased the NaCl concentration to 1.5 M. The pH optimum was found to be at pH 5 in both SQ-R

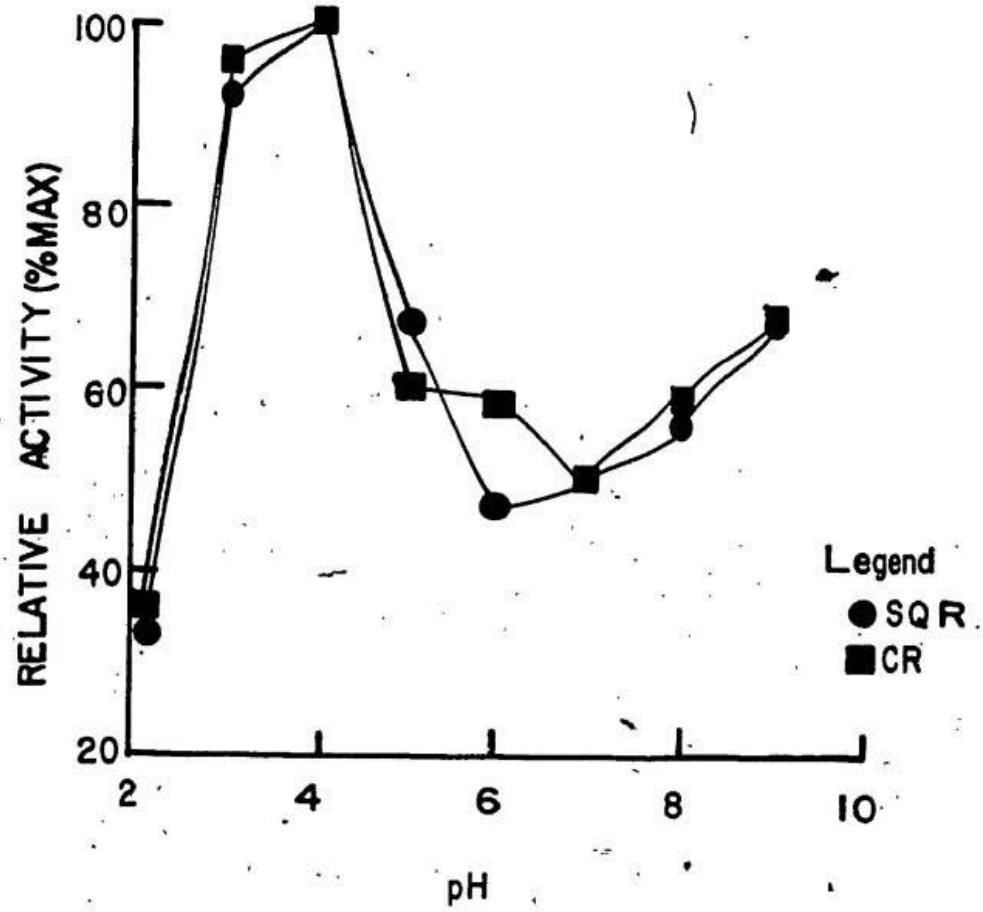


Figure 3-27: Effect of pH on protease activity of fish sauce retentate on azocasein substrate

Values plotted are averages of duplicate analyses. Sodium acetate-HCl buffer pH 6.0 was replaced by citric acid-phosphate buffer pH 2.2-8.0 (McIlvaine, 1921). NaCl concentration in the assay was 0.2 M

and C-R (Fig 3-28). This result was consistent with the results in section 3.2.3, where the pH optimum for fish sauce fermentation was found to be at initial pH of 6.0. The maximal activity at pH 4 in the presence of low concentration of salt was consistent with a previous report (Gildberg, 1982) that capelin proteases exhibited maximal activity on hemoglobin at pH 4.0. It was also reported that salt inhibits protease activity, especially the activity of pepsin at low pH (Gildberg *et al*, 1984). The shift in optimum pH might be due to the fact that salt suppressed those enzymes which are most active at pH 4 or it may be a direct effect of salt on enzyme substrate interaction causing a shift in pH optimum of certain enzyme(s).

3.6.3. Effect of NaCl on protease activity

The effect of salt on protease activity of the retentate fraction from fish sauce prepared with SHP (SQ-R) at pH 5 and 6 is shown in Fig 3-29. At pH 5.0, the activities of enzymes (DSQ-R) with the addition of salt to give final concentrations of 1, 2, 3 and 4 M (5.8, 11.7, 17.5 and 23.4%) were 56, 23, 12 and 5% of control (with no salt added), respectively. At pH 6.0 the activities were 74, 42, 23 and 13% of control, respectively. When salt was not added, the activity was higher at pH 5. The activity at pH 6 was higher with the addition of salt. Salt may inhibit the activity of certain enzymes, e.g. pepsin, at pH 4 more than others. Salt may also shift pH optimum of a given enzyme-substrate reaction from 4 to 6. The enzyme activity was more affected by NaCl at pH 5 than at pH 6.0. It can be seen that at 23.4% salt the protease activity was markedly inhibited. This probably explains why fermentation of fish sauce requires a very long time

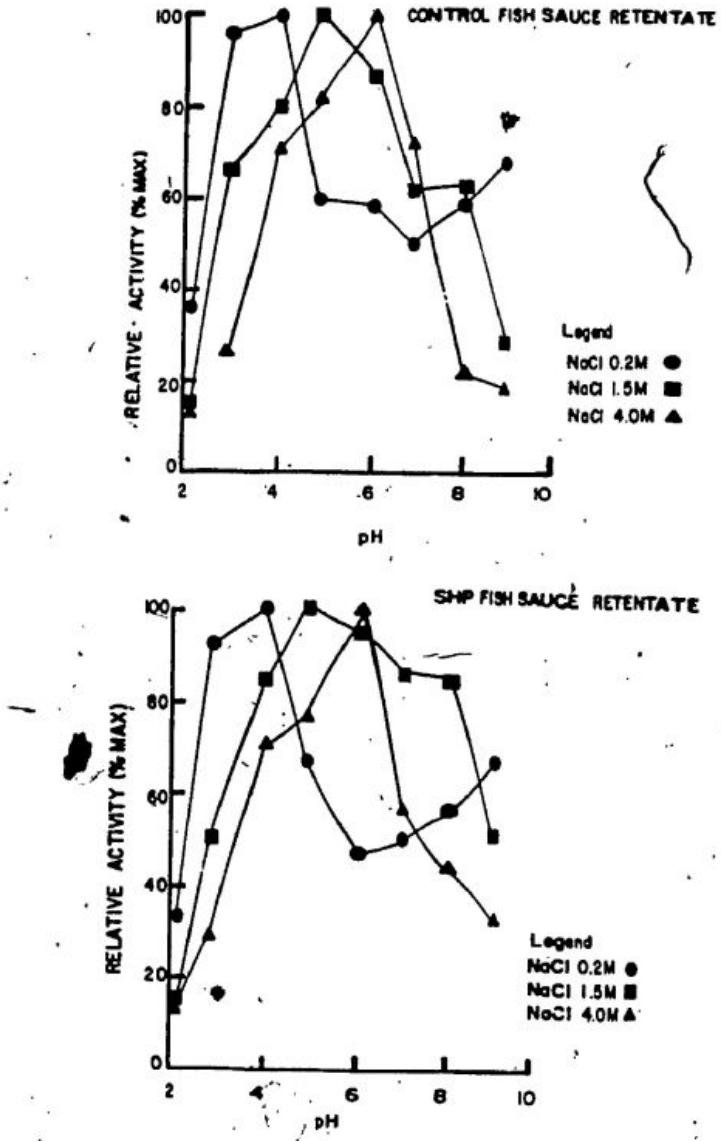


Figure 3-28: Effect of salt on pH optimum of protease activity on azocasein

Values plotted are averages of duplicate analyses.
Salt was added to give the final concentration of 4 M in the assay-buffer.
The conditions of the assay were the same as in Fig 3-27.
$\Delta A_{366\ nm}$/h-ml for retentate prepared with SHP (SQ-R)
at pH 4 without added salt = 0.761
$\Delta A_{366\ nm}$/h-ml for retentate prepared without SHP (C-R)
at pH 4 without salt = 0.401

since the NaCl content in fish sauce may be as high as 30% (w/v). At the beginning of the fermentation of capelin fish sauce, the pH of the fish-salt mixture was close to pH 6 and this is near the optimum for the fermentation.

3.6.4. Effect of inhibitors on protease activity of fish sauce

Various compounds known to inhibit specific groups of proteases were employed. Ethylene diamine tetra acetic acid (EDTA) inhibits metalloprotease activity. Soybean trypsin inhibitor (SBTI) inhibits serine proteases. Iodoacetate, mercuric chloride and PCMB inhibit sulfhydryl proteases. Results of the inhibition study are summarized in Table 3-31. In the presence of 4M NaCl alone, the activity of control (no inhibitor) was reduced by 86 and 72% in C-R and SQ-R, respectively. The inhibition by EDTA, SBTI, PCMB, iodoacetate, and $HgCl_2$ at the concentration used in the study, at low salt concentration, were 5, 29, 30, 8, and 21% for SQ-R; and 7, 24, 31, 8 and 19% for C-R, respectively. In the presence of a mixture of all three groups of inhibitors, the inhibition was found to be more than 50% at low NaCl in the reaction mixture. It was also found that at a higher concentration of SBTI (0.25 mg in the assay mixture), the inhibition was only 35%. At higher concentration of EDTA (4 mM) in the assay mixture, the proteolytic activity increased. It was apparent that the residual enzyme activity in the presence of NaCl was not sensitive to inhibitors other than those of sulfhydryl proteases.

The hepatopancreas of marine invertebrates was reported to contain high collagenolytic activity. Nip *et al.* (1985) characterized a collagenolytic enzyme from the hepatopancreas of freshwater prawn. Collagenolytic protease from

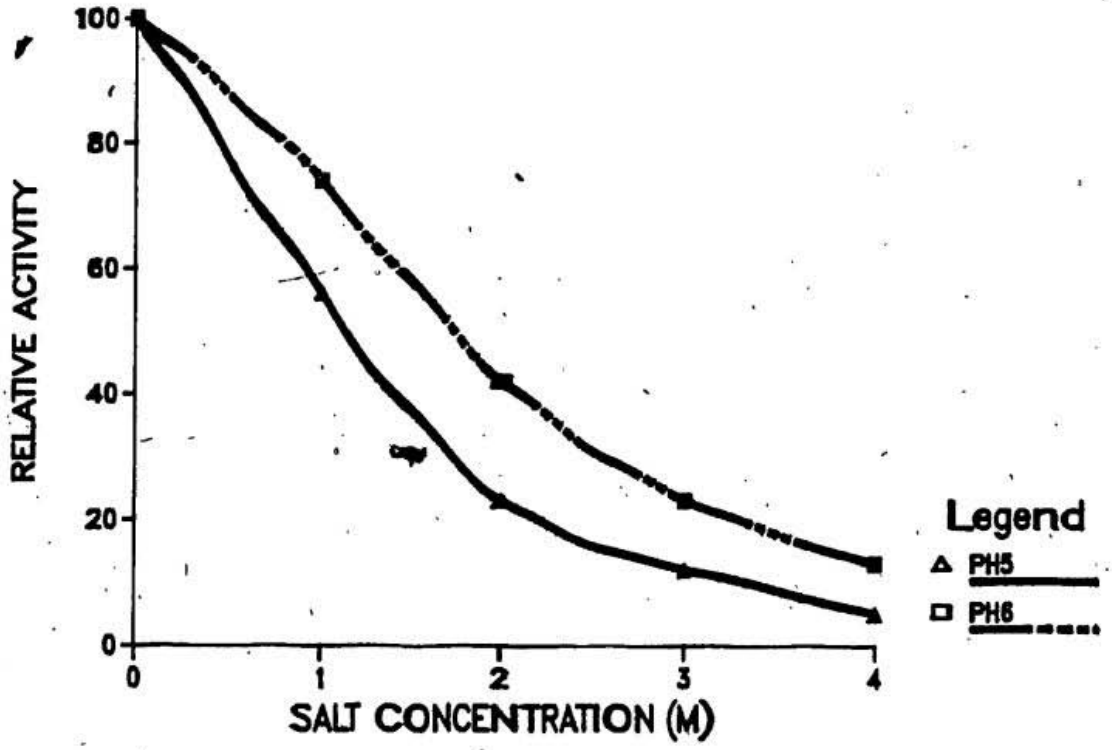


Figure 3-29: Effect of salt on the protease activity of fish sauce retentate

Values plotted are averages of duplicate analyses
Assay was carried out in 1.0 ml of 0.2 M sodium acetate-HCl buffer (pH 5 and 6), 1.0 ml dialyzed retentate prepared with SHP (DSQ-R), and 1.0 ml of 2% azocasein substrate.

$\Delta A_{366\ nm}$/h-ml at pH 5 without NaCl = 0.237

$\Delta A_{366\ nm}$/h-ml at pH 6 without NaCl = 0.191

Table 3-31: Effect of inhibitors on protease activity of fish sauce

Inhibitor	Conc. in assay mM	Activity (% Control)[1] SQ-R 0.2 M NaCl	SQ-R 4 M NaCl	C-R 0.2 M NaCl	C-R 4 M NaCl
Control	-	100	100	100	100
EDTA	1.0	95	217	93	180
	4.0	115	207	120	188
SBTI	0.05mg	71	152	76	138
	0.25mg	65	98	73	111
PCMB	1.0	70	75	69	84
Iodoacetic acid	1.0	92	118	92	106
$HgCl_2$	0.15	79	89	81	105
Mixture[2]	-	46	101	42	111

[1] Values are averages of duplicate analyses. Assay was at pH 6.0 on 0.75%-azocasein substrate, with 0.1 ml retentate, at 30°C. Activity without inhibitor of SQ-R and C-R was inhibited by NaCl at 72 and 86%, respectively. $A_{366\ nm}$ for SQ-R and C-R in the presence of 0.2 M NaCl were 0.276 and 0.110, respectively.

[2] Mixture contained 0.1 ml of SBTI, PCMB and EDTA to give a final concentration of inhibitor in the assay mixture of 0.05 mg SBTI and 1.0 mM of PCMB and 1.0 mM of EDTA.

hepatopancreas of fiddler crab was found to be different from other animal collagenases, in which its activity was not inhibited by EDTA, cysteine and o-phenanthroline and it is activated by $HgCl_2$ (Grant *et al.*, 1981). Cathepsin C hydrolase activity from squid hepatopancreas was slightly increased in the presence of EDTA but completely inhibited by $HgCl_2$, PCMB and iodoacetic acid (Hameed and Haard, 1985). Interpretation of the inhibition study with fish sauce retentate is complicated, since it is a mixture of several enzymes and it is apparent that there are natural inhibitor(s) present in fish sauce. It is possible that the marked activation of protease activity at high salt concentration in the assay is due to interaction of EDTA or SBTI with naturally ocurring inhibitors, e.g. peptides formed during fermentation.

3.6.5. Cathepsin C activity

3.6.5.1. Hydrolase activity

Gly-Arg-MNA was reported to be a specific substrate for hydrolase activity of cathepsin C (Hameed and Haard, 1985) and both SQ-R and C-R hydrolyzed Gly-Arg-MNA substrate. Fig 3-30 shows the hydrolase activity as a function of time. The finding of higher activity in SQ-R (2.8-fold) than in C-R is consistent with the previous report that SHP is a rich source of cathepsin C. Hydrolase activity of cathepsin C isolated from SHP is Cl^- dependent and the optimum pH is at pH 5.6 (Hameed and Haard, 1985).

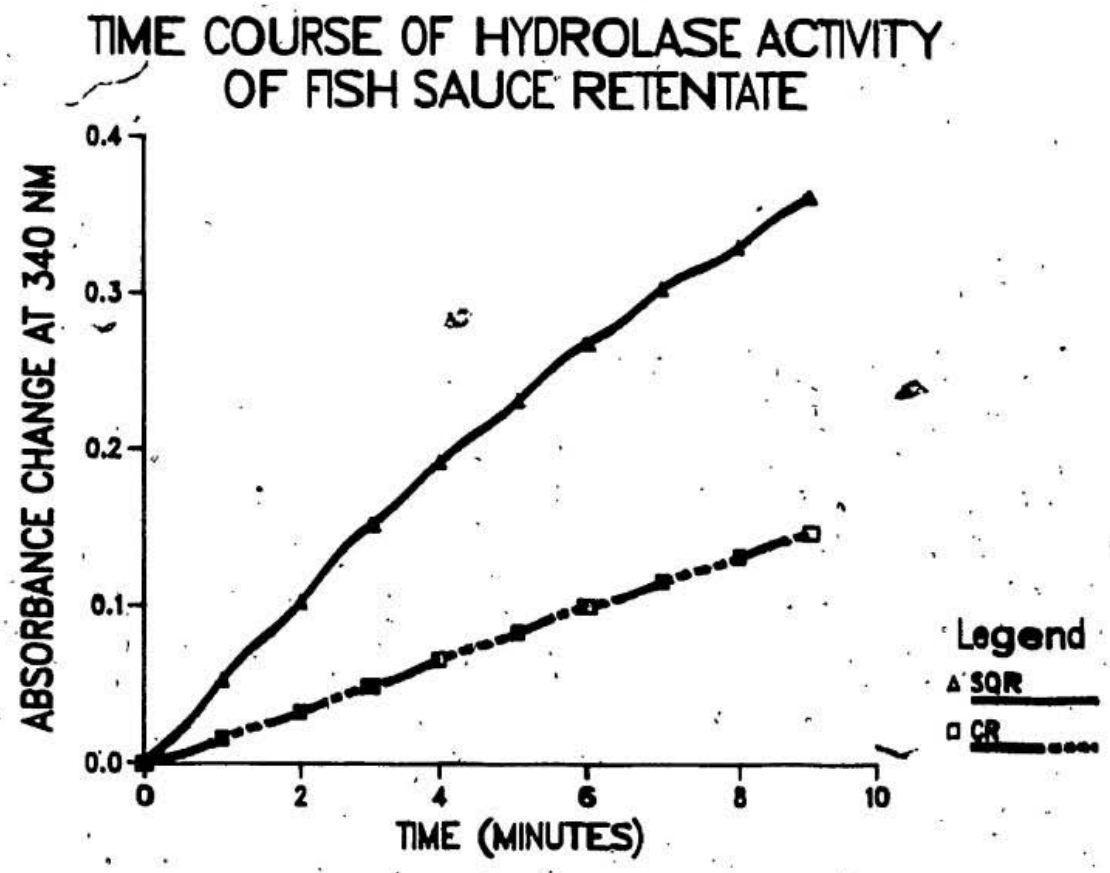


Figure 3-30: Effects of time on cathepsin C hydrolase activity of fish sauce retentate

Values plotted are averages of duplicate analyses. Assay was at pH 6.0 with 0.1 ml fish sauce retentate on Gly-Arg-MNA substrate

Slope for retentate prepared with SHP (SQ-R)=0.040, r^2=0.9949

Slope for retentate prepared without SHP (C-R)=0.016, r^2=0.9999

3.6.5.2. Transferase activity

Both SQ-R and C-R showed transferase activity with the cathepsin C specific substrate, Gly-Phe-NH_2 (Fig 3-31). The activity of SQ-R was found to be about 7-fold greater than the activity of C-R. Transferase activity of cathepsin C isolated from squid hepatopancreas was reported to be dependent on Cl^-, the maximal activity was found at pH 7 (Hameed and Haard, 1985).

3.6.5.3. Effect of NaCl on hydrolase activity

Addition of NaCl to the assay mixture to provide the concentrations of 10-40 mM increased the hydrolase activity of the dialyzed retentate (against distilled water for 72 h at 4°C, with external solution changed every 24 h) to 112% of the control (Table 3-32). The results were consistent with previous report (Hameed and Haard, 1985) that hydrolase activity of cathepsin C required Cl^-. At a higher concentration of salt (20%) in the assay mixture, the activities in non-dialyzed SQ-R and C-R reduced to 66% and 40%, respectively (Fig 3-32). It was apparent that SQ-R contained residual enzymes which were more tolerant to NaCl than were C-R. It also appeared that cathepsin C activity is more salt tolerant than the total proteolytic activity in fish sauce.

3.6.5.4. Inhibition studies

The results for inhibition of cathepsin C hydrolase activity of the fish sauce retentate are summarized in Table 3-33.

$HgCl_2$

At the concentration of 0.1 mM of $HgCl_2$ in the assay mixture, the activity

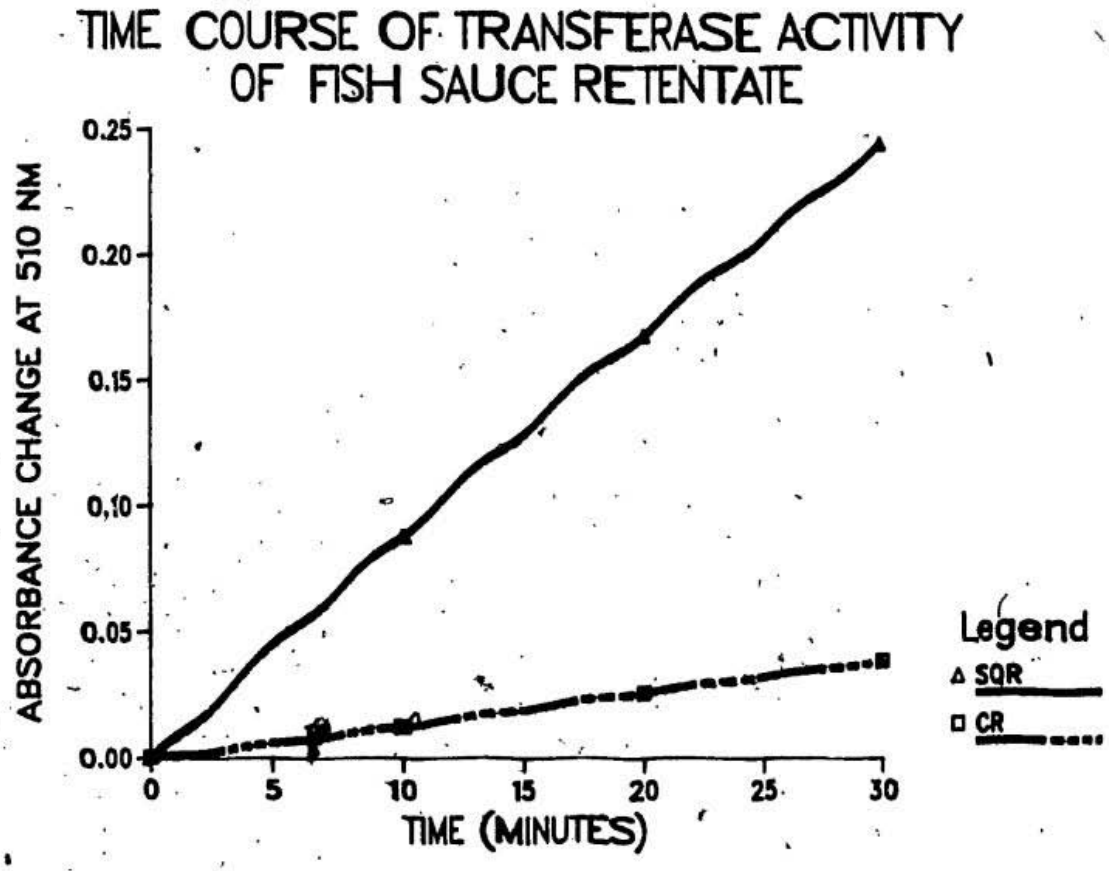


Figure 3-31: Time course of transferase activity of fish sauce retentate

Values plotted are averages of duplicate analyses.
Assay was at pH 6.8 with 0.1 ml fish sauce retentate
on Gly-Phe-NH_2

Slope for retentate prepared with SHP (SQ-R)=0.007, r^2=0.9844

Slope for retentate prepared without SHP (C-R)=0.001, r^2=0.9965

Table 3-32: Effect of NaCl on cathepsin C hydrolase activity in the retentate fraction of fish sauces prepared with and without SHP

NaCl (mM)	Relative activity[1] SQ-R[2]	C-R[2]
0	100	100
10	111	108
20	112	110
30	112	110
40	110	112

[1] Values are averages of duplicate analyses.
Assay was at pH 6 with 0.1 ml retentate on Gly-Phe-NA substrate.
Mercaptoethanolamine-HCl was replaced by mercaptoethanol.
Enzyme solutions were dialyzed against distilled water for 72 h at 4°C, with external solution changed every 24 h before used in the assay.
$\Delta A_{340\ nm}$ for dialyzed SQ-R at 0 mM NaCl = 0.022
$\Delta A_{340\ nm}$ for dialyzed C-R at 0 mM NaCl = 0.007

[2] SQ-R = SHP-supplemented retentate; C-R = Control retentate.

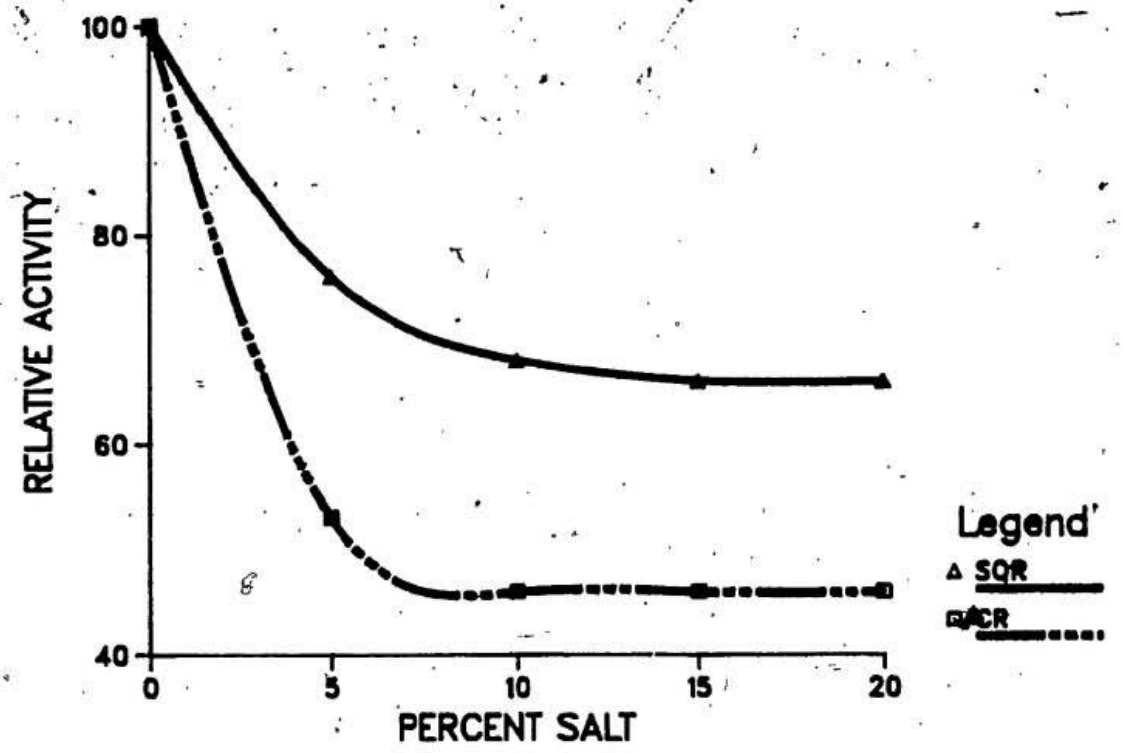


Figure 3-32: Effect of NaCl on cathepsin C hydrolase activity

Values plotted are averages of duplicate analyses.
Assay was at pH 6.0 with 0.1 ml retentate on Gly-Arg-MNA
$\Delta A_{340\ nm}$/min with 0% salt were 0.041 and 0.016
for SQ-R and C-R, respectively.

Table 3-33: Effect of inhibitors on cathepsin C hydrolase activity in the retentate fraction of fish sauce prepared with and without SHP

Inhibitor	conc (mM)	% Relative activity[1]	
		SQ-R	C-R
None	-	100	100
$HgCl_2$	0.1	91	96
Iodoacetic acid	1	12	38
PCMB	1	46	33
SBTI	0.02 mg	120	113

[1] Values are averages of duplicate analyses.
Fish sauce retentate was preincubated with inhibitors for 30 minutes prior to assay at pH 6.0 with 0.1 ml fish sauce retentate on Gly-Arg-MNA substrate. NaCl concentration in the assay mixture was 0.15 M.
$\Delta A_{340\ nm}$ for SQ-R without inhibitor = 0.045
$\Delta A_{340\ nm}$ for C-R without inhibitor = 0.018

on Gly-Arg-MNA was inhibited by 9 and 4% for SQ-R and C-R, respectively. However, when the concentration of $HgCl_2$ was increased to 1 mM, it was found that the $A_{340\ nm}$ increased to 145 and 280% of the control for SQ-R and C-R, respectively. Mercuric chloride was reported to inactivate the activity of purified cathepsin C, however, in fish sauce retentate which might contain more than one enzyme, $HgCl_2$ might react with other components e.g., endogenous inhibitors in the retentate and no longer available as an inhibitor, also, impurities in the retentate fraction may react with $HgCl_2$ more rapidly than with cathepsin C.

Iodoacetic acid

For SQ-R, at 1 mM concentration of iodoacetic acid, the activity of enzyme was inhibited by 88% but when the concentration was increased to 2 mM the inhibition was only 83%. A similar pattern of inhibition was observed with C-R, at 1 mM, the activity was inhibited by 62% and at 2 mM the inhibition was only 36%.

PCMB

After 30 min preincubation of retentate with 1 mM PCMB, the activity for SQ-R was inhibited by approximately 54% and for C-R the activity was inhibited by 67% of the control.

Soybean trypsin inhibitor

Soybean trypsin inhibitor at the concentration of 0.025 mg in the assay mixture increased the activity of SQ-R and C-R to 120 and 113%, respectively.

The ability of fish sauce retentate to hydrolyze a cathepsin C specific substrate and its inhibition by thiol inhibitors is consistent with the conclusion that fish sauce retentate contains cathepsin C. At high concentration of NaCl (20%) in the assay mixture, the maximum inhibition of hydrolase activity in SQ-R was found to be only 44%, but the protease activity on azocasein was found to be inhibited by 72% in the presence of 4 M NaCl. C-R appeared to be less tolerant to high salt. The hydrolase activity was inhibited by 54% and the protease activity was inhibited by 86%. The cathepsin C activity was higher in SQ-R than C-R. Rosario and Maldo (1984) reported on catheptic activity in fish sauce fermentation. It was found that in fish sauce prepared from fresh fish, cathepsin D activity was greater than from stale fish. Cathepsin D activity decreased when the amount of high molecular weight protein, which served as substrate for the enzyme, decreased. Cathepsin A and C were found to play an important role in proteolysis during patis fermentation. Both cathepsin A and C activities were found in the brine after 4 months' fermentation at relatively high rates compared to cathepsin B activity. Activity of cathepsin A had a positive correlation with amino nitrogen in the brine, while cathepsin C activity had a positive correlation with TCA soluble protein.

3.6.5.5. Aminopeptidase activity

Aminopeptidases isolated from viscera of sardine were reported to be stable in the presence of 15% NaCl (Vo Van *et al.*, 1983). Aminopeptidase activity was also found in fish sauce (Vo Van *et al.*, 1984). Analysis of aminopeptidases in fish sauce retentate at pH 7 showed that the activity of SQ-R was about 90% that of C-R on Leu-NA substrate. The effect of NaCl on aminopeptidase activity is

shown in Table 3-34. Aminopeptidase, like cathepsin C, appears to be a fairly salt tolerant enzyme.

3.6.6. General discussion on protease activity

The protease activities of fish sauce retentate and fish sauce prepared with and without SHP supplementation are summarized in Tables 3-35 and 3-36, respectively. General proteolytic activity with azocasein as substrate were 3.0- and 2.8-fold more active in SHP-supplemented retentate (SQ-R) and SHP-supplemented fish sauce (SQ-FS) than in control retentate (C-R) and control fish sauce (C-FS), respectively. The rate of protein hydrolysis during the first 4 weeks of fermentation, measured as mg formol nitrogen/ml fish sauce, indicated that protein hydrolysis in SHP-supplemented sauce was 4-fold the activity in the control (Fig 3-2, from 0-4 weeks, slope for control=0.83, r^2=0.9882; slope for SHP-supplemented sample=3.35, r^2=0.9732). After 4 weeks, the rate of protein hydrolysis was at a slower rate than the initial rate; this may be due to end product inhibition and/or depletion of protein and peptides which served as substrate for the enzymes.

The rate of azocasein hydrolysis by fish sauce retentate prepared with or without SHP was greatest at pH 4 in the presence of low concentration of NaCl (Fig 3-27). This was consistent with previous studies of capelin autolysis (Gildberg, 1982) or SHP hydrolysis (Hameed, 1984) which showed that acidic proteases are more active than neutral or alkaline proteases. The proteolytic enzymes identified in the hepatopancreas of *Illex illecebrosus*, namely cathepsins B, D and E by LeBlanc and Gill (1982), are acid protease, which are normally

Table 3-34: Effect of NaCl on aminopeptidase activity in the retentate fraction of fish sauces prepared with and without SHP

NaCl (%)	Relative activity[1] SQ-R[2]	C-R[2]
0	100	100
5	88	93
10	74	74
15	64	59
20	56	47
25	35	40

[1] Values are averages of duplicate analyses.
$\Delta A_{405\ nm}$/min at 0% salt for SQ-R and C-R were 0.140 and 0.150, respectively.

[2] Fish sauce was concentrated 3.5-fold by a Millipore ultrafiltration unit. Assay was at pH 7 on Leu-NA with 0.1 ml fish sauce retentate at 30°C. SQ-R = SHP-supplemented retentate; C-R = Control retentate.

Table 3-35: Protease activity of the retentate fraction of fish sauces prepared with and without SHP

Substrate	SQ-R	C-R	SQ/C
Hide powder azure ($\Delta A_{570\ nm}$/h-ml)	0.617	0.118	5.2
Azocasein ($\Delta A_{366\ nm}$/h-ml)	0.480	0.160	3.0
Hydrolase (Unit/ml) (nmoles 2-naphthylamine formed/min-ml)	710.22	290.37	2.4
Transferase (unit/ml) (μmoles dipeptide hydroxamate formed/min-ml)	139.51	19.93	7.0
Aminopeptidase (unit/ml) (μmoles p-anilide formed/min-ml)	145.1	155.5	0.9

Values are averages of duplicate analyses.
Enzyme solution was concentrated 3.5-fold by a Millipore ultrafiltration unit, with poly sulfone membrane cassette, M.W. cut off 10^4.
Assays were carried out at pH 6 except for transferase at pH 6.8, and for aminopeptidase at pH 7.
SQ-R=SHP-supplemented retentate; C-R=Control retentate.

Table 3-36: Protease activity of fish sauces prepared with and without SHP

Substrate	SQ-FS	C-FS	SQ/C
Hide powder azure ($\Delta A_{570\ nm}$/h-ml)	0.50	0.011	4.5
Azocasein ($\Delta A_{366\ nm}$/h-ml)	0.218	0.077	2.8
Hydrolase (Unit/ml) (nmoles 2-naphthylamine formed/min-ml)	78.65	39.89	2.0
Transferase (unit/ml) (μmoles dipeptide hydroxamate formed/min-ml)	54.66	14.00	3.9
Aminopeptidase (unit/ml.min) (μmoles p-anilide formed/min-ml)	13.10	16.00	0.8

Values are averages of duplicate analyses.
Assays were carried out at pH 6 except for transferase activity at pH 6.8, and aminopeptidase at pH 7.0.
C-FS=Control fish sauce; SQ-FS=SHP-supplemented fish sauce.
Fish sauce was at filtration stage after fermentation for 6 months.

most active at pH 3-4. However, acidification of SHP-supplemented salted mince resulted in a lesser degree of protein hydrolysis and free amino acid content when compared to the sample fermented at the natural fermentation pH. On the basis of the results obtained, the acid proteases appear to be of lesser importance in fish sauce fermentation than proteases active near neutral pH. It is also possible that the pH optimum of these enzymes is shifted to pH 5-6 by high salt. However, since high salt inhibits much of the enzymic activity in fish sauce, it is reasonable to conclude that these acid proteases are inhibited by salt.

In the presence of 4M salt it was found that the pH optimum of the residual enzyme activity was at pH 5-6. It appears that the neutral proteases are more stable than acid proteases in the high concentration of NaCl present in the fish sauce brine. This result was consistent with the rate of protein hydrolysis during fermentation which was highest at the initial fermentation pH of 6.

Hydrolysis of hide powder azure was 5-fold greater with retentate prepared with SHP than it was in the control retentate. The hepatopancreas of marine invertebrates appear to be a rich source of collagenolytic activity (Eisen *et al.*, 1973; Grant *et al.*, 1981; Nip *et al.*, 1985). The results of free amino acid analyses of control and SHP-supplemented sauces indicated that after 8 weeks fermentation, there was only a trace of hydroxyproline (the major amino acid in connective tissue) in the control fish sauce and it remained more or less constant. In SHP-supplemented sauce, the concentration of hydroxyproline after 8 weeks was approximately 900 nmoles/ml and increased to about 1,000 nmoles/ml at 20

weeks. The results indicate a higher collagenolytic activity in fish sauce supplemented with SHP than in the control.

The results of the inhibition studies indicated that there were more than one group of proteolytic enzymes in fish sauce. The proteolytic activity was inhibited by EDTA, PCMB and soybean trypsin inhibitor, which indicated the activity of metalloproteases, sulfhydryl proteases and serine proteases. However, on the basis of the activity expressed with high concentration of NaCl, it was found that activity with a cathepsin C specific substrate was more tolerant to salt than that with protein substrate. Cathepsin C is a salt tolerant, neutral protease previously identified in SHP (Hameed and Haard, 1985) Cathepsin C is reported to contribute to the formation of end products with a positive effect on sensory quality of fermented squid (Lee *et al.*, 1982b). Also in high salt concentration, the only effective inhibitors of general proteolytic activity were those for sulfhydryl proteases.

It is apparent that cathepsin C and other sulfhydryl proteases are the major proteolytic enzymes which remain active during the later stage of fermentation of fish sauce, especially in SHP-supplemented sauce. It is also apparent that the assay of enzyme activity in fish sauce or its retentate is quite complex due to the anomalous behavior of the system with respect to assay at a high concentration of retentate or the response of the system to a high concentration of inhibitors (EDTA, SBTI etc.).

3.7. Aging or ripening studies

Aging or ripening is the process employed after fish sauce is recovered from the fermentation tank. The process involves exposing the sauce to the sunlight for a period of 1-4 months. Saisithi (1983) stated that during the ripening period volatile compounds such as ammonia are liberated and the flavor is improved. Sedimentation also occurs at this time making the sauce remain clear after bottling. Browning reactions occur during this stage as well. In South East Asia, the temperature of fish sauce when exposed to the sun can be $> 40^{\circ}C$. Such high temperatures are known to accelerate the non-enzymatic browning reaction (Berk, 1976). Orejana (1978) reporting on the effect of aging of patis and showed there was a slight decrease in soluble protein and amino acid content with a corresponding increase in browning. Aging is also claimed to improve the flavor of patis.

After 4 months, the unaged (frozen at $-20^{\circ}C$) and aged fish sauce were not significantly different in amino acid composition (Fig 3-33 , Tables A-11 and A-12) or in sensory evaluation (Preference and triangle test), (Table 3-37). The tristimulus color measured by a Hunter-Gardner Colorimeter did not show any significant change (Table 3-38) although there was a trend toward darkening after aging.

It could be concluded that under the aging conditions employed in this study, 4-months storage did not have significant effect on the quality of fish sauce. However, the aging process which had been carried out was different from the

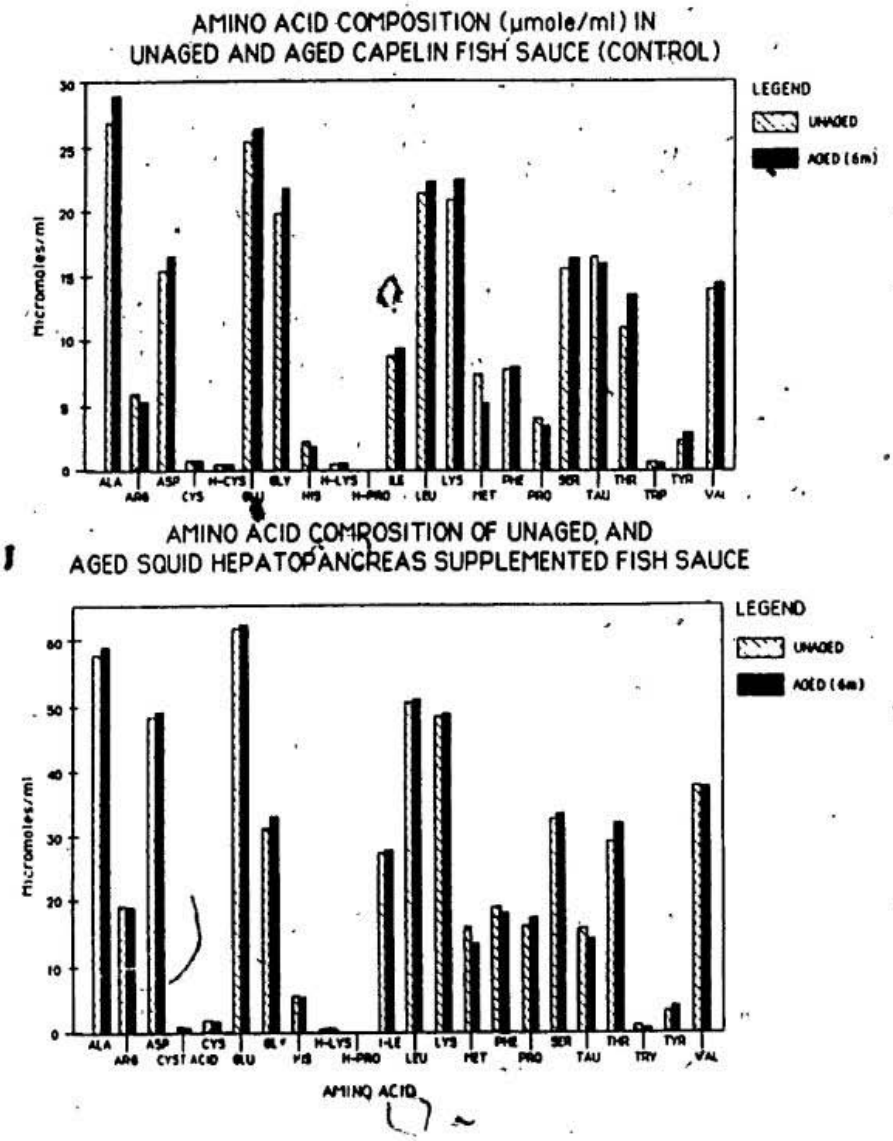


Figure 3-33: Free amino acid composition of aged and unaged fish sauce

Values plotted are averages of duplicate analyses.
Samples were fermented for 6 months, unaged sample was kept frozen at -20°C; aged sample was kept in sealed jar at ambient temperature.

Table 3-37: Sensory evaluation of aged and unaged fish sauces prepared with and without SHP

Sample[1]	Triangle test[2]		Preference score[3]
	Correct	Incorrect	
Aged C-FS vs unaged C-FS	5	3	Aged C-FS 5.4 a unaged C-FS 5.4 a
Aged SQ-FS vs unaged C-FS	4	4	Aged SQ-FS 8.1 b unaged SQ-FS 8.2 b

[1] Aged fish sauce was kept at ambient temperature for 4 months.
Unaged fish sauce was kept frozen at -20°C.
C-FS=Control; SQ-FS=Squid hepatopancreas supplemented.

[2] Samples are not significantly different ($P<0.05$), $n = 8$.

[3] Values followed by the same letter are not significantly different ($P<0.05$), $n = 8$.

Table 3-38: Changes in tristimulus color indices during aging of fish sauces prepared with and without SHP.

Sample[1]	L[2]	a[2]	b[2]	E[3]
C-FS				
Unaged	31.5	9.9	20.2	64.5
Aged 2 m	30.7	8.4	20.1	65.0
Aged 6 m	29.1	12.2	18.4	66.7
SQ-FS				
Unaged	19.0	17.5	12.2	76.2
Aged 2 m	18.6	17.4	12.2	76.6
Aged 6 m	17.6	18.0	11.5	77.6

[1] C-FS=Control fish sauce; SQ-FS=SHP-supplemented fish sauce.
Unaged fish sauce was kept at -20°C.
Aged fish sauce was kept in sealed jar at ambient temperature.

[2] Values are averages of duplicate analyses.

[3] Values calculated from average values.

process used in Thailand. Ok *et al.* (1982b) reported that at 30°C and under reciprocal shaking, the ripening process was accelerated but at 37°C or higher temperatures the typical aroma was not developed. Thus it could be that the ripening process might involve incorporation of air into the fish sauce to promote oxidation reactions.

3.7.1. Influence of the retained enzyme activity on aging process

To test the hypothesis that residual enzymes in filtered fish sauce contribute to flavor development during aging, the fish sauce retentate was heat-treated to inactivate enzymes prior to its addition back to the filtrate and incubation at ambient temperature for 4 months. Free and total amino acid contents of the mixtures (R + F and Heated R + F) are shown in Fig 3-34 and Table A-13. The two samples contained approximately the same concentration of amino acids, the amino acid profiles were also similar. Sensory evaluation using triangle test also showed that there was no significant difference between R + F and Heated R + F (Table 3-39).

3.8. Separation of peptides in fish sauce

3.8.1. HPLC

The use of HPLC (TSK 125 column) was employed to fractionate fish sauce prepared with and without SHP. According to the manufacturer, the molecular weight range of the column is 500-60,000. The Bio-Rad gel filtration standard of thyroglobulin (M.W. 670000), gamma globulin (158000), ovalbumin (47000), myoglobin (17000) and vitamin B-12 (1350) had retention times of 12.2, 14.8, 17.4,

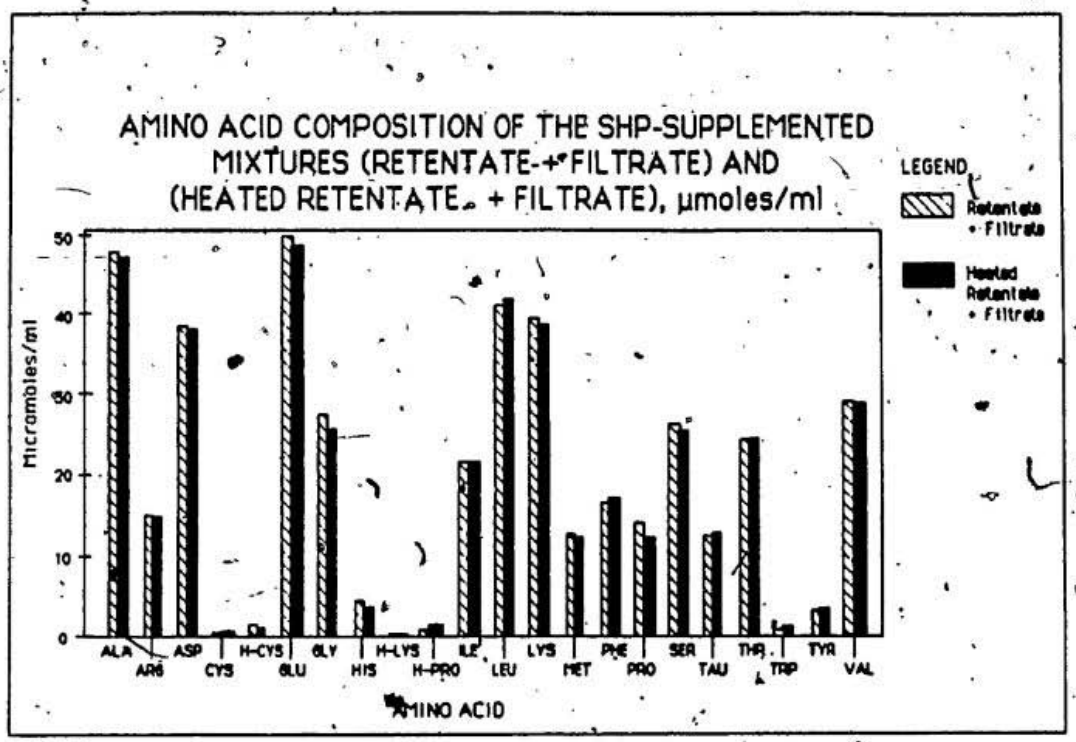


Figure 3-34: Free amino acid concentration of fish sauce mixture

Values plotted are averages of duplicate analyses. Mixtures of filtrate and retentate (6:1) were kept in sealed jar for 4 months at ambient temperature

Table 3-39: Sensory evaluation of retentate and filtrate mixture, with and without heating, after 4 months aging

Fish sauce	Fraction compared	Triangle test[3]	
		Correct	Incorrect
Control	Filtrate + retentate[1] vs filtrate + heated retentate[2]	4a	4
SHP supplemented	Filtrate + retentate[1] vs filtrate + heated retentate[2]	3a	5

[1] Fish sauce retentate and filtrate was mixed at the ratio of 1:6 and kept at ambient temperature for 4 months.

[2] Fish sauce retentate was heated at 100°C for 10 min.

[3] Values followed by "a" show that the fractions compared are not significantly different, ($P<0.05$), n=8.

23.8 and 28.2 min, respectively, with coefficient of determination of 0.98. The low molecular weight standard employed were ,Bacitracin C (M.W. 1400, retention time 29.7 min), N-acetyl-L-phenylalanyl-L-diiodotyrosine (M.W. 622, r.t. 21.9), Gly-Pro-Ala (M.W. 243, r.t. 23.2) Trp (M.W. 204, r.t. 30.8) and Tyr (M.W. 181, r.t. 25.3), with coefficient of determination=0.12. Analysis of fish sauce by HPLC, resulted in the separation of different peaks but, since there was no reliable standard curve available, the approximate molecular weight of the compounds could not be accurately obtained. HPLC does not yet appear to have been used in the direct estimation of peptides in foods, although several methods have been published for use in sequence studies (Williams, 1982). Chromatograms of fish sauce at different fermentation time are shown in Figs 3-35 and 3-36. The chromatograms of different fractions of fish sauce are shown in Fig 3-37.

Squid hepatopancreas supplemented mince appears to be depleted of high molecular weight substance (retention time $<$ 20 min) at 4 weeks fermentation (Fig 3-36), while higher molecular weight substances were retained in control fish sauce even after 33 weeks of fermentation (Fig 3-35).

Fractionation of fish sauce prepared with SHP by ultrafiltration with a Millipore unit, Pellicon cassette system into retentate and filtrate fractions appeared to selectively remove high molecular weight components from the filtrate fraction (Fig 3-37).

Interpretation of these results is limited since it appears that, for low molecular weight amino acids and peptides, the relationship between molecular size and retention time is not readily predictable.

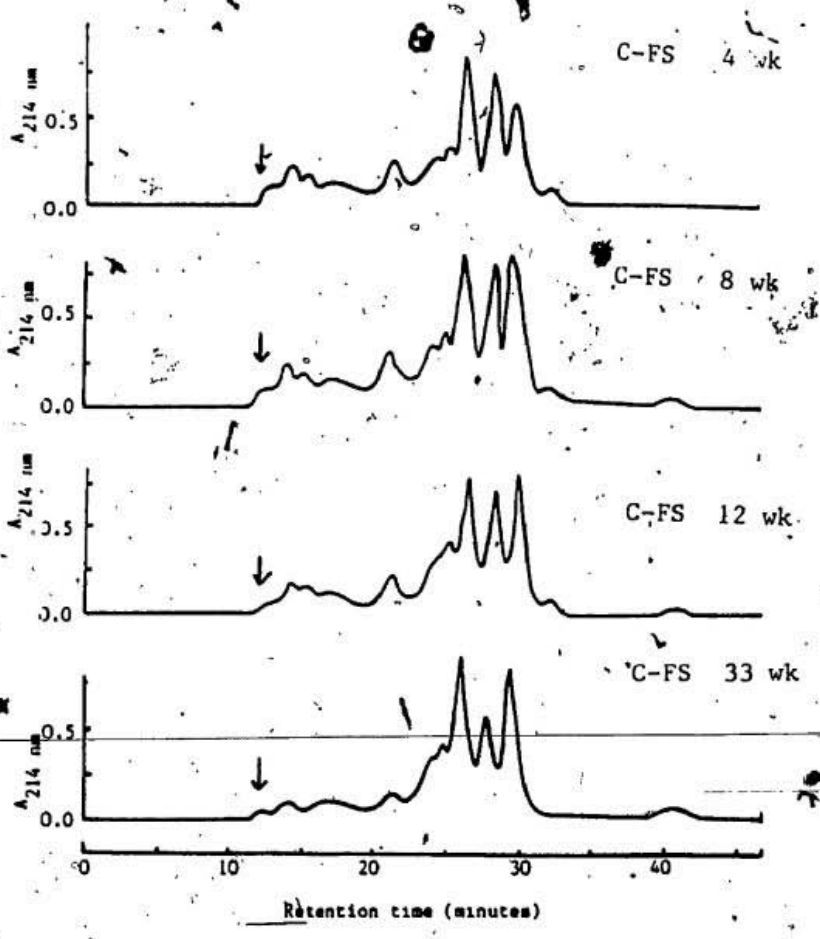


Figure 3-35: HPLC chromatograms of fish sauce at different fermentation times

Sample: Control fish sauce at different fermentation times.
Diluted 1:1 with 0.1 M Na_2SO_4, 0.02 M NaH_2PO_4 (pH 6.8).

Sample Size: 10 µl.
Column: 2 x Bio-Sil TSK-125 HPLC Gel Filtration Column, 300 x 7.5 mm.
Eluant: 0.1 M Na_2SO_4, 0.02 M NaH_2PO_4 (pH 6.8).
Flow rate: 1.0 ml/min.
↓: Void volume

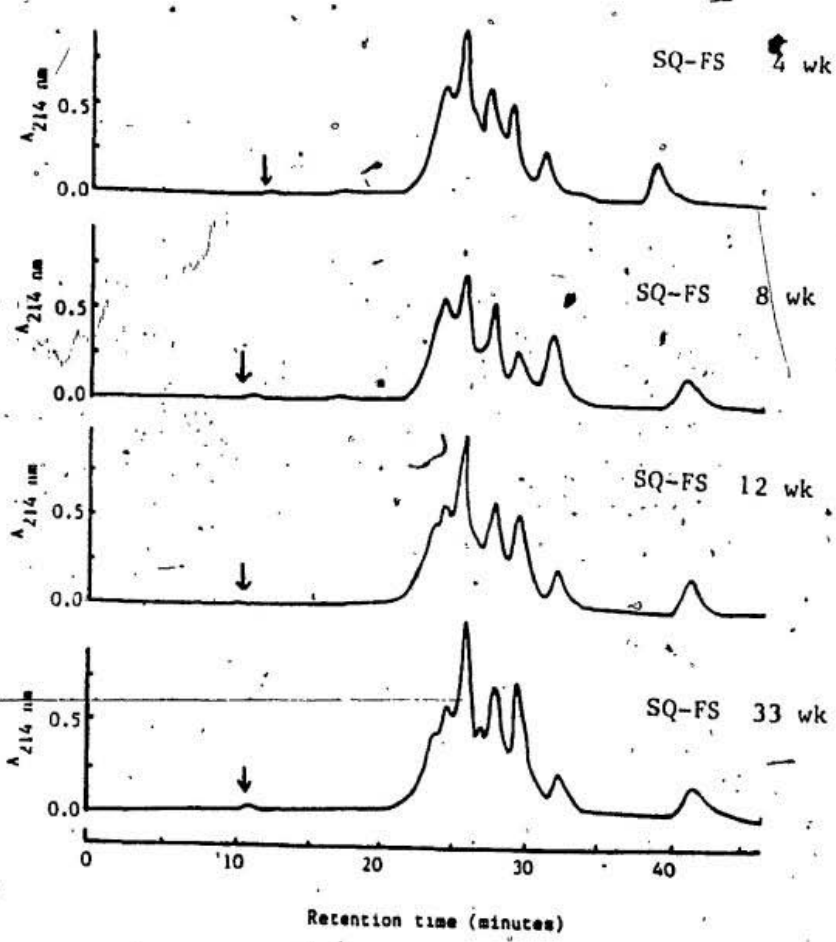


Figure 3-36: HPLC chromatograms of fish sauce at different fermentation times

Sample: SHP-supplemented fish sauce at different fermentation times. Diluted 1:2 with 0.1 M Na_2SO_4, 0.02 M NaH_2PO_4 (pH 6.8).

Sample Size: 10 μl.

Column: 2 x Bio-Sil TSK-125 HPLC Gel Filtration Column, 300 x 7.5 mm.

Eluant: 0.1 M Na_2SO_4, 0.02 M NaH_2PO_4 (pH 6.8).

Flow rate: 1.0 ml/min.

↓ : Void volume

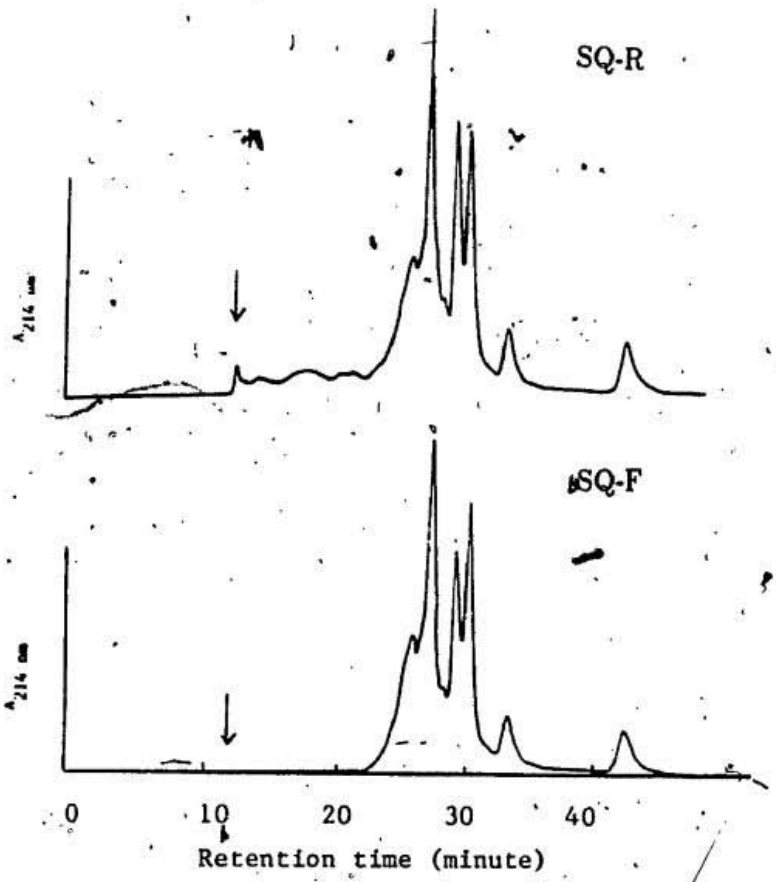


Figure 3-37: HPLC chromatograms of different fractions of fish sauce

Sample: SHP-supplemented fish sauce, fractionated by a Millipore ultrafiltration, M.W. cut off 10,000.
SQ-R = Retentate fraction; SQ-F = Filtrate fraction.
Samples were diluted 1:2 with 0.1 M Na_2SO_4, 0.02 M NaH_2PO_4 (pH 6.8).
Sample Size: 10 µl.
Column: 2 x Bio-Sil TSK-125 HPLC Gel Filtration Column, 300 x 7.5 mm.
Eluant: 0.1 M Na_2SO_4, 0.02 M NaH_2PO_4 (pH 6.8).
Flow rate: 1.0 ml/min.
↓: Void volume

3.8.2. Bio Gel P-2

The retention time for gel filtration chromatography of standards on Bio Gel P-2 and log molecular weight showed the coefficient of determination (r^2) = 0.99 (Fig 3-38). Molecular size separation range of Bio Gel P-2, according to the manufacturer, is 100-1800. It was found that for aromatic amino acids or peptides containing aromatic amino acids, there was no correlation between molecular weight and retention time. Glutamic acid, an acidic amino acid, had a longer retention time than did basic or neutral amino acids on the basis of molecular weight.

Chromatograms of the control and SHP-supplemented fish sauce are shown in Fig 3-39. The chromatograms of different ultrafiltration fractions of control and SHP-supplemented fish sauce are shown in Figs 3-40 and 3-41, respectively. The estimations of the molecular weights of the peaks obtained are summarized in Tables 3-40 and 3-41. Amino acid analysis of isolated peaks is summarized in Table 3-42. Bio Gel P-2 separated the components in fish sauce into 6 major fractions. It was found that control fish sauce contained more material of M.W. > 1300 daltons than the SHP-supplemented sauce (peak 1, Fig 3-39). This first fraction was negative to ninhydrin reaction. A small peak of estimated M.W. between 500-1,000 daltons, was resolved only in SQ-FS (retention time app. 200 min, Fig 3-39). The result of amino acid analysis indicated that this peak was peptides containing aspartic acid, threonine, serine, glutamic acid, glycine, alanine and lysine. Apparently, M.W. of the largest peak (peak 3, retention time 270 min, Fig 3-39) in both control and SQ-FS fell between the range of 100 and 300

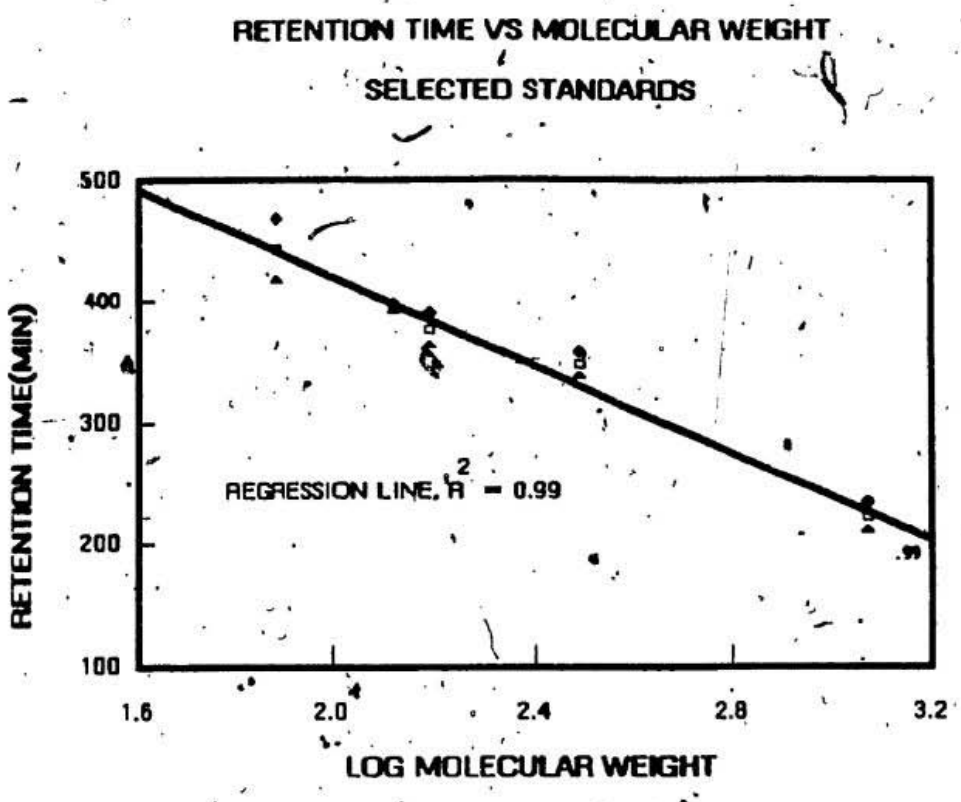


Figure 3-38: Standard curve of molecular weight vs retention time

Values plotted are averages of 3 determinations. Standards were glycine (75), leucine (131), histidine (155), glutathione (307), cyanocobalamine (1165).

Sample Size: 0.25 ml.
Column: Bio-Gel P-2 Gel Filtration chromatography column, 80 x 1.5 cm.
Eluant: 0.05 M HCl containing 0.1 M NaCl.
Flow rate: 20 ml/h.

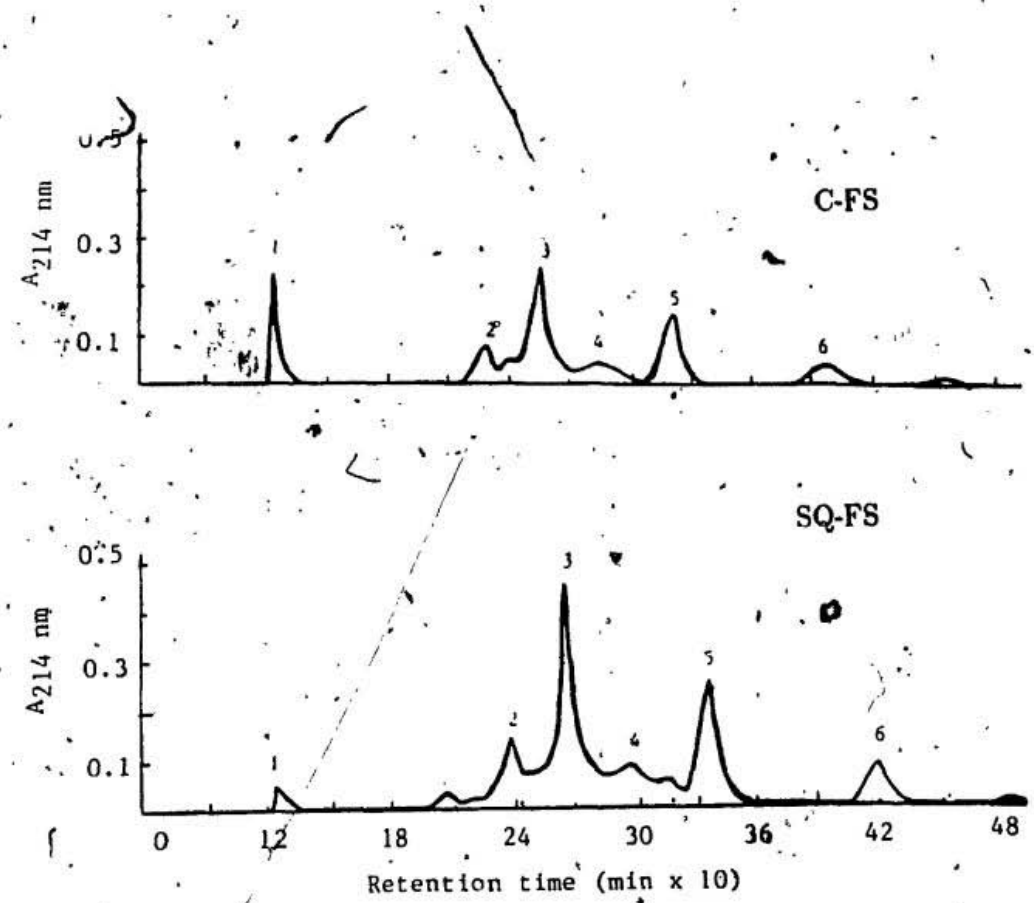


Figure 3-39: Bio-gel P-2 column chromatography of control and SHP-supplemented fish sauce after fermentation for6 months

Sample: Fish sauce prepared with (SHP) or without SHP (Control)
Diluted 1:9 with 0.05 M HCl containing 0.1 M NaCl.
Sample Size: 0.25 ml.
Column: Bio-Gel P-2 Gel Filtration chromatography column, 80 x 1.5 cm.
Eluant: 0.05 M HCl containing 0.1 M NaCl.
Flow rate: 20 ml/h.

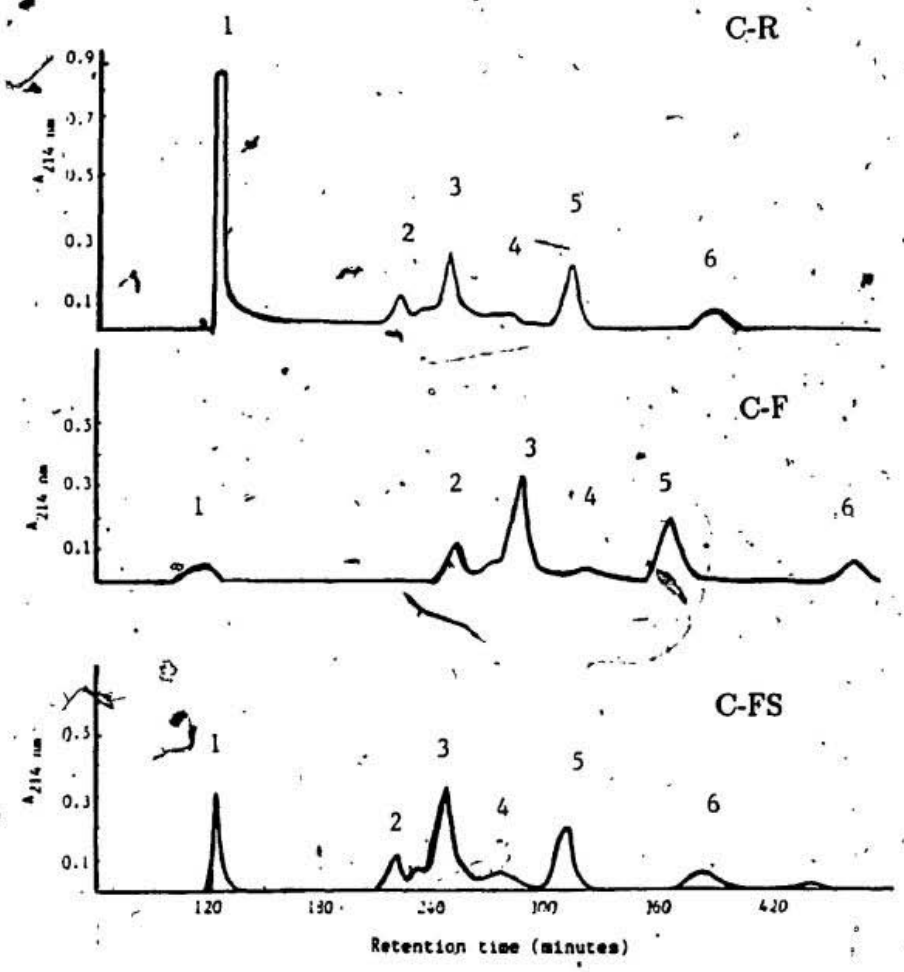


Figure 3-40: Bio-gel P-2 column chromatography of different fractions of fish sauce after 6 months fermentation

Sample: Control fish sauce was fractionated with a Millipore ultrafiltration unit, M.W. cut off 10,000 and diluted 1:9 with 0.05 M HCl containing 0.1 M NaCl.
Sample Size: 0.25 ml.
Column: Bio-Gel P-2 Gel Filtration chromatography column, 80 x 1.5 cm.
Eluant: 0.05 M HCl containing 0.1 M NaCl.
Flow rate: 20 ml/h.

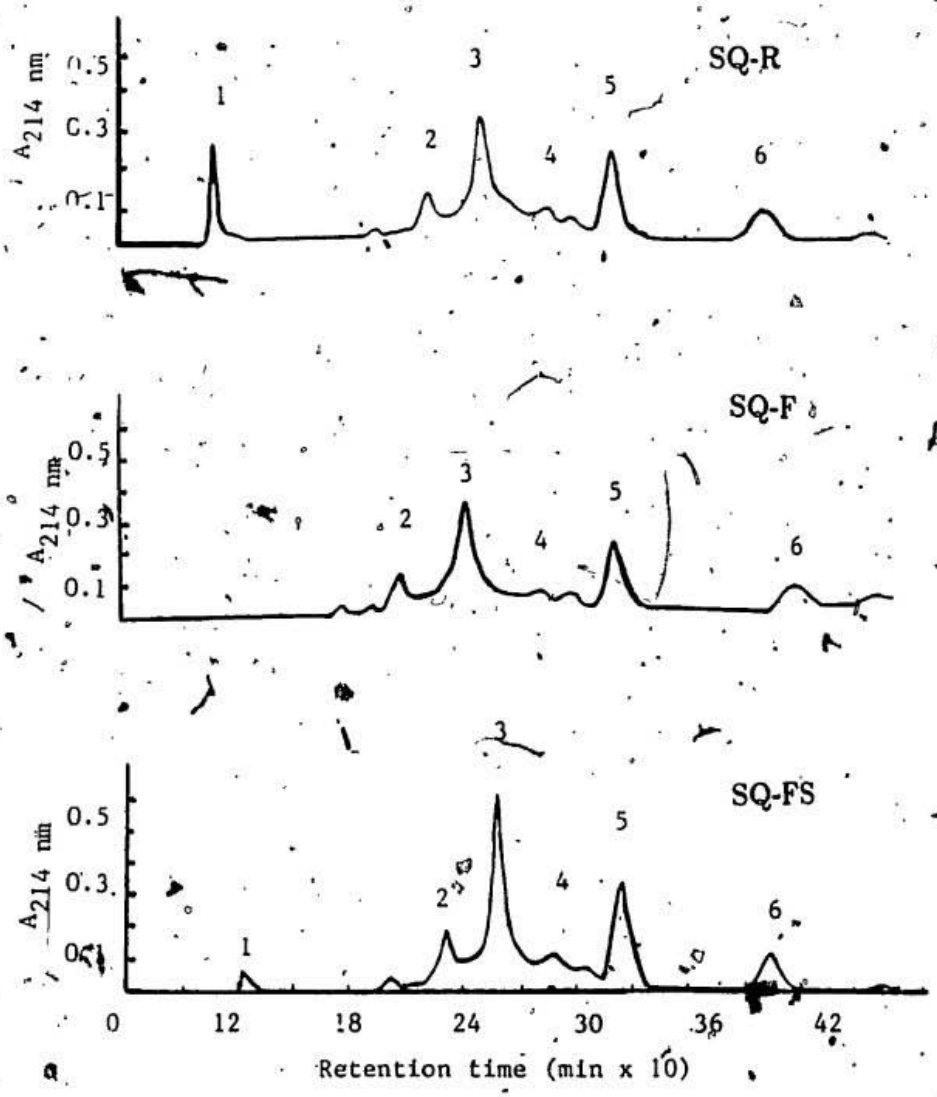


Figure 3-41: Bio-gel P-2 column chromatography of different fractions of fish sauce after 6 months fermentation

Sample: SHP-supplemented fish sauce was fractionated with a Millipore ultrafiltration unit, M.W. cut off 10,000 and diluted 1:9 with 0.05 M HCl containing 0.1 M NaCl.
Sample Size: 0.25 ml.
Column: Bio-Gel P-2 Gel Filtration chromatography column, 80 x 1.5 cm.
Eluant: 0.05 M HCl containing 0.1 M NaCl.
Flow rate: 20 ml/h.

Table 3-40: Estimated molecular weights of fish sauce and fractions from fish sauce prepared without SHP

M.W. range	Peak[3]	Area (% Total) C-FS	C-F	C-R
>1300[1]	1	13.3	2.3	43.6
500-1000	-	-	-	-
300-500	2	9.1	8.6	6.2
100-300	3	28.8	34.1	15.8
<100	4	9.6	6.0	3.5
?[2]	5	27.0	29.0	21.0
?	6	7.2	9.7	7.6
?[1]	-	4.9	10.4	2.3

[1] Negative ninhydrin reaction
[2] Probably glutamic acid and peptide containing glutamic acid
[3] Figure 3-39
C-FS=Control fish sauce; C-F=Control filtrate; C-R=control retentate.

Table 3-41: Estimated molecular weights of fish sauce and fractions prepared with SHP

M.W. range	Peak[3]	Area (% Total) SQ-FS	SQ-F	SQ-R
>1300[1]	1	1.3	-	10.0
500-1000	-	0.7	1.2	0.7
300-500	2	3.8	5.2	5.7
100-300	3	34.7	35.4	24.5
<100	4	3.7	1.1	4.1
?	-	2.9	5.3	2.6
?[2]	5	26.7	30.6	22.3
?	6	6.5	10.8	10.4
?[1]	-	7.1	10.4	8.9
?[1]	-	12.6	-	10.4

[1] Negative ninhydrin reaction.

[2] Probably glutamic acid and peptides containing glutamic acid.

[3] Figure 3-39.

SQ-FS=SHP-supplemented fish sauce; SQ-F=SHP-supplemented filtrate; SQ-R=SHP-supplemented retentate.

Table 3-42: Major amino acids in fractions from Bio-gel P-2 chromatography of fish sauces prepared with and without SHP

	C-FS		SQ-FS	
	Amino acid (Mole %)			
Peak	Free amino acid	Hydrolysate amino acid	Free amino acid	Hydrolysate amino acid
1		Asp(11) Thr(6) Ser(12) Glu(19) Pro(6) Gly(11) Ala(10) Leu(10) Lys(9)		Asp(8) Thr(5) Ser(14) Glu(12) Gly(41) Leu(13) His(4)
2	Lys(98)	Pro(7) Gly(7) Lys(60) 1-Met-His(5)	Pro(18) Lys(80)	Pro(20) Gly(4) Lys(65)
3	Pro(19) Ala(48) Lys(32)	Pro(19) Gly(7) Ala(34) Lys(22)	Gly(12) Ile(44) Leu(26)	Ser(37) Glu(11) Gly(11) Ile(16) Leu(10) His(11)
4	Gly(26) Ala(50) Val(34) Leu(21)	Thr(10) Pro(6) Gly(21) Ala(26) Val(18) Leu(14)	Asp(9) Glu(82)	Asp(11) Glu(72) Gly(5)
5	Glu(100)	Asp(30) Glu(57) Gly(4)	Asp(59) Glu(37)	Asp(53) Glu(34) Gly(5)

[1] C-FS=Control fish sauce.

[2] SQ-FS=SHP-supplemented fish sauce.

daltons. The major components of this peak in control fish sauce were free proline, alanine and lysine along with peptides containing glycine. In SHP-supplemented fish sauce, the major free amino acids were glycine, isoluecine and leucine and the major amino acid residues in the peptide fraction were serine, glutamic acid and histidine (Table 3-42). The fifth peak was found to be predominantly free aspartic and glutamic acids and peptides containing these two amino acids. The results from amino acid analysis indicated that the sixth peak did not contain amino acids or peptides. The percentages of free and peptide amino acid in each peak are shown in Appendix Table A-14. The amino acid concentrations in each peak are shown in Appendix Table A-15.

Fractionation of fish sauce with a Millipore ultrafiltration unit, Pellicon cassette system M.W. cut off 10000, into retentate and filtrate fractions appeared to remove most of the substances with M.W.>1300 from the filtrate (Figs 3-40 and 3-41). The control retentate fraction contained very high concentrations of substances with M.W.>1300 compared to the control fish sauce and the SHP-supplemented retentate. However, similar patterns of resolution in each fraction were obtained.

The molecular size of the major polypeptides and oligopeptides in fish sauce fermented for 40 days was reported to be between 700-1500 daltons (Orejana and Liston, 1979). The previous workers used Sephadex G-10 gel filtration chromatography.

3.8.3. Importance of peptides to the flavor of fish sauce

The results from sensory evaluation (Table 3-43) indicated that the fish sauce retentate and the filtrate were significantly different. All the panelists could detect the difference between the filtrate and the retentate in both control and SHP supplemented samples and all preferred the retentate. According to the panelists, fish sauce retentate had a more brothy taste than the filtrate. The filtrate was also thought to be more salty than the retentate. The results of chemical analyses indicated that there was no significant difference between NaCl content of retentate and filtrate. Peptide amino acids (hydrolyzate-free amino acids) in control and SHP retentate fractions were, respectively, 70 and 47% but peptide amino acids in control and SHP filtrate fractions were only 50 and 36% of the total amino acids. Biuret soluble protein and total nitrogen were also higher in retentate than in filtrate fraction (Table 3-30). C-R contained a higher concentration of soluble protein (38 mg/ml Biuret) than SQ-R (21 mg/ml), and a higher amount of substances with M.W.$>$1300; however, the sensory preference score of SQ-R was higher than C-R ($P<0.05$). It was apparent that larger molecules in retentate contribute to the brothy taste as indicated by sensory preference score, but the typical flavor was a combination effect of both free amino acids and peptides. Taste of peptides from fish protein concentrate (FPC) hydrolyzate was studied by Fujimaki *et al.* (1973). It was found that the fraction of M.W.$<$1000 was responsible for the acceptable taste of the hydrolyzate. After free glutamic and aspartic acids were removed from this fraction, it exhibited a brothy taste nearly as intensive as before the removal. Acidic oligopeptides of M.W.$<$1000 were found to have a strong brothy taste and have a favorable after-

Table 3-43: Sensory evaluation of retentate and filtrate of fish sauce prepared with and without SHP

Fish sauce	Fraction compared	Triangle test[2]		Preference score[3]
		Correct	Incorrect	
Control	Retentate vs filtrate	8a	0	Retentate 5.6a
	Retentate vs fish sauce	6c	2	Filtrate 3.0b
	Filtrate vs fish sauce	6c	2	Fish sauce 5.4a
SHP supplemented	Retentate vs filtrate	8a	0	Retentate 8.5c
	Retentate vs fish sauce	7b	1	Filtrate 5.5a
	Filtrate vs fish sauce	7b	1	Fish sauce 7.8c

[1] Samples were fermented for 6 months, filtered through a Millipore filtration unit with polysulfone filter Pellicon Cassette system, M.W. cut off 10,000, and kept at ambient temperature for 4 months.

[2] Values followed by a, b or c indicate the significant difference between the fractions compared at $P<0.001$, 0.01 and 0.05, respectively; n=8.

[3] Values followed by the same letter are not significantly different ($P<0.05$); n=8.

taste effect. Fraction of M.W.>5000 had very weak brothy taste. They concluded that the brothy taste of Pronase-treated FPC was due not only to free glutamic acid but also due to acidic peptides of M.W.<1000.

3.9. Use of fish sauce as a flavoring agent in a surimi-based product

Surimi is a Japanese term meaning minced fish flesh that has been water-washed. Cryoprotectants such as sugar, sorbitol and polyphosphate are used to retain the functional properties of the mince when surimi is kept frozen. It is used as a base material for the manufacture of imitation shellfish products, e.g., crab leg, shrimp, scallop, lobster. Synthetic flavoring agents as well as shellfish meat have been used to prepared a product with a flavor similar to shellfish. Since fish sauce supplemented with SHP has been reported to have a strong characteristic flavor with a brothy taste, there is the possibility to use fish sauce as a flavoring agent to reduce the cost of production of these imitation products.

The result from the triangle test using deep fat fried products (Table 3-44) showed that there was a significant difference between the sample with salt and the sample with heat-treated fish sauce ($P<.01$). Seven out of eight judges chose the correct sample and 6 out of 8 preferred fish sauce to salt. However, when preference test was conducted, the sensory score on the hedonic scale did not show any statistically significant difference among the samples (Table 3-45). Fried samples prepared with fish sauce rather than salt had a higher preference score, however, these results were not significantly different ($P<0.05$).

Sensory evaluation of steamed surimi by preference test showed that the sample with salt was not significantly different from the sample with fish sauce ($P<0.05$), but the heat-treated and untreated fish sauce were significantly different ($P<0.05$). The steamed product received a lower score than the fried one. The color of the dough with fish sauce was off-white thus fish sauce cannot be used in a product in which whiteness is a criterion for quality; e.g., imitation crab leg. It can be used in breaded type products.

Results of a fold test showed that enzymes in fish sauce affected the texture of the product (Table 3-45). The texture of the steamed fish cake with unheated fish sauce deteriorated to grade B while the products with salt and heat-treated fish sauce retained grade AA texture.

Table 3-44: Comparison of surimi products using the triangle test[1]

Sample[2]	Correct	Incorrect	Preference
Surimi with salt vs surimi with heat-treated fish sauce	7 a	1	6 preferred surimi with fish sauce 2 preferred surimi with salt

[1] Value followed by a is significantly different ($P<0.01$), $n = 8$.
[2] Samples were deep-fat fried.

Table 3-45: Sensory evaluation of surimi products

Sample	Preference score[1]	Folding test
Fried surimi		
with salt	6.0 a	-
with fish sauce	7.0 a	-
with heated fish sauce	6.5 a	-
Steamed surimi		
with salt	6.00 ab	AA
with fish sauce	5.62 a	B
with heated fish sauce	6.12 b	AA

[1] Values followed by the same letter are not significantly different ($P<0.05$), $n = 8$.

3.10. Comparison of different batches of fish sauce prepared at different times

To compare the results from different batches of fish sauce prepared at different times, including the effect of frozen storage of capelin prior to salting, data of different batches of fish sauce prepared from fresh capelin caught in 1983, 1984 and frozen capelin (caught in 1983 and kept frozen at -20°C, 3 months) were analyzed. Statistical analyses of the degree of protein hydrolysis during fermentation indicated that there were no significant differences ($P<0.05$) between replicates within each treatment (Table 3-46). Thus it is apparent that frozen capelin can be used as raw material for fish sauce preparation without any adverse effect on the rate of protein hydrolysis during the first 20 weeks of fermentation.

Table 3-46: Comparison of the degree of proteolysis of different batches of fish sauce

Sample[1]	Degree of protein hydrolysis (mg formol N/ml fish sauce) Time (weeks) 4	8	12	20
Control				
1983 A	2.97	3.58	4.13	5.19
1983 L	2.87	3.33	4.13	5.25
1983 FA	2.80	3.47	4.02	4.90
1984 A	2.97	3.69	4.28	5.32
$\bar{X}$	2.90 ± .07	3.51 ± .13	4.14 ± .09	5.17 ± .16
SHP-Supplemented				
1983 B	9.02	9.34	9.55	10.56
1983 FB	8.82	9.38	9.52	10.22
1984 B	8.90	9.86	10.28	10.68
$\bar{X}$	8.91 ± .10	9.52 ± .29	9.78 ± .35	10.49 ± .19
Delayed-Salting				
1983 E	2.97	3.67	4.48	4.44
1983 FE	3.08	3.92	4.20	4.86
1984 E	3.23	4.03	4.67	4.96
$\bar{X}$	3.09 ± .13	3.87 ± .18	4.45 ± .24	4.75 ± .28

[1] Number before code letter indicates year of preparation.
F = Capelin was frozen at -20°C for 3 months.
E = Delayed salting for 24 hours.
$\bar{X}$ = Mean values followed by standard deviation.

Chapter 4

Conclusions

Fish sauce having excellent quality can be obtained by fermentation of male capelin with salt in the presence of squid hepatopancreas.

This study focussed on the hydrolysis of protein during the fermentation process, conditions most favorable for the fermentation, involvement of proteolytic enzymes in both fermentation and aging periods, and the importance of peptides and amino acid to the sensory acceptability of the finished product.

The conclusions from this study are as follows :

1. A method for preparation of capelin fish sauce was developed. On the basis of variables tested, the following conditions are recommended for the fermentation of capelin, supplementation with 2.5% (w/w) of squid hepatopancreas, fermentation at natural fermentation pH (5-7) with 25% (w/w) salt and at ambient temperature (20-25°C). The chemical changes of protein during fish sauce fermentation occur mostly during the first four weeks as evidenced by increases in free amino acids, formol nitrogen and soluble protein. The aging of capelin fish sauce in sealed jars at ambient temperature does not improve the quality of the finished product. After aging for 4 months, there is no significant difference in sensory score, free amino acid content and color between the aged and unaged fish sauce samples. Squid hepatopancreas supplemented capelin fish sauce is highly acceptable after fermentation for 6 months and can be used without further aging.

2. Squid hepatopancreas aids fermentation of capelin-salt mixture to fish sauce by virtue of its enzymes. This conclusion is based on the finding

that the heat-treated squid hepatopancreas did not improve the fermentation process either by increasing (i) the degree of protein hydrolysis, (ii) the formation of free amino acid or (iii) the sensory evaluation score. Squid hepatopancreas contains proteolytic enzymes which exhibit optimum proteolytic activity in the presence of high NaCl concentration (25 % NaCl) at about pH 6, the natural pH of fish sauce fermentation. It was found that proteases from squid hepatopancreas-supplemented sauce retain considerable activity at the high NaCl concentration of the fermentation.

3. Enzymes associated with fish viscera contribute significantly to protein hydrolysis during the fermentation of capelin fish sauce. However, the sensory evaluation of fish sauce prepared from round or gutted capelin by a triangle test did not show any significant difference between the two samples.

4. Viable bacteria do not play a beneficial role with respect to sensory quality and rate of protein hydrolysis in the fermentation process. This conclusion is based on the finding that (i) the rate of protein hydrolysis of the antibiotic treated salted mince sample and the control were not significantly different, (ii) the total viable bacterial count decreased after the addition of salt. It is apparent that when bacterial count approached 10^7 CFU/g, the microbial enzymes produced prior to the addition of salt can contribute significantly to the protein hydrolysis. This is shown by the increase in protein hydrolysis in the 24 h delayed-salting sample which is significantly higher than the control. However, histamine, the biologically active amine, was found in the delayed-salting sample. Although the level of histamine was very low (29 nmole/ml) compared to the values previously reported in fermented fish, it indicated the potential of amine formation in delayed salting.

5. The enzymes which contribute most to the protein hydrolysis in fish sauce fermentation are salt tolerant, neutral proteases. Partial characterization of residual enzymes after fermentation showed that squid hepatopancreas-supplemented sauce contained more proteolytic activity than control sauce. The proteolytic activity on azocasein was inhibited by EDTA, PCMB iodoacetate and soybean trypsin inhibitor. Fish sauce contained enzymes which hydrolyzed hide powder azure substrate and the activity was found to be higher in sample prepared with squid hepatopancreas. Dipeptidyl aminopeptidase I activity appeared to be present in fish sauce since a specific substrate for cathepsin C is hydrolyzed. Cathepsin C hydrolase activity is found to be more tolerant (than the other proteases) to salt. It is apparent that

cathepsin C contributes to the proteolysis in fish sauce fermentation. Based on the results obtained from the fermentation of fish sauce at different pHs, fermentation of gutted fish, fermentation of antibiotic-treated fish-salt mixture and delayed-salting fish, it is apparent that enzymes associated with the flesh are important to the protein hydrolysis, enzymes associated with the viscera of the fish although contribute significantly to the protein hydrolysis, they do not significantly improve sensory evaluation. Enzymes from squid hepatopancreas contribute to both the protein hydrolysis and the formation of delicious taste.

6. The free amino acid content of fish sauce is directly related to the sensory preference score. The correlation coefficient between amino acid content and preference score is 0.851 ($P<0.05$). It is apparent that larger molecular weight components present in fish sauce also contribute to the typical flavor of the sauce. Removal of the larger molecular weight components lowered the preference score. It can be concluded that the typical flavor of fish sauce is influenced by a combination of oligopeptides and free amino acids, especially glutamic acid and glycine. Linear regression of the preference score with glutamic acid or glycine concentration showed positive correlation which was significant ($P<0.05$). The results of gel filtration chromatography suggested that the apparent molecular weight of the major components ranged between 100-300. However, it is also possible that certain low M.W. components, e.g. fatty acids, do not pass the membrane.

7. The use of fish sauce as a flavoring agent in kamaboko products is possible, provided that the fish sauce is heat treated to inactivate the enzymes before addition to the surimi.

Suggestion for further study

More complete characterization of enzymes which contribute to protein hydrolysis in fermentation of fish sauce will provide a better understanding of the fermentation process. The optimum conditions for these enzymes to react on the protein will accelerate the process and shorten the time for fermentation. The amino acid sequence of the peptides which appear to contribute to the flavor of

fish sauce will be of importance to understand how structure of the peptides reflect flavor. Since cathepsin C was reported to catalyze transpeptidation reaction, and since transferase activity was also detected in fish sauce retentate especially when the fish-salt mixture was supplemented with squid hepatopancreas, the occurrence of transpeptidation or plastein reaction to form the delicious peptides in fish sauce deserves further investigation.

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Appendix A

Biuret method for soluble proteins

Biuret reagent : 1.5 g $CuSO_4.5H_2O$, 6.0 g sodium-potassium tartate ($NaKC_4O_6.4H_2O$) were added to 500 ml distilled water and stirred well before adding 300 ml of 10% (w/v) NaOH and diluted to 1 L with water.

Sample : 0.1 to 0.2 ml of fish sauce were diluted to 1 ml with water.

Procedure : To 1 ml sample, 4 ml biuret reagent was added, mixed and allowed to stand for 30 min at room temperature. Absorbance at 540 nm was read against blank of 1 ml water and 4 ml reagent.

Standard curve : 6 mg/ml Bovine serum albumin standard in distilled water was used to develop a standard curve of 0.6-6.0 mg protein.

Appendix B

MicroKjeldahl determination of total nitrogen

Reagent :

1. Digestion mixture. 40 g of K_2SO_4 and 2 ml of selenium oxychloride was mixed and diluted to 250 ml with deionized water. A final volume of 500 ml was made by adding 250 ml conc. (specific gravity 1.84). Prior to using, the mixture was heated and mixed well.

2. Nessler's reagent (Folin and Wu) was obtained from BDH Chemicals.

3. Ammonium sulfate standard was prepared by dissolving 1.179 g of previously dried $(NH_4)_2SO_4$ in 250 ml of 0.2 N H_2SO_4. The concentration of the nitrogen was 1 mg/ml.

Sample : Fish sauce was diluted with distilled water (1:99).

Procedure : To 1 ml of diluted fish sauce in a 1.5 x 13 cm test tube, 0.2 ml of the digestion mixture was added. The tubes were inserted to a depth of 2.5 cm into a sandbath. The temperature of the sandbath was raised to 310-320°C until the digestion was completed, which was indicated by a clear solution remaining in the tubes. The heat was turned off and the tubes were cooled to room temperature, the acid digest was diluted to 10 ml with deionized water. Aliquots of 1 ml or less were transferred to 12 x 75 mm tubes. To each tube water was added to bring the volume to 1.33 ml and 0.67 ml of Nessler's reagent was then added. The solution was mixed thoroughly after each addition and left at room temperature in the dark for 10 min. Absorbance at 420 nm was read against blank prepared with no ammonium sulfate.

Standard curve : Standard curve of 1-10 ug nitrogen was prepared.

Appendix C

Salt determination (Volumetric Method)

Reagents : 0.1 N silver nitrate

0.1 N ammonium thiocyanate

6 N nitric acid

Saturated ferric alum indicator

Sample : Fish sauce was diluted with distilled water (1:19).

Procedure : To 10 ml diluted fish sauce, 30 ml of 0.1 N $AgNO_3$, 5 ml of 6 N HNO_3 and 5 ml of ferric alum indicator were added. The remaining $AgNO_3$ was titrated with 0.1 N NH_4SCN standard solution until the solution became permanent light brown. Subtract ml of 0.1 N NH_4SCN used from ml of $AgNO_3$ and calculated difference as NaCl.

$$X = 117\ (30\ N_1 - Y\ N_2)$$

X, NaCl in mg/ml

Y, ml of NH_4SCN

N_1, normality of $AgNO_3$

N_2, normality of NH_4SCN

Appendix D-1

Scoresheet for the assessment of fish sauce quality

NAME : _____ DATE : _____

INSTRUCTION :

You are receiving samples of fish sauce for evaluation of color, flavor and overall acceptability. Taste these samples and check how much you like or dislike each one.

PRODUCT CODE :

_____	_____	_____
__ like extremely	__ like extremely	__ like extremely
__ like very much	__ like very much	__ like very much
__ like moderately	__ like moderately	__ like moderately
__ like slightly	__ like slightly	__ like slightly
__ neither like nor dislike	__ neither like nor dislike	__ neither like nor dislike
__ dislike slightly	__ dislike slightly	__ dislike slightly
__ dislike moderately	__ dislike moderately	__ dislike moderately
__ dislike very much	__ dislike very much	__ dislike very much
__ dislike extremely	__ dislike extremely	__ dislike extremely

Comments : _____

Appendix D-2

QUESTIONNAIRE FOR RANKING

NAME : _____ DATE : _____

PRODUCT : _____

INSTRUCTION :

Please rank these samples for preference. Rank the sample you like best as first.

Taste the samples in the following order: _____

First _____

Second _____

Third _____

Fourth _____

Fifth _____

Comments : _____

Appendix D-3

TRIANGLE TEST

NAME : _____ DATE : _____

PRODUCT : _____

Two of these three samples are identical, the third is different.

1. Taste the samples in the order indicated and identify the odd sample.

Code	Check odd sample
_____	_____
_____	_____
_____	_____

2. Indicate the degree of difference between the duplicate samples and the odd sample.

Slight _____

Moderate _____

Much _____

Extreme _____

3. Acceptability :

Odd sample more acceptable _____

Duplicates more acceptable _____

4. Comments : _____

Appendix E

Fold test for surimi

Slices of 3 mm thick is folded, the grade is judged as :

Grade AA: no cracks on folding in quarters, extremely elastic.

Grade A: no cracks on folding in half; cracks on folding in quarters, moderately elastic.

Grade B: some cracks on folding in half, slightly elastic.

Grade C: breaks into pieces on folding in half, not elastic.

Grade D: breaks into fragments on finger pressure.

Table A-1: Amino acid composition of fish sauces prepared by different methods

	μmoles/ml[1]							
	A[2]		B[3]		C[4]		D[5]	
Amino acid	Free a.a.	Total a.a.	Free a.a.	Total a.a.	Free a.a.	Total a.a.	Free a.a.	Total a.a.
Ala	31.68	40.86	57.50	67.08	28.04	43.76	41.84	54.03
Arg	5.60	7.96	18.80	20.06	8.24	11.63	0	26.79
Asp	14.04	34.87	48.62	67.53	14.64	37.73	20.56	53.90
H-Cys	0.72	0	0.60	0.71	0.74	0.93	0.40	0.96
Cys	0.66	1.14	1.48	3.27	0.06	1.43	0.74	2.36
Glu	25.82	57.27	60.36	91.90	24.30	61.24	34.42	75.15
Gly	20.68	46.95	28.40	71.08	16.46	51.11	23.24	62.39
His	2.02	2.96	4.74	6.74	1.28	2.60	6.34	9.60
H-Lys	0.30	1.10	0.32	1.28	0.16	1.24	0.32	1.12
H-Pro	0	4.35	0.10	4.59	0	4.67	1.00	4.13
Ile	10.82	11.91	28.60	30.01	9.92	12.91	25.78	24.78
Leu	23.34	28.70	47.90	50.40	21.50	30.36	45.56	44.28
Lys	23.54	32.18	48.70	56.99	21.68	34.86	30.94	45.20
Met	6.20	8.49	12.42	18.02	5.08	9.35	12.50	15.01
Phe	8.20	7.89	18.70	17.50	7.20	8.26	16.60	14.29
Pro	5.40	19.02	19.60	33.70	5.60	19.43	17.76	26.92
Ser	16.04	25.06	31.92	42.29	14.00	26.31	19.76	35.04
Tau	13.84	13.86	14.14	15.48	16.56	17.28	15.04	13.91
Thr	12.96	18.47	30.94	38.62	11.68	20.11	22.14	30.31
Trp	0	0	3.06	0	0.20	0	0	0
Tyr	4.94	4.53	4.38	3.41	3.84	4.37	3.40	3.49
Val	15.56	19.45	38.08	43.49	14.08	21.10	31.60	34.36
Total	242.14	387.02	520.26	684.15	225.26	420.68	369.94	578.02

[1] Values are averages of duplicate analyses. Samples were fermented for 6 months, aged for 6 months.
[2] A=Control
[3] B=SHP supplemented
[4] C=Heat treated SHP supplemented
[5] D=SHP supplemented (pH 4.5)

Table A-2: Free amino acid composition of control capelin fish sauce during fermentation

Amino acid	μmoles/ml[1] Time (weeks) 1d	4	8	20
Ala	4.26	17.14	20.46	29.32
Arg	1.00	4.35	5.03	6.44
Asp	0.57	5.94	9.12	13.95
Cysteic acid	0.08	0.35	0.64	0.81
Cys	0	0.23	0	0
Glu	2.75	12.20	16.15	24.50
Gly	2.33	10.39	14.38	19.44
His	0.46	1.44	1.64	1.15
H-Lys	0	0.10	0.09	0.13
H-Pro	0	0	0.19	0.15
Ile	0.72	4.60	5.90	4.10
Leu	1.42	12.32	15.64	22.69
Lys	1.64	10.50	14.93	21.87
Met	0.55	4.81	5.52	8.29
Phe	0.54	3.30	4.80	7.26
Pro	0.77	1.70	2.01	3.68
Ser	1.50	8.36	11.02	15.45
Tau	13.11	17.73	15.31	16.26
Thr	1.13	5.46	7.36	10.94
Trp	0.12	0.33	0.26	0.21
Tyr	0.42	2.39	3.41	4.90
Val	1.41	7.72	10.30	14.62
Total	34.76	131.29	164.12	230.77

[1] Values are averages of duplicate analyses.

Table A-3: Free amino acid composition of squid hepatopancreas supplemented sauce during fermentation

Amino acid	µmoles/ml[1] Time (weeks) 1d	4	8	20
Ala	17.96	46.95	51.58	54.46
Arg	6.69	15.37	18.69	17.81
Asp	2.04	45.60	46.13	50.90
Cysteic acid	0.18	0.51	0.67	0.63
Cys	0.33	2.52	3.27	2.67
Glu	16.48	48.99	55.20	57.72
Gly	6.91	19.90	24.46	27.22
His	2.33	5.20	5.60	4.17
H-Lys	0.05	0.22	1.08	0.47
H-Pro	0	0	0	0
Ile	6.77	23.56	25.44	27.03
Leu	15.70	48.12	50.49	53.57
Lys	12.58	38.72	43.93	46.59
Met	5.29	15.87	16.71	17.11
Phe	4.96	15.85	18.28	18.11
Pro	2.44	7.68	11.89	14.67
Ser	9.24	23.64	27.09	30.01
Tau	14.24	17.52	15.41	14.17
Thr	7.18	21.54	23.99	27.00
Trp	0.75	2.31	0.68	1.14
Tyr	4.04	4.40	4.01	3.01
Val	10.91	30.13	35.56	36.03
Total	147.08	434.56	480.86	506.04

[1] Values are averages of duplicate analyses.

Table A-4: Free amino acid composition during fish sauce (20% salt w/w) fermentation

Amino acid	mole%[1] Time (weeks) 1d	4	8	20
Ala	12.6	12.2	11.9	12.1
Arg	3.0	3.2	2.8	3.0
Asp	2.5	6.3	7.2	7.9
H-Cys	0.2	0.2	0.2	0.2
Cys	0.1	0.1	0.2	0.1
Glu	8.8	10.6	11.0	11.6
Gly	7.4	8.2	8.9	8.6
His	1.4	1.4	1.1	0.8
H-Lys	0	0.1	0	0.1
H-Pro	0	0	0	0
Ile	2.5	3.8	4.0	4.3
Leu	5.1	9.5	9.7	9.7
Lys	5.6	8.3	8.6	9.4
Met	2.0	3.5	3.5	3.4
Phe	2.0	2.7	3.3	3.0
Pro	2.3	1.3	1.0	1.5
Ser	5.3	6.9	6.8	6.8
Tau	29.4	8.7	5.6	4.5
Thr	3.7	4.7	5.0	5.0
Trp	0.2	0.4	0.2	0.2
Tyr	1.5	2.0	2.3	1.3
Val	4.6	6.2	6.5	6.8

[1] Values are averages of duplicate analyses.

Table A-5: Free amino acid composition during fish sauce (25% salt w/w) fermentation

Amino acid	mole%[1] Time (weeks) 1d	4	8	20
Ala	12.3	12.5	13.1	12.8
Arg	2.6	3.4	3.3	2.9
Asp	1.2	4.1	5.1	5.9
H-Cys	0.2	0.2	0.4	0.3
Cys	0.1	0.2	0	0
Glu	7.9	9.0	9.6	10.6
Gly	6.6	7.7	8.9	8.3
His	1.2	1.2	0.9	0.5
H-Lys	0	0.1	0.1	0.1
H-Pro	0	0	0	0
Ile	1.9	3.6	3.5	3.9
Leu	3.7	9.4	10.0	9.8
Lys	4.4	8.0	8.8	9.4
Met	1.5	3.7	3.5	3.6
Phe	1.5	2.7	2.7	2.7
Pro	2.1	1.3	0.3	1.6
Ser	4.1	6.2	6.5	6.5
Tau	40.4	14.5	10.2	7.5
Thr	3.2	4.0	4.3	4.4
Trp	0.1	0.3	0.1	0.1
Tyr	1.2	1.7	2.0	2.6
Val	3.9	6.2	6.6	6.5

[1] Values are averages of duplicate analyses.

Table A-6: Free amino acid composition during fish sauce (30% salt w/w) fermentation

Amino acid	mole%[1] Time (weeks) 1d	4	8	20
Ala	12.2	12.6	13.6	13.3
Arg	2.3	3.4	3.4	2.9
Asp	0.9	2.8	3.8	4.3
H-Cys	0.2	0.3	0.6	0.4
Cys	0	0.1	0	0.2
Glu	7.3	7.5	8.5	8.8
Gly	8.4	8.1	9.1	8.3
His	1.2	1.0	1.3	0.4
H-Lys	0	0.1	0.1	0.1
H-Pro	0	0	0	0
Ile	2.0	3.0	2.5	3.2
Leu	3.4	8.0	9.3	9.5
Lys	3.8	7.7	8.8	8.8
Met	1.3	3.5	3.3	6.2
Phe	1.4	2.2	2.5	2.4
Pro	2.0	1.4	0.4	2.2
Ser	3.7	5.2	5.5	5.6
Tau	44.0	22.7	15.7	10.6
Thr	3.0	3.4	3.6	4.2
Trp	0.1	0.3	0	0
Tyr	1.1	1.4	2.2	2.2
Val	3.6	5.2	5.7	6.1

[1] Values are averages of duplicate analyses.

Table A-7: Effect of salt concentration during fermentation on amino acid composition of fish sauces

Amino acid	20% salt		25% salt		30% salt	
	μmoles/ml[1]					
	Free a.a.	Total a.a.	Free a.a.	Total a.a.	Free a.a.	Total a.a.
Ala	50.22	63.90	31.10	41.02	18.90	29.30
Arg	11.40	13.37	6.53	8.57	4.20	5.49
Asp	30.12	54.62	17.79	38.95	5.86	22.07
Cyt	0.40	0.34	0.67	2.39	0.20	0.62
Cys	0.06	1.79	0.61	0.60	0.18	1.03
Glu	47.42	87.18	24.64	59.13	12.84	38.89
Gly	35.72	68.30	22.34	49.34	12.04	37.52
His	3.06	4.81	1.62	3.61	0.96	1.80
HLys	0.32	1.50	0.24	1.21	0.10	0.99
HPro	0.60	5.65	0.19	4.10	0.40	3.13
Ile	18.90	21.16	11.57	14.94	5.26	6.60
Leu	38.84	46.88	25.32	30.70	12.96	18.19
Lys	32.96	50.80	25.54	34.91	13.20	21.19
Met	11.62	14.73	8.77	13.52	3.90	5.55
Phe	14.00	13.31	12.19	8.92	5.00	4.68
Pro	8.08	25.98	6.58	22.68	2.98	12.96
Ser	28.20	36.48	16.49	23.50	8.44	17.90
Tau	17.22	18.33	9.90	11.34	12.62	22.07
Thr	22.50	30.37	13.40	25.14	6.52	12.54
Trp	0.60	0	0	0	0	0
Tyr	5.20	6.17	3.88	3.22	3.00	2.85
Val	27.40	33.47	16.92	22.33	7.94	12.04
Total	411.14	599.14	256.27	420.12	137.50	277.41

[1] Values are averages of duplicate analyses.
Samples were fremented for 6 months, aged for 6 months.

Table A-8: pH change during fermentation of capelin after adjustment of initial pH

Initial pH	pH[1] Time (Weeks)						
	2	4	8	12	16	36	40
3	3.53	3.63	3.60	3.55	3.54	3.68	3.50
4	3.99	4.04	4.04	4.02	4.02	4.04	4.01
5	4.71	4.77	4.84	4.75	4.76	4.77	4.75
6	5.58	5.66	5.70	5.63	5.65	5.70	5.63
7	6.20	6.28	6.31	6.25	6.25	6.35	6.28
8	7.60	7.63	7.61	7.57	7.59	7.60	7.55

[1] Values are averages of duplicate batches.

Table A-9: Free amino acid composition of 24 h delayed-salting capelin fish sauce during fermentation

Amino acid	μmoles/ml[1] Time (weeks) 1 d	4 wk	8 wk	20 wk
Ala	7.98	15.08	19.94	31.28
Arg	0	0	0	0
Asp	0.18	5.11	8.64	16.00
H-Cys	0.03	0.08	0.26	0.40
Cys	0.01	0.12	0.38	0.29
Glu	2.65	10.56	16.30	29.31
Gly	3.86	10.02	16.48	25.30
His	0.44	1.62	2.60	2.94
H-Lys	0.01	0.08	0.11	0.36
H-Pro	0	0	0	0.18
Ile	0.60	2.98	4.04	7.98
Leu	1.20	8.28	12.36	20.90
Lys	0.92	8.18	12.50	21.96
Met	0.58	3.20	4.80	6.99
Phe	0.46	2.40	3.52	5.18
Pro	1.44	1.92	1.92	3.2[illegible]
Ser	0.36	5.41	8.59	14.75
Tau	12.04	15.46	12.18	15.12
Thr	0.52	3.56	5.86	10.96
Trp	0.02	0.19	0.40	0.51
Tyr	0.08	1.04	1.32	3.47
Val	1.44	5.76	8.02	14.48
Total	32.22	100.79	140.25	231.64

[1] Values are averages of duplicate analyses.

Table A-10: Effect of delayed salting on amino acid composition of fish sauce

Amino acid	μmoles/ml[1]			
	Control		Delayed salting (24 h)	
	Free a.a.	Total a.a.	Free a.a.	Total a.a.
Ala	31.38	40.86	39.82	52.10
Arg	5.60	7.96	0.80	2.11
Asp	14.04	34.87	19.30	37.04
Cyt	0.72	0	0.28	0.38
Cys	0.66	1.14	0.38	1.73
Glu	25.82	57.27	38.48	68.78
Gly	20.68	46.95	33.54	61.41
His	2.02	2.96	3.76	4.96
HLys	0.30	1.10	0.48	1.52
HPro	0	4.35	0.40	5.70
Ile	10.82	11.91	11.32	12.89
Leu	23.34	28.70	26.34	32.86
Lys	23.54	32.18	28.26	36.77
Met	6.20	8.49	5.78	10.15
Phe	8.20	7.89	11.66	9.04
Pro	5.40	19.02	6.94	21.19
Ser	16.04	25.06	19.22	25.61
Tau	13.84	13.86	17.92	19.46
Thr	12.96	18.47	16.06	20.70
Trp	0	0	0.40	0
Tyr	4.94	4.53	5.12	5.47
Val	15.56	19.45	18.20	22.38
Total	242.14	387.02	304.46	452.25

[1] Values are averages of duplicate analyses, fish sauce were fermented for 6 months and aged for 6 months.

Table A-11: Amino acid composition in unaged and aged capelin fish sauce (Control)

Amino acid	Free amino acids (μmoles/ml[1])		Acid hydrolyzate (μmoles/ml[1])	
	Unaged	Aged (6 m)	Unaged	Aged (6 m)
Ala	26.74	28.92	43.53	42.24
Arg	5.98	5.26	8.66	9.90
Asp	15.40	16.46	38.87	36.95
Cyst acid	0.64	0.64	0.44	0.50
Cys	0.32	0.30	1.58	1.70
Glu	25.34	26.30	64.32	63.10
Gly	19.86	21.72	51.53	50.35
His	2.06	1.80	4.25	3.52
HLys	0.34	0.37	2.32	4.02
HPro	0	0	3.20	2.67
Ile	8.91	9.50	12.54	12.97
Leu	21.34	22.26	31.93	31.40
Lys	20.76	22.36	35.58	34.98
Met	7.58	5.24	8.71	8.75
Phe	7.93	8.05	9.00	8.53
Pro	3.19	3.36	17.62	17.79
Ser	15.68	16.28	25.58	25.52
Tau	16.42	15.98	16.11	15.84
Thr	11.06	13.60	18.60	19.11
Trp	0.68	0.65	0	0
Tyr	2.23	2.80	1.64	1.81
Val	14.02	14.53	20.05	20.09
Total	226.48	236.38	416.06	411.94

[1] Values are averages of duplicate analyses.
Samples were fermented for 6 months.
Unaged = kept frozen at -20°C
Aged = kept at ambient temperature

Table A-12: Amino acid composition of unaged and aged squid hepatopancreas supplemented fish sauce

Amino acid	Free amino acids (μmoles/ml[1])		Acid hydrolyzate (μmoles/ml[1])	
	Unaged	Aged (6 m)	Unaged	Aged (6 m)
Ala	58.10	59.24	66.76	66.32
Arg	19.38	19.18	20.64	20.95
Asp	48.34	49.28	71.62	70.14
Cyst acid	0.71	0.50	0.47	0.48
Cys	1.81	1.61	1.60	2.46
Glu	61.88	62.51	94.03	94.56
Gly	31.46	33.36	77.15	72.39
His	5.33	5.18	7.33	6.04
HLys	0.32	0.39	1.61	1.47
HPro	0	0	3.77	4.01
Ile	27.62	27.90	30.41	29.20
Leu	50.60	51.02	53.33	53.55
Lys	47.94	48.84	57.38	56.03
Met	16.02	13.44	18.69	17.07
Phe	19.10	18.73	17.14	16.97
Pro	16.30	17.70	31.37	32.90
Ser	32.90	33.73	40.08	43.54
Tau	15.80	14.80	16.16	15.40
Thr	29.33	32.32	42.81	36.58
Trp	0.98	0.76	0	0
Tyr	3.18	3.94	2.41	3.47
Val	37.86	37.39	42.14	40.95
Total	524.94	531.82	697.17	684.48

[1] Values are averages of duplicate analyses.
Samples were fermented for 6 months
Unaged = kept frozen at -20°C
Aged = kept at ambient temperature

Table A-13 : Amino acid composition of the retentate-filtrate mixtures, with and without heat-treatment, from fish sauce prepared with SHP

Amino acid	μmoles/ml[1]			
	R + F		Heated R + F	
	Free a.a.	Total a.a.	Free a.a.	Total a.a.
Ala	48.03	46.81	47.72	48.98
Arg	15.01	14.08	14.92	14.57
Asp	38.87	48.59	38.14	49.40
Cyt	0.42	0	0.59	0.89
Cys	1.46	2.26	1.37	3.12
Glu	50.20	66.62	48.64	68.60
Gly	27.84	53.46	25.98	57.71
His	4.42	5.02	3.90	5.15
HLys	0.29	0.91	0.25	0.98
HPro	0.76	3.80	1.52	3.64
Ile	22.01	20.91	22.01	21.69
Leu	41.28	39.22	42.11	40.34
Lys	39.63	41.18	38.97	43.10
Met	12.63	12.57	12.18	10.68
Phe	17.18	12.47	17.39	12.73
Pro	13.98	23.51	12.32	25.07
Ser	26.70	29.73	25.83	29.76
Tau	11.90	10.89	13.00	11.67
Thr	24.57	26.32	24.65	26.21
Trp	0.80	0	1.14	0
Tyr	3.23	2.60	3.39	0.41
Val	29.14	29.51	29.14	30.61
Total	430.26	490.43	425.17	505.32

[1] Values are averages of duplicate analyses.
Retentate and Filtrate were mixed at the ratio of 1:6 and stored at ambient temperature for 4 months before analysis.
R=Retentate; F=Filtrate.

Table A-14: % Free and peptide amino acid in fractions from Bio Gel P-2 chromatograpy of fish sauce prepared with and without SHP

	C-FS[1]		SQ-FS[2]	
Peak	Free amino acid(%)	Peptide amino acid(%)	Free amino acid(%)	Peptide amino acid(%)
1	0	100	0	100
2	55	45	64	36
3	56	44	27	73
4	57	43	64	36
5	56	44	68	32

[1] C-FS=Control fish sauce

[2] SQ-FS=Squid hepatopancreas supplemented fish sauce

Table A-15 : Amino acid concentration in fractions from Bio-gel P-2 chromatography of fish sauce prepared with and without SHP

	nmole/ml eluant			
	C-FS		SQ-FS	
Peak	Free amino acid	Total amino acid	Free amino acid	Total amino acid
1	-	Asp(8) Thr(4) Ser(9) Glu(14) Pro(4) Gly(8) Ala(7) Ile(2) Leu(7) Lys(6) Arg(2)	-	Asp(2) Thr(1) Ser(4) Glu(3) Gly(11) Ala(1) Leu(3) His(10)
2	Lys(98)	Asp(5) Thr(6) Ser(6) Glu(7) Pro(12) Gly(12) Ala(4) Val(3) Ile(1) Leu(3) Phe(1) Lys(109) 1-Met-His(10)	Pro(55) Lys(242)	Asp(5) Thr(8) Ser(6) Glu(11) Pro(95) Gly(20) Ala(8) Ile(1) Leu(3) H-Lys(3)
3	Pro(20) Ala(53) Lys(35)	Asp(5) Thr(3) Ser(5) Glu(10) Pro(36) Gly(13) Ala(66) Val(3) Ile(2) Leu(5) Lys(42) Arg(2)	Ser(4) Gly(23) Ile(82) Leu(48) His(21)	Asp(13) Thr(29) Ser(244) Glu(73) Pro(4) Gly(74) Ala(5) Val(10) Ile(106) Leu(69) Lys(8) His(21)
4	Gly(55) Ala(106) Val(73) Leu(46)	Thr(51) Ser(6) Glu(9) Pro(31) Gly(111) Ala(134) Val(94) Ile(5) Leu(72) Lys(7) His(1)	Asp(20) Glu(183) Met(6)	Asp(36) Thr(6) Ser(7) Glu(240) Pro(8) Gly(16) Ala(5) Ile(2) Leu(3) His(2) Arg(2)
5	Glu(141)	Asp(82) Thr(4) Ser(6) Glu(157) Pro(4) Gly(11) Ala(4) Ile(1) Leu(3) Tyr(2)	Asp(235) Glu(146) Arg(7)	Asp(315) Glu(199) Pro(4) Gly(28) Ala(3) Cys(3) Ile(2) Leu(2) Arg(10)

C-FS = Control fish sauce. SQ-FS = SHP-supplemented fish sauce.

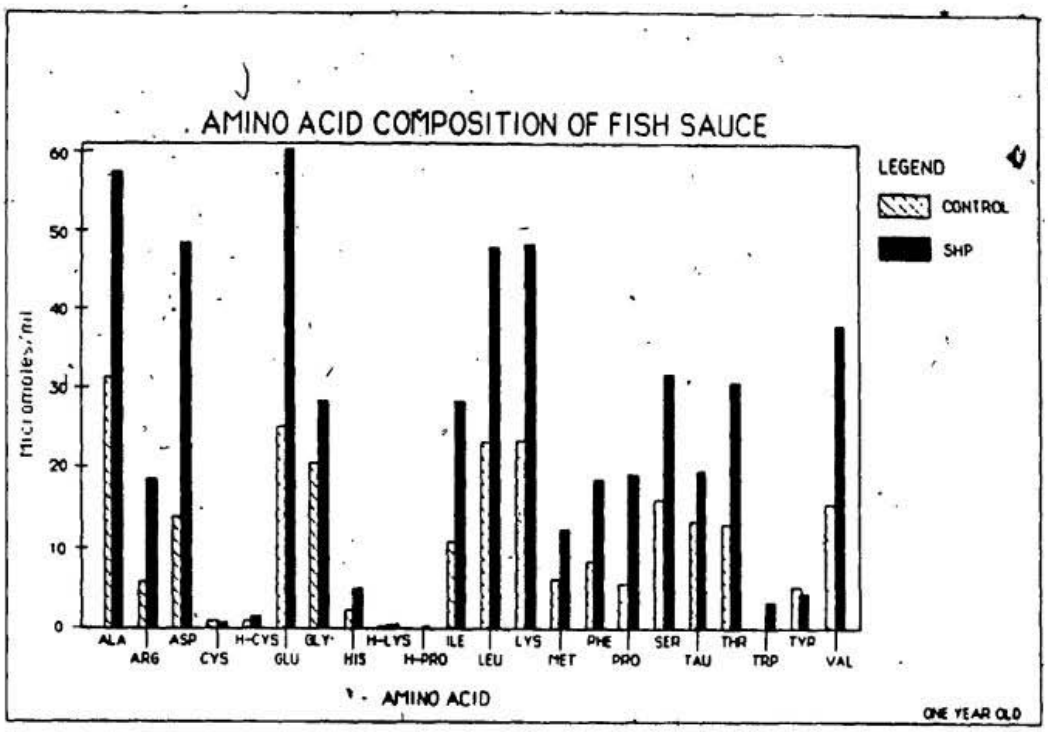

Figure A-1: Free amino acid content of control and squid hepatopancreas supplemented fish sauce

Values plotted are averages of duplicate batches
Samples were fermented for 6 months and aged for 6 months

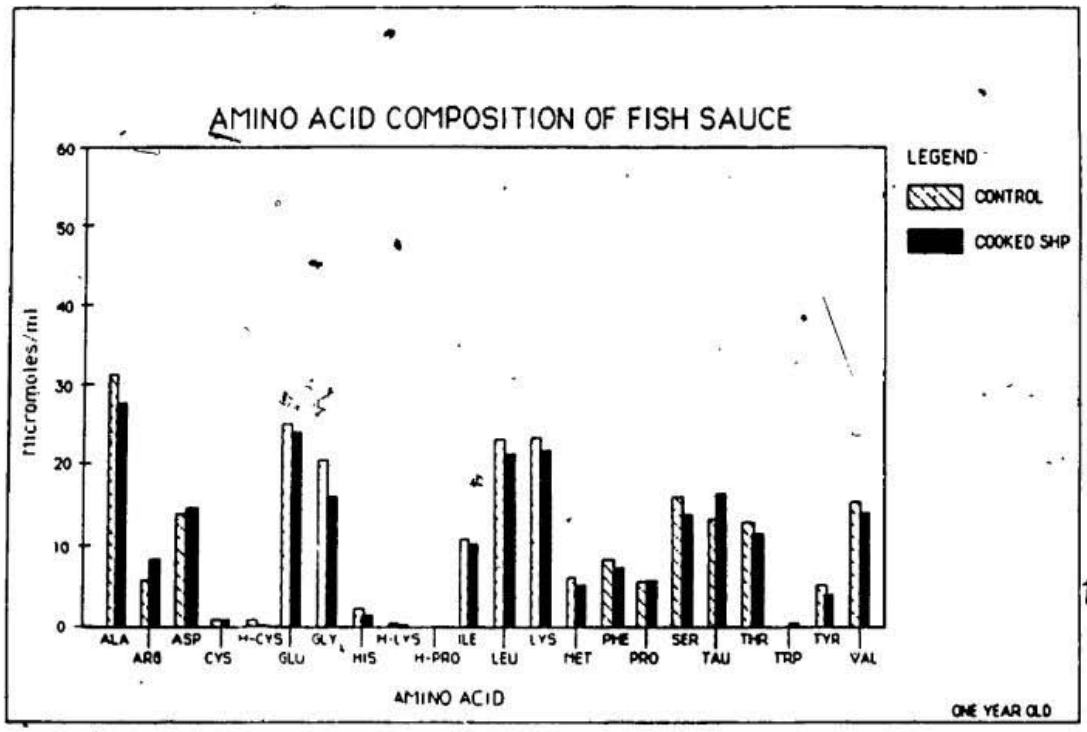

Figure A-2: Free amino acid content of control and heat treated squid hepatopancreas supplemented fish sauce

Values plotted are averages of duplicate batches
Samples were fermented for 6 months and aged for 6 months

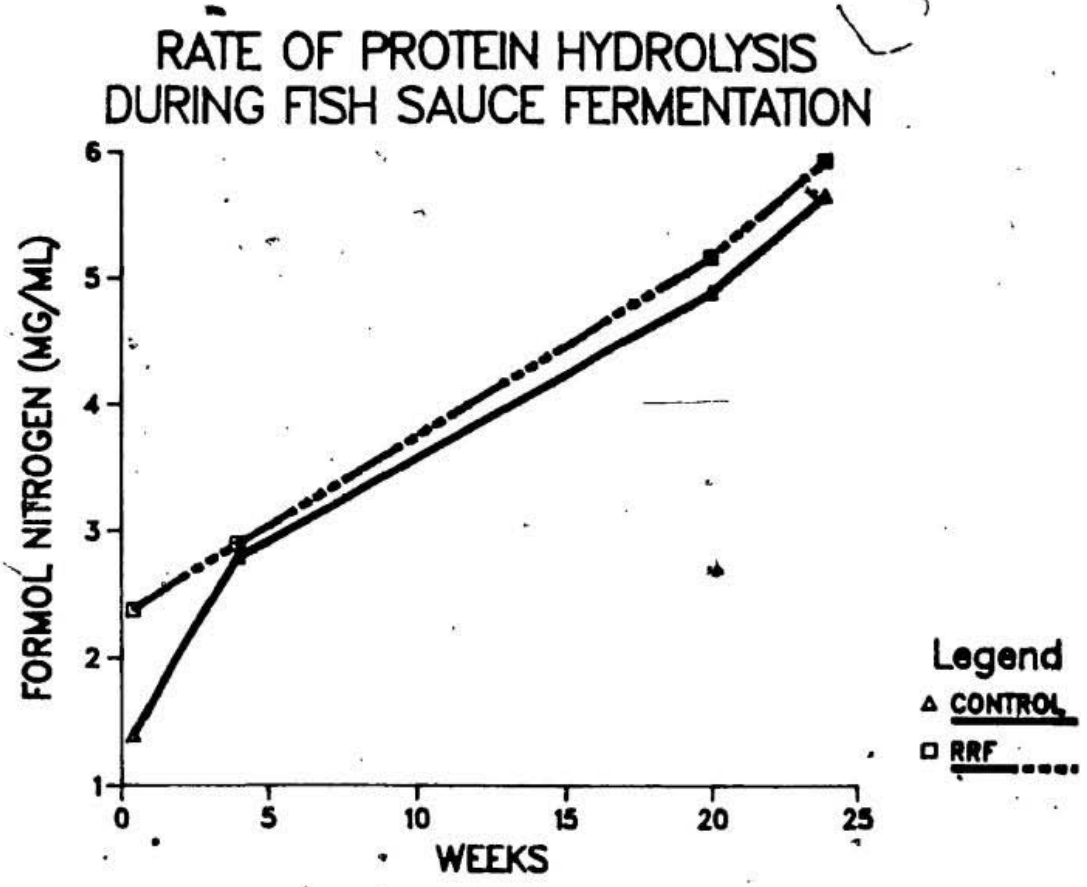

Figure A-3: Protein hydrolysis in capelin during fermentation

Values plotted are averages of duplicate determinations for 2 lots of fish sauce

Control= capelin caught at Outer Cove in 1983, kept at -20°C for 3 months. RRF= Redfeed capelin obtained from Fogo Island.