Chapter 3

Results and discussions

1. Characterization of proteinases in internal organs of skipjack tuna

1.1 Temperature and pH profiles

The proteolytic activities of various skipjack tuna internal organs assayed at different temperatures are shown in Figure 2. The optimal temperature of proteolytic enzymes from spleen, bile sac, intestine, stomach, liver, pancrease and pooled organs of skipjack tuna determined at pH 9 was 55°C. Proteinases from stomach and pancreas assayed at pH 3 also had the optimal temperature of 55°C. Nevertheless, liver proteinase assayed at pH 3 exhibited the highest activity at 45°C. The sharp decrease in proteolytic activity of all internal organs tested was found at temperature above 55°C, suggesting that proteinases might be inactivated by thermal denaturation. At high temperature, the conformational change of enzymes might occur, leading to the loss in their activity (Simpson 2000) .

The pH profiles of proteinases from various internal organs revealed that proteinases from intestine, spleen, pooled organs and bile sac had the optimal activity at pHs 8, 9, 9 and 9.5, respectively (Figure 3). On the other hand, proteinases from pancreas and liver showed the maximal activity at pH 3.5 and those from stomach had the optimal pH of 3. From the results, proteinases from tuna internal organs could be classified as acidic and alkaline proteinases, based on their optimal pHs. At very acidic or alkaline pHs, most of proteinases lost their activities,

presumably due to the denaturation of enzymes in the harsh condition. The differences in optimal pH are attributed to the different accessibility of the substrate to the active site as affected by charge on the substrate and on the active site at the particular pH of the medium (Mason *et al.,* 1984). Areekijseree *et al.* (2004) reported that pH and temperature could play a vital role on their activities and stability of enzymes. Moreover, each enzyme has its own specific structure upon which a slight change of these factors could drastically affect its catalytic performance. The presence of acid and alkaline proteinases in individual internal organs of various fish species has been reported (Simpson 2000). Acidic proteases from fish stomach display high activity between pH 2 and 4, while alkaline digestive proteases are most active at pH between 8 and 10 (Simpson 2000). Pepsin and trypsin are two main groups of proteinases found in fish viscera. Pepsin is found in fish stomach and is active at acidic condition (Gridberg *et al.,* 1990), while trypsin is concentrated in pyloric caeca and is active at neutral and alkaline conditions (Asgirsson *et al.,* 1989).

Usually, fish synthesize two pepsins, pepsin I and pepsin II. Pepsin I was most abundant in species like sardine (*Sardinops melanostica*) and capelin (*Mallotus villosus*), whereas pepsin II seems to be most abundant in species like cod (*Gadus morhua*) and salmom (*Oncorhynchus keta*) (Gildberg 1988). Fish pepsins have very low activity on small peptide substrates, and like other aspartic proteases, exhibit high activity on haemoglobin (Sanchez-Chiang *et al.,* 1987). Gildberg *et al.* (1989) reported that the affinity of the cod pepsin, especially pepsin I, was lower at pH 2 than at pH 3.5 towards haemoglobin. Furthermore, pH optimum was highly dependent on substrate concentration. Pepsin I and II showed similar pH optimal at pH 3.0 at high concentration of haemoglobin; whereas pepsin I had a maximal activity

at pH 3.5-4.0 at low substrate concentration. Acidic proteases from Monterey sardine (*Sardinops sagax carulea*) viscera exhibited optimal activity at 45°C and pH 2.5 when heamoglobin was used as a substrate (Castillo-Yonez 2004).

From the result, stomach was the crucial source of acidic proteinase, presumably pepsin. Pooled organs also consisted of acid proteinases, which were mainly from stomach. The results also revealed that most internal organs studied comprised the alkaline proteinases, which were active at high temperature (55 °C).

Alkaline proteinase from the intestinal section of discus fish (*Symphysodon aequifasciata*) had an optimum pH ranging from 11.5 to 12.5 when casein was used as a substrate (Chong *et al.,* 2002). Trypsin and chymotrypsin from anchovy viscera had the maximal activity at pH 9.0 and 8.0 when casein and synthetic substrates (BAPNA and BTEE) were used as substrates, respectively (Heu *et al.,* 1995). Intestinal extract of discus showed two activity peaks in the pH range of 7.5-9.0 and at a higher pH range of 11.5-12.5, suggesting the existence of two groups of alkaline proteases functioning in the intestinal regions of the discus (Chong *et al.,* 2002). Maximal proteolytic activities were observed in pH range of 8.0-10.0 for carp (Jonas *et al.,* 1983), striped and European seabass (Eshel *et al.,* 1993), and goldfish (Hidalgo *et al.,* 1999).

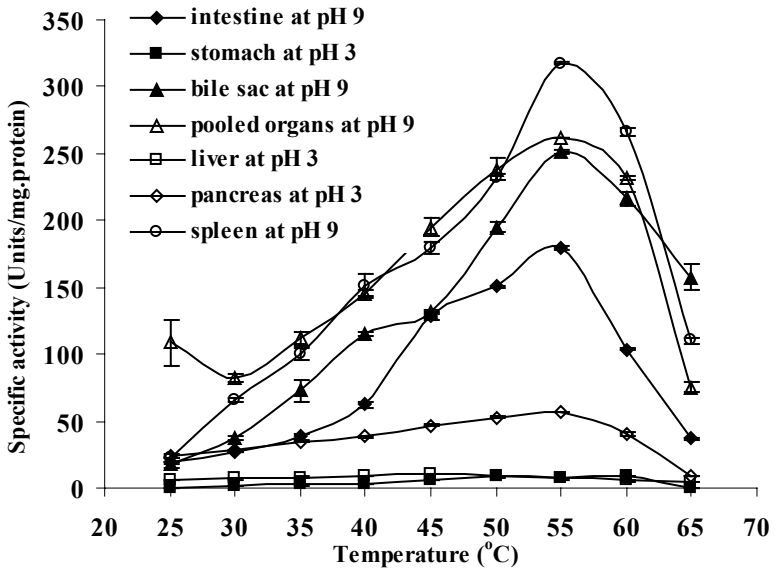

Figure 2 Temperature profiles of individual and pooled internal organs from skipjack tuna. Bars represent the standard deviation from triplicate determinations.

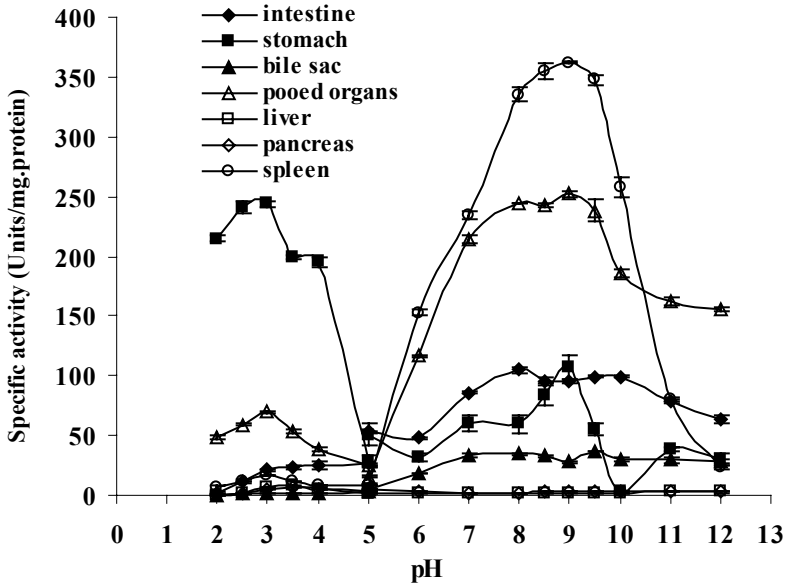

Figure 3 pH profiles of individual and pooled internal organs from skipjack tuna. Bars represent the standard deviation from triplicate determinations.

1.2 Distribution of proteolytic activity

Proteolytic activities of individual and pooled internal organs are shown in Table 1. When the activity was assayed under the optimal temperature and pH of each organ or pooled organs, spleen had the highest proteinase activity when casein was used as a substrate, followed by pooled organs, intestine, stomach, bile sac, pancreas and liver, respectively. Gastrointestinal organs including stomach and intestine also showed the proteolytic activity, mainly for digestive progress. Among all organs tested, liver showed the lowest proteolytic activity. Therefore, the distribution of proteinases varied with organs. For discus fish proteinase activity in intestine was higher than that in stomach (Chong *et al.,* 2002). Additionally, proteolytic activity of the same organ can be varied with species. Torrissen (1984) reported that proteinase activity from intestine of rainbow trout (*Salmo gairdneri*) was higher than that of Atlantic salmon (*Salmo salar*). Spleenic extract of yellowfin tuna had the higher specific activity than those of skipjack tuna (*Thunnus albacares*) and tongol tuna (*Thunnus tonggol*) (Klomklao *et al.,* 2004).

Table 2 Distribution of proteinase activity in different internal organs of skipjack tuna.

Organs	Proteinase activity (U/ g tissue)	Assay condition
spleen	36,495.12 $\pm$ 1.26*	55°C, pH 9.0
pooled organs	8,754.23 $\pm$ 5.65	55°C, pH 9.0
stomach	3,615.82 $\pm$ 2.74	55°C, pH 3.0
intestine	2,219.37 $\pm$ 6.09	55°C, pH 8.0
bile sac	1,445.60 $\pm$ 1.40	55°C, pH 9.5
pancreas	716.50 $\pm$ 2.24	55°C, pH 3.5
liver	521.68 $\pm$ 1.56	45°C, pH 3.5

*Mean $\pm$ S.D. from triplicate determinations.

1.3 Effect of NaCl on proteolytic activity

The effect of NaCl at different concentrations on proteinase activity in individual and pooled tuna internal organs is depicted in Figure 4. The proteinase activity in all organs tested decreased with increasing NaCl concentration. Among proteinases from all organs, that from spleen was more susceptible to activity loss as evidenced by the highest decreasing rate. Loss in activity might be due to denaturation of proteinases caused by "salting out" effect. At 30% NaCl, negligible activity was noticeable for all extracts tested. Klomklao *et al.* (2004) reported that the activity of alkaline proteinase from yellowfin tuna spleen was reduced with the addition of 25-30% NaCl. From the result, regardless of proteinase types or source, the activity was reduced as the salt concentration increased. The activity of acid proteinases from sardine was reduced with the addition of 3.42 M NaCl (Noda and Murakami 1981). Nevertheless, Fang and Chiou (1989) reported that NaCl up to 3.42 M had no effect on pepsin, trypsin and chymotrypsin activities from tilapia. Porcine pepsin was slightly inhibited at higher levels of NaCl, while cod protease was unaffected by the presence of NaCl (Squires *et al.,* 1986). Yatsunami and Takenaka (2000) found that viscera proteolytic activity of sardine (*Etrumeus micropus*) decreased when the NaCl concentration increased up to 14%. Hernàndez *et al.* (1999) reported that the proteolytic activity of the acid and alkaline proteinases is inhibited strongly in the presence of 15-20% NaCl.

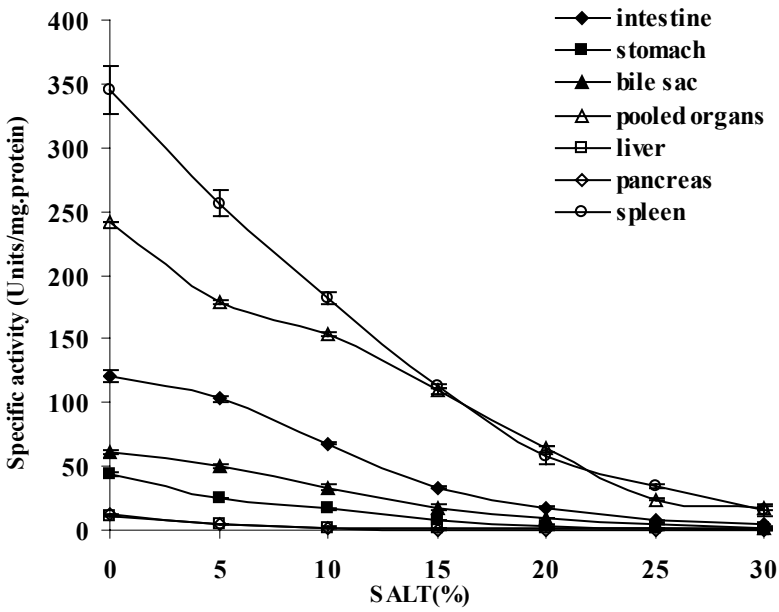

Figure 4 Effect of NaCl concentrations on proteinase activity of different internal organs from skipjack tuna. Bars represent the standard deviation from triplicate determinations.

1.4 Changes in proteolytic activity of internal organs during fermentation

The proteolytic activity of each internal organ including spleen, stomach, liver, pancrease, bile sac and pooled organs in the presence of 25%salt during fermentation of 12 months are presented in Figure 5. Proteolytic activity of each internal organ decreased with increasing fermentation time. The activity of each internal organ decreased rapidly during the first 0.25 months of fermentation. However, the rate of decrease varied, depending upon organ. For spleen, which contained the highest proteolytic activity, approximately 94% of activity was remained after 0.25 month of fermentation. However, the sharp decrease was observed at month 0.5 and almost complete loss in activity was found. During

fermentation stomach showed the highest relative activity, compared with other organs at the same fermentation time. Stomach consists of a high amount of connective tissue and smooth muscle. As a consequence, the concentration of salt into the tissue could be lower and the inactivation of pepsin could be retarded. Noda *et al*. (1982) found that the proteolytic activity of the acid and alkaline proteinases is inhibited strongly in the presence of 15-20% NaCl. Simpson (2000) reported that salt concentration has also been shown to have a major effect on the proteolytic activity, in which alkaline and acid proteinases were inhibited by >15% NaCl. Yasunami and Takenaka, (2000) found that the activity of viscera from sardine during fermentation decreased when the NaCl concentration increased. Vega-Villasante *et al*. (1995) also reported that the addition of NaCl did not enhance the protease activity in the whole digestive tract of the Pacific brown shrimp (*Penaeus californiensis*). Munilla-Moràn and Rey (1996) reported that the intestinal proteolytic activity of redfish was reduced in the presence of 0.5 M NaCl. Shih *et al.* (2003) found that the activities of fish sauce were quickly reduced in the rest of the fermentation period. The activity of crude pepsin from Atlantic salmon (*Salmo salar*) was very low at 10% and was completely inhibited at 15% salt concentration (Gildberg 1989). The decrease in activity might be due to the denaturation of enzymes, particularly in presence of high salt (Orejana and Liston, 1981). From the result, proteinases in internal organs would play an essential in hydrolysis of proteins or autolysis during the early stage. However, the remaining activity in some organs might hydrolyze the protein substrates during the extended fermentation time.

2. Effect of storage condition on changes of individual tuna internal organs

2.1 Chemical changes

2.1.1 Changes in TCA-soluble peptide

The changes in TCA-soluble peptides in individual tuna internal organs including pooled organ, spleen, pancreas, stomach, intestine, liver and bile sac during storage in ice and at room temperature for up to 8 h are shown in Figure 6. A greater increase in TCA-soluble peptides in tuna internal organs stored at room temperature was observed, compared with samples stored in ice. The lower TCA-soluble peptides in sample stored in ice indicated that low temperature retarded the degradation of fish proteins caused by either endogenous or microbial proteinases during storage. Internal organs contain a variety of proteinases and a number of bacteria (Haard, 1994). Recenly, Klomkloa *et al.* (2004) reported that spleen of those species of tuna, including skipjack, yellowfin and tongol contained the typsin-like serine proteinase with the optimal activity at pH 9.0 and 55 ^{o}C. Therefore, the lowered keeping temperature possibly resulted in the decrease in proteolytic activity of those endogenous enzymes as well as the retarded degradation as evidenced by the lower TCA-soluble peptides. Therefore, storage in ice could reduce protein degradation caused by digestive enzymes and microbial spoilage.

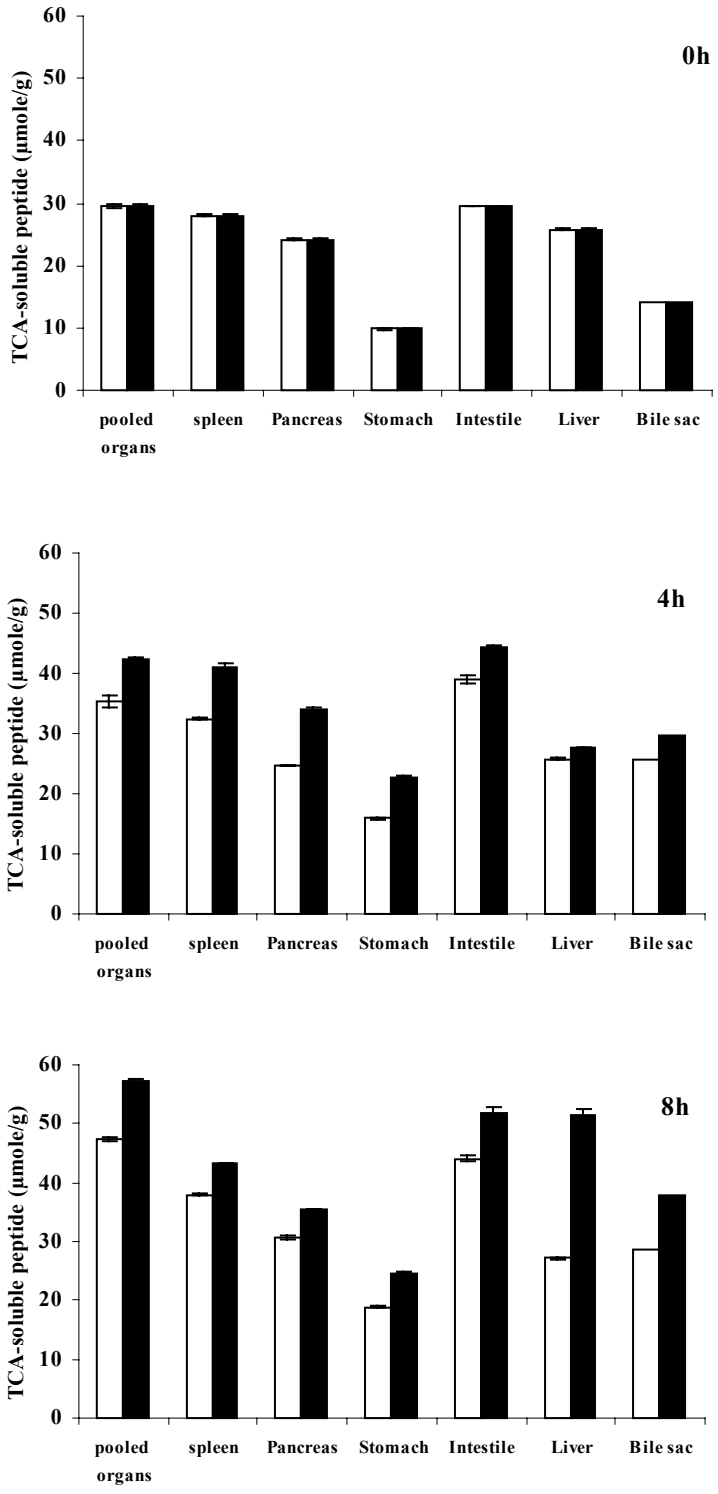

Figure 6 Changes in TCA-soluble peptide of individual internal organs during storage for up to 8 h. Bars represent the standard deviation from triplicate determinations. (□) storage in ice, (■) storage at room temperature.

2.1.2 Changes in pH

The changes in pH of individual tuna internal organs including pooled organ, spleen, pancreas, stomach, intestine, liver and bile sac during storage in ice and at room temperature for up to 8 h are shown in Figure 7. The pH values of tuna individual internal organs stored under both conditions tended to decrease throughout the storage period. After 8h of storage the pH values of each organ stored under both conditions decreased. The pH value of samples stored at room temperature was greater than that of samples kept in ice. The decrease in pH might be associated with organic acid such as lactic acid produced by lactic acid bacteria (Aquerreta *et al.,* 2001). Conversely, the increases in pH are related to the accumulation of basic substances such as ammonia and TMA produced during fish muscle spoilage (Hebard *et al.,* 1982). Benjakul *et al.* (2002) reported that the decomposition of nitrogenous compounds caused an increase in pH of fish flesh.

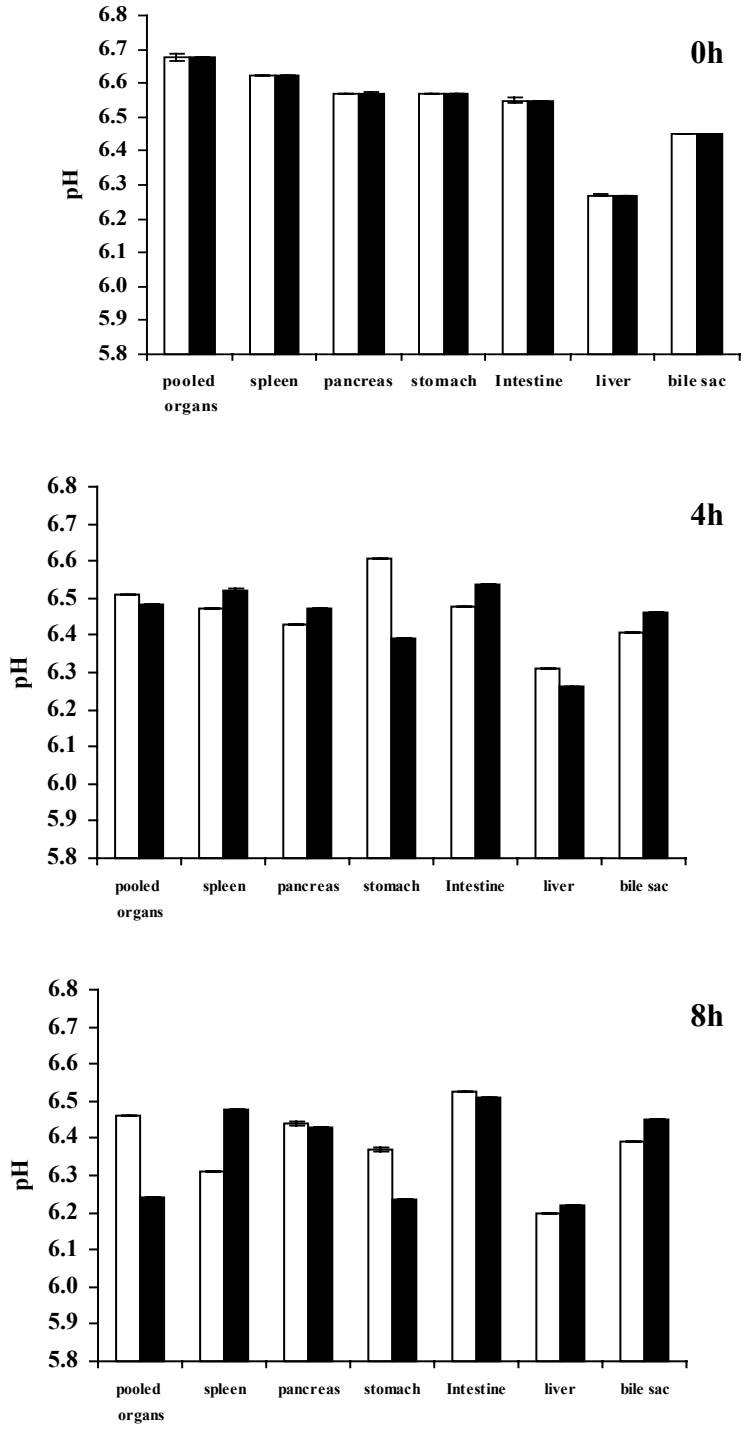

Figure 7 Changes in pH of individual internal organs during storage for up to 8 h. Bars represent the standard deviation from triplicate determinations. (□) storage in ice, (■) storage at room temperature

2.1.3 Changes in TVB and TMA

The changes in TVB and TMA contents of individual tuna internal organs including pooled organ, spleen, pancreas, stomach, intestine, liver and bile sac during storage in ice and at room temperature for up to 8 h are shown in Figure 8 and 9, respectively. TVB and TMA contents of tuna internal organs stored at room temperature were generally higher than those of samples stored in ice. The increases in TVB and TMA contents were observed throughout the storage time. Under the same condition after 8h of storage, spleen had the highest TVB, TMA followed by pancreas, pooled organ, intestine, and stomach and bile sac. After 8h of storage, TVB contents in individual tuna internal organs stored in ice and at room temperature ranged from 19.77 to 52.36 and from 21.91 to 57.17 mg N/100g, respectively. TMA contents in individual tuna internal organs stored for 8h in ice and at room temperature ranged from 1.9 to 2.94 and from 2.32 to3.56 mg N/100g, respectively. The increase in TVB and TMA contents over the entire storage period reflected the deterioration of viscera. TMA is produced by the decomposition of TMAO due to bacterial spoilage and enzymatic activity (Hebard *et al.,* 1982). TVB levels of 20-25 mg N/100g and above 25 mg N/100g indicate that fish are slightly decomposed/edible and decomposed/inedible, respectively (Lannelongue *et al.,* 1982). TVB content was a good index of quality of black-skipjack during iced storage (Mazorra-Manzano *et al.,* 2000). TMA concentration is normally used as a limit for acceptability of fish. The rejection limit is usually 5 to 10 mgN/100g. (Sikorki *et al.,* 1990). This result suggested that the spoilage caused by bacteria occurred, particularly when the storage time at high temperature increased. Srikar *et al.* (1993) pointed out that storage at higher temperature accelerated the rate of growth and metabolism of microorganisms.

Moreover, Karacam *et al.* (2002) reported that the TVB contents of brined anchovies increased throughout the period of storage both at refrigerator and room temperature conditions. However, the increasing rate of TVB content at room temperature was more pronounced than that at refrigerator condition. The formation of TVB and TMA is generally associated with the growth of specific spoilage bacteria such as *Shewanella putrefaciens*, *Photobacterium phosphoreum*, and *Vibrioaceae* (Gram and Huss, 1996; Huss, 1995). The increase in both TVB and TMA were in accordance with the increase in pH (Table 2). Those volatile compounds are known to cause the offensive odor in the fish muscle (Sikorki *et al.,* 1990).

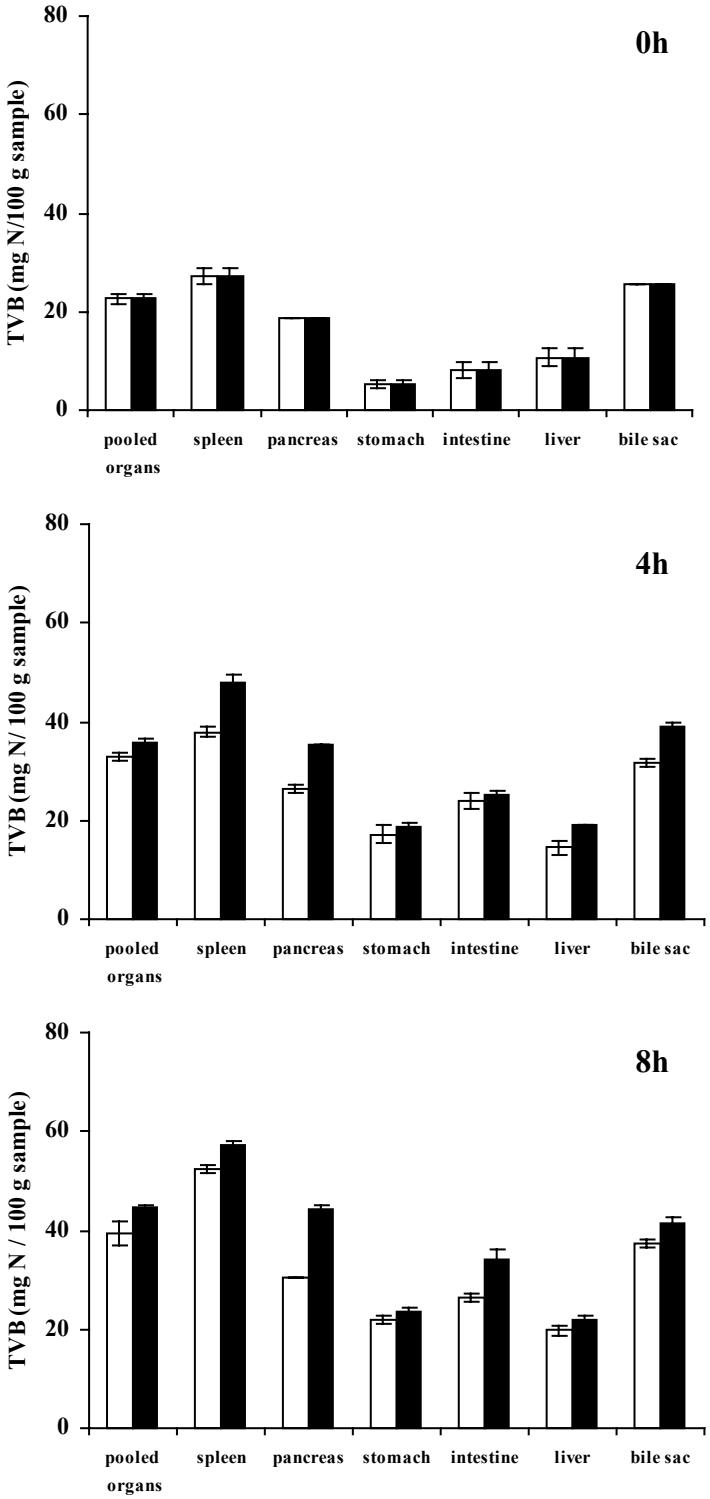

Figure 8 Changes in TVB content of individual internal organs during storage for up to 8 h. Bars represent the standard deviation from triplicate determinations. (□) storage in ice, (■) storage at room temperature.

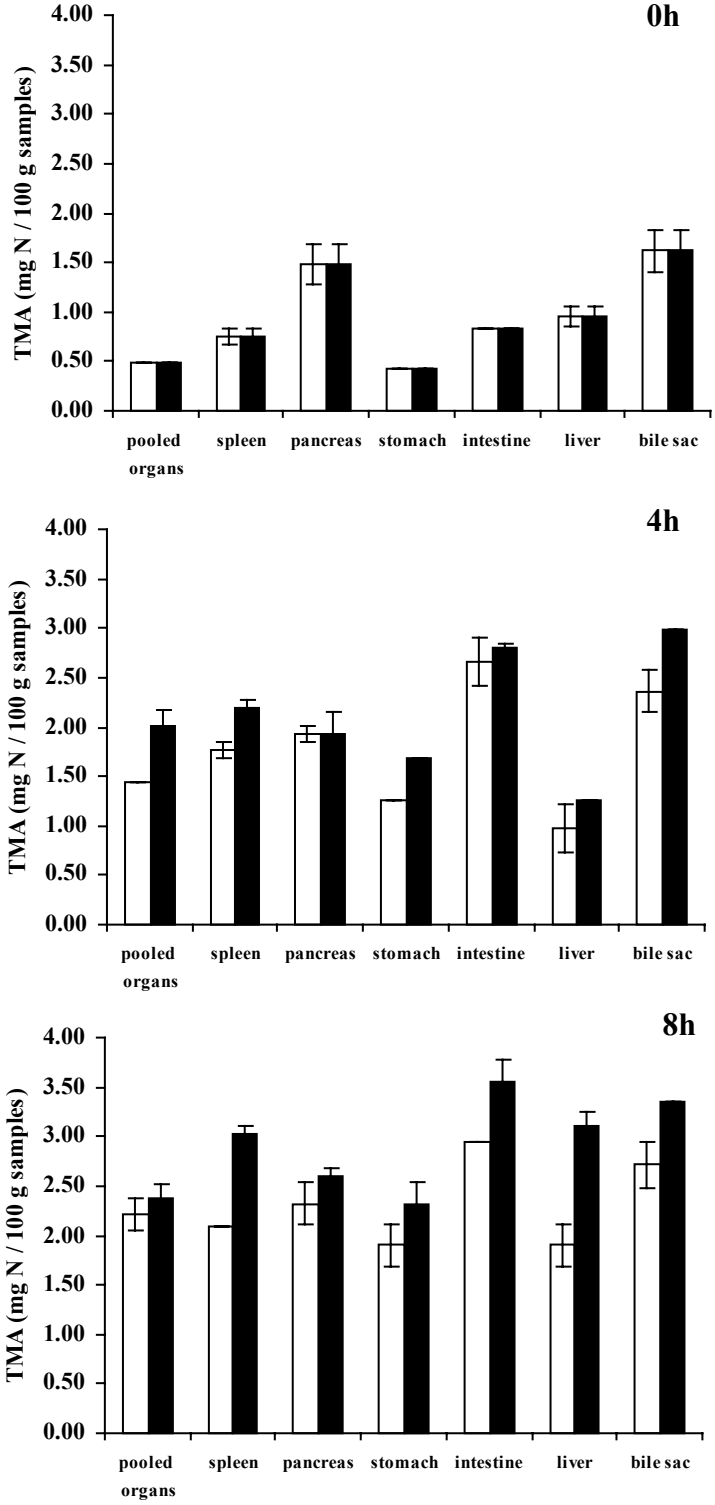

Figure 9 Changes in TMA content of individual internal organs during storage for up to 8 h. Bars represent the standard deviation from triplicate determinations. (□) storage in ice, (■) storage at room temperature.

2.1.4 Changes in histamine

Histamine content in individual tuna internal organs including pooled organ, spleen, pancreas, stomach, intestine, liver and bile sac during storage in ice and at room temperature for up to 8 h are shown in Figure 10. Tuna internal organs kept at room temperature had lower histamine content than that found in the sample stored in ice and fresh sample. From the results, intestine of tuna internal organs showed the highest histamine content than those organs. It has been known that abused temperature during handing or storage caused the induced formation of histamine in fish and fish product (Yongsawatdigul *et al.,* 2004). From the result, the greater depletion of histidine, a histamine precursor, caused by some microorganism at room temperature was postulated. Dapkevicius *et al.* (2000) found that histidine can be degraded by lactic acids bacteria such as *Lactobacillus* sp., *Leuconostoc mesenteroides*, and *Lactobacillus sake* isolated from naturally fermented mackerel-sucrose (88:12, w/w) pastes.

Additionally, histamine formed could be degraded by microorganisms with diamine oxidase (DAO) activity (Ishizuka *et al.,* 1993). Diamine oxidase activity is found in various mammalian tissues, especially placenta, kidney and intestine (Argento – Ceru and Autuori, 1985). Schwelberg and Bodner (1997) also found diamine oxidase activity in porcine, kidney and intestine. Leuschner *et al.* (1998) reported that *Lactobacillus plantarum*, *Lactobacillus sake*, *Lactobacillu pentosus*, *Pediococcus acidilactici*, *Rhodococcus* sp., *Arthrobacter* sp., *Micrococcus* sp., *Brevibacterium linens* and *Geotrichum candidum* were potential food-fermenting microorganisms for histamine degradation. The most effective strain for histamine reduction among lactic acid bacteria was *Pediococcus acidilactici* (Leuschner *et al.,*

1998). Since the rate of decomposition of histamine at room temperature was presumably greater than the rate of formation, the lower histamine content was found in the viscera stored at room temperature.

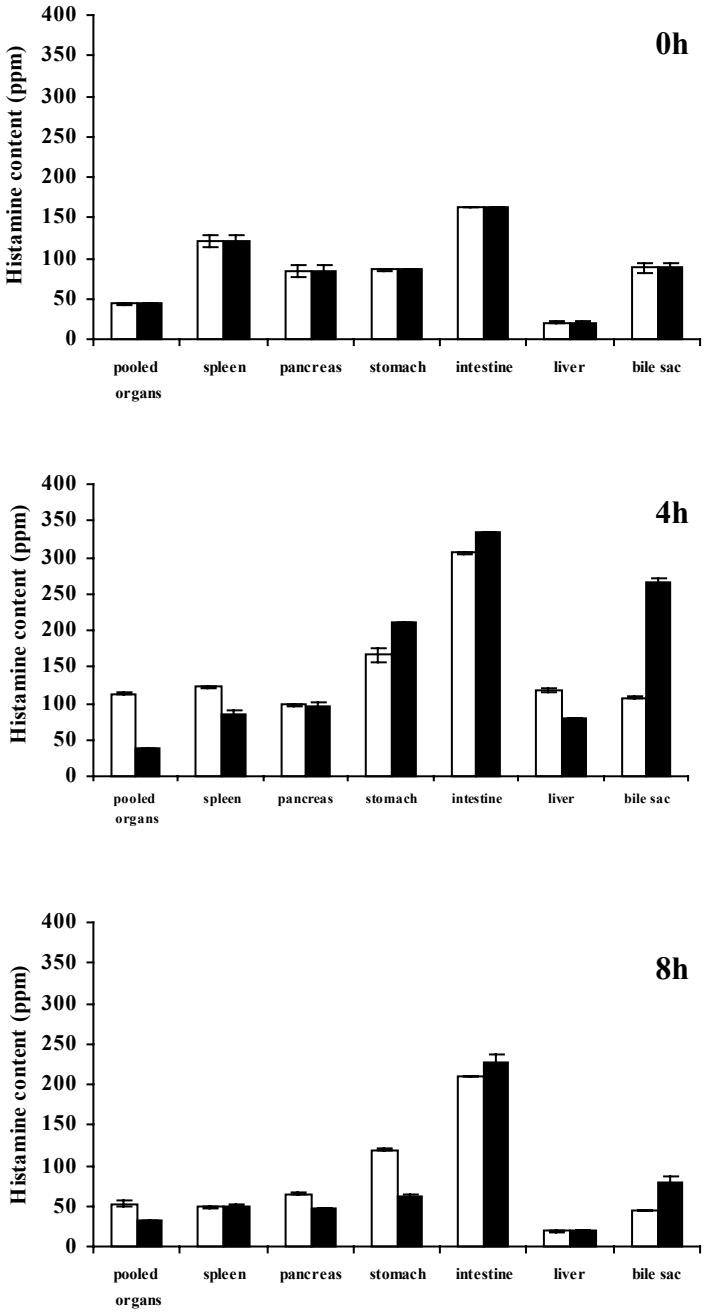

Figure 10 Changes in histamine content of individual internal organs during storage for up to 8 h. Bars represent the standard deviation from triplicate determinations. (□) storage in ice, (■) storage at room temperature

2.1.5 Changes in biogenic amines

The contents of biogenic amines in tuna internal organs during storage in ice and at room temperature for up to 8 h are given in Table 2. Changes in individual biogenic amines were varied with the storage temperatures, indicating the pronounced effect of storage temperature on the formation or degradation of biogenic amines. From the result, tryptamine was not found in fish samples and samples stored at either 4 °C or room temperature for both storage times. Additionally, ß - phenylethylamine was not detected in samples store at room temperature. Generally, amine contents present in tuna internal organs kept changing as the storage time in creased, depending on the fermentation. Storage of tuna internal organs at room temperature lowered the concentration of putrescine, tyramine, but the contents of cadaverine and spermidine increased. The greater biogenic amines content were obtained in tuna internal organs stored in ice for a longer time (8 h). For histamine, sample stored in ice for 8 h had the increased histamine content. Conversely, the substantial decrease was observed in the samples kept at room temperature for 8 h, presumably due to the decomposition of histamine at a higher rate at room temperature. Yamanaka (1984) reported that the histamine production rate at 20 °C of mackerel and yellowtail was greater than at 35 °C, whereas the higher rate was observed at 35 °C in skipjack tuna. The lower levels of histamine at high temperatures in the former species were attributed either to histaminases (Diamine oxidase) or to histidine decarboxylation bacteria. Dapkevicius *et al.,* (2000) reported that the highest degradation rate of biogenic amines was observed at 37 °C, but at 22 and 15 °C, the degradation was still considerable. Dapkevicius *et al*. (2000) found that lactic acid bacteria could degrade biogenic amines. Histamine and biogenic amines are degraded

by two enzymes: diamine oxidase (histaminase) and histamine *N* - methyltransferase. Diamine oxidase catalyzes the oxidative deamination of histamine to imidazoleacetaldehyde. Histamine *N* – methyltransferase catalyzes the *N* - methylation of histamine to *N* – methyl histamine. Leuschner (1998) reported that biogenic amines can be degraded by amino oxidases. Diamines, such as histamine and putrescine, are degraded by diamine oxidase (DAO).

From the result, the levels of these biogenic amines in sample stored in ice increased progressively throughout storage time. The levels of histamine and tyramine in sample stored at room temperature decreased progressively throughout storage time. Putrescine, Cadaverine and Spermidine in sample stored at room temperature increased with increasing storage time. As the storage time progressed, cadaverine became the dominant amine reaching 137 and 231 ppm, respectively, after 8h of storage in ice and at room temperature. Biogenic amines, such as cadaverine and putrescine, are also very important in food, especially in fish and fish products, because they have been shown to potentate the toxicity of histamine (Stratton *et al.,* 1991; Taylor and Sumner 1986; Chu and Bjeldanes 1981).

Biogenic amines are formed by decarboxylation of their precursor amino acids, as a result of the action of either endogenous amino acid decarboxylase activity (Halasz *et al.,* 1994) or by the growth of decarboxylase positive microorganisms (Silla Santos, 1996). Once bacterial spoilage began, the concentration of biogenic amine especially cadaverine and putrescine tended to increase (Ozögul *et al.,* 2005). From the result, the increase in putrescine and cadaverine contents was coincidental with TCA-soluble peptide (Figure 5). It appears that proteolysis might provide the nutrient for spoilage microorganisms, leading to the promoted growth of

those microorganisms. Fernandez–Salguero and Mackie (1987) indicated that cadaverine and putrescine showed a steady rise when bacterial spoilage begins, hence, these amines are considered as potential indicators of fish quality. Dawood *et al* (1988) also indicated that cadaverine and putrescine levels are potential indicators of fish quality. The decrease in biogenic amines content could be explained by the fact that these compounds can be used by microorganisms as a nitrogen source (Bardócz 1995).

Table 3 Changes in biogenic amine contents (ppm) in tuna internal organs (pooled organs) during storage in ice and at room temperature for up to 8 h.

Biogenic amines	Fresh	Ice		Room temperature	
		4 h	8h	4 h	8h
Tryptamine	0 ± 0.00[a]	0 ± 0.00[a]	0 ± 0.00[a]	0 ± 0.00[a]	0 ± 0.00[a]
ß-Phenylethylamine	20 ± 1.89[b]	20 ± 4.11[b]	27 ± 1.49[a]	0 ± 0.00[c]	0 ± 0.00[c]
Putrescine	72 ± 10.73[a]	58 ± 4.81[b]	77 ± 19.61[a]	41 ± 6.35[c]	46 ± 2.77[bc]
Cadaverine	116 ± 2.00[c]	119 ± 16.35[c]	137 ± 4.86[b]	134 ± 5.91[b]	231 ± 1.9[a]
Histamine	44 ± 3.05[b]	33 ± 3.36[c]	61 ± 3.05[a]	53 ± 6.11[a]	19 ± 7.32[d]
Tyramine	44 ± 10.30[b]	44 ± 1.23[b]	60 ± 4.61[a]	36 ± 3.18[b]	29 ± 8.09[c]
Spermidine	45 ± 5.15[b]	48 ± 2.34[b]	51 ± 2.66[b]	49 ± 1.15[b]	57 ± 2.55[a]

Mean ± Standard deviation from triplicate determinations.

Different letters in the same row indicate the significant differences ($p < 0.05$)

2.2 Microbiological changes

2.2.1 Changes in the microbial count

The changes in the microbial count of individual tuna internal organs including pooled organ, spleen, pancreas, stomach, intestine, liver and bile sac during storage in ice and at room temperature for up to 8 h are shown in Figure 11. Total viable count (TVC) of different tuna internal organs stored at room temperature was generally higher than those stored in ice at all storage time tested ($p<0.05$). TVC of samples stored at both conditions increased with increasing storage time ($p<0.05$). Under the same condition, pooled organ had the highest TVC than that of other organs. Initial TVC for pooled organs was 2.93 log CFU/g. After 8 h of storage, TVC reached 5.46 log CFU/g and 5.73 log CFU/g for sample stored in ice and at room temperature, respectively. Shewan, (1962) reported that the gill and the intestines of seabass contain TVC between 3.0 and 9.0 log CFU/g. Chytiri *et al.* (2004) reported that TVC in rainbow trout fillets kept in ice increased when the storage time increased. Omar (2002) found that TVC of mackerel (*Rastrelliger kanagurta*) stored at room temperature was greater than that kept in ice. Since the viscera consisted of the microbial flora, those microorganisms could grow rapidly in the samples containing the great amount of nutrient, especially nitrogen sources. The increase in TVC could be enhanced at higher temperature.

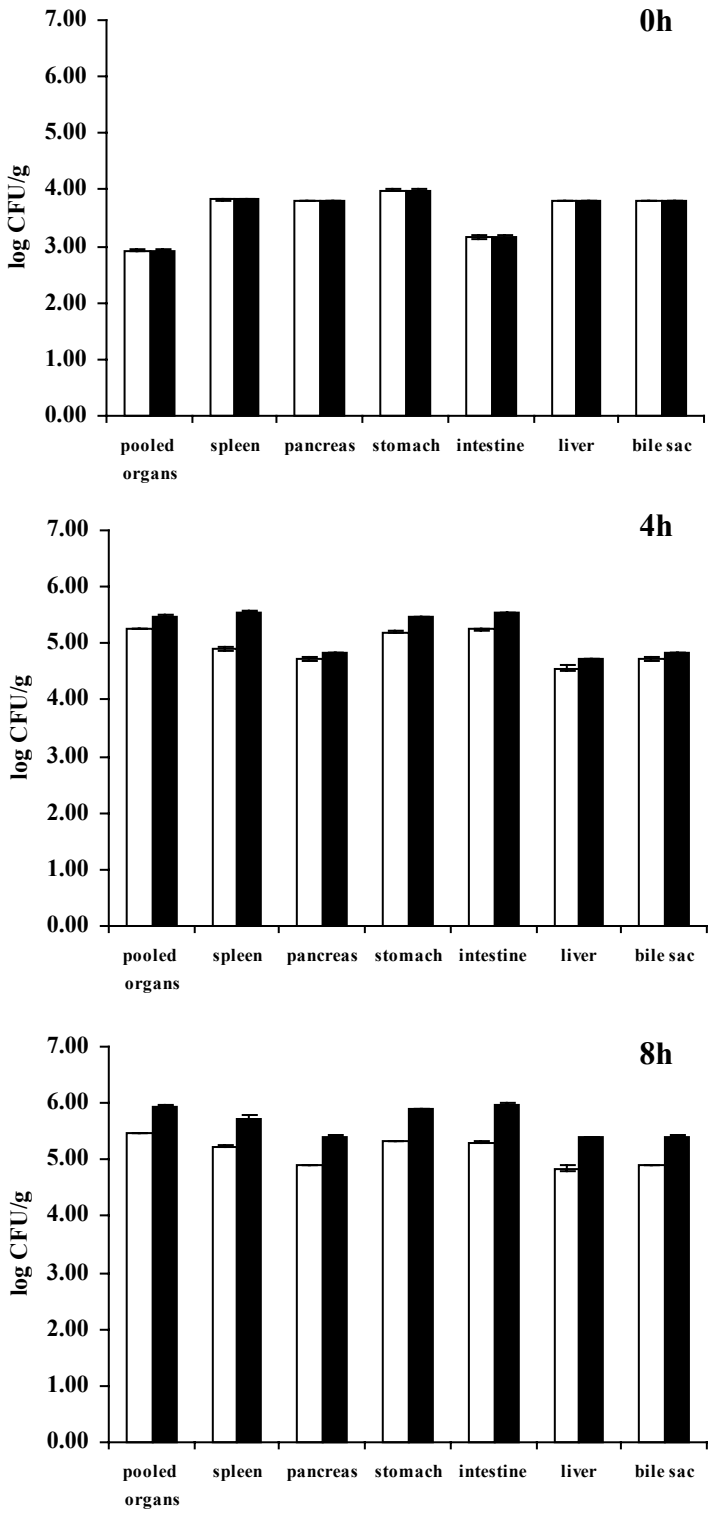

Figure 11 Changes in microbial count of individual internal organs during storage for up to 8 h. Bars represent the standard deviation from triplicate determinations. (□) storage in ice, (■) storage at room temperature.

2.2.2 Changes in total halophilic bacteria

The changes in total halophilic bacteria during storage in ice and at room temperature of different tuna internal organs including pool organ, spleen, pancreas, stomach, intestine, liver and bile sac are shown in Figure 12. The initial count of the total halophilic bacteria in each organ ranged from 0.0 to 2.2 log CFU/g. During the extended storage, total halophilic bacteria increased for both storage conditions ($p<0.05$). Pooled organs of skipjack tuna viscera stored at room temperature showed the highest total halophilic bacteria. After 8 h of storage, total halophilic bacteria counts were 4.16 and 4.36 log CFU/g for pooled organs stored in ice and at room temperature, respectively. The presence of high level of total halophilic bacteria in tuna internal organs was mainly due to a large number of halophilic bacteria in the visceral region of marine fish. Lakshmanan *et al.* (2002) reported that the total halophilic bacteria in whole sardine were higher than in the eviscerated sardine. Lower halophilic bacteria in different tuna internal organs stored in ice compared with ambient storage pointed out the retardation effect of bacterial growth by the low temperature.

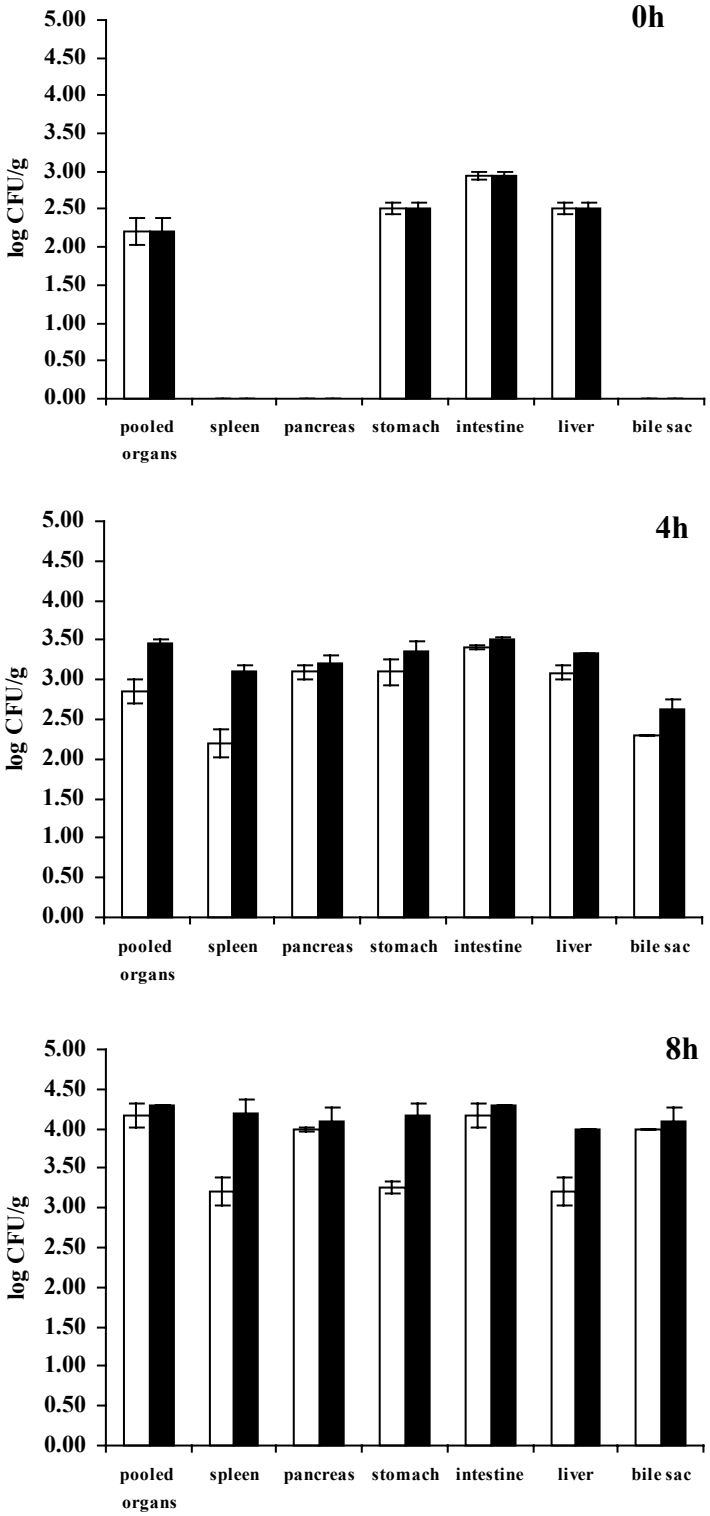

Figure 12 Changes in total halophilic bacteria of individual internal organs during storage for up to 8 h. Bars represent the standard deviation from triplicate determinations. (□) storage in ice, (■) storage at room temperature.

2.2.3 Changes in proteolytic bacteria

The changes in proteolytic bacteria during storage in ice and at room temperature of individual tuna internal organs including pooled organ, spleen, pancreas, stomach, intestine, liver and bile sac are shown in Figure 13. *Proteolytic bacteria also increased as the storage time increased.* After 8h of storage, pooled organs and intestine had the highest proteolytic bacteria, followed by pancreas, stomach, liver, bile sac and spleen, respectively. *The increasing rate was more* pronounced *at room temperature, suggesting that the growth of those microorganism*s *was more flavorable at high temperature. Those microorganisms are know*n *to involve in protein degradation of various perishable muscle foods including fish and meat* (Holtmann., 2004; Ashie., 1996; Ando *et al.,* 1995).

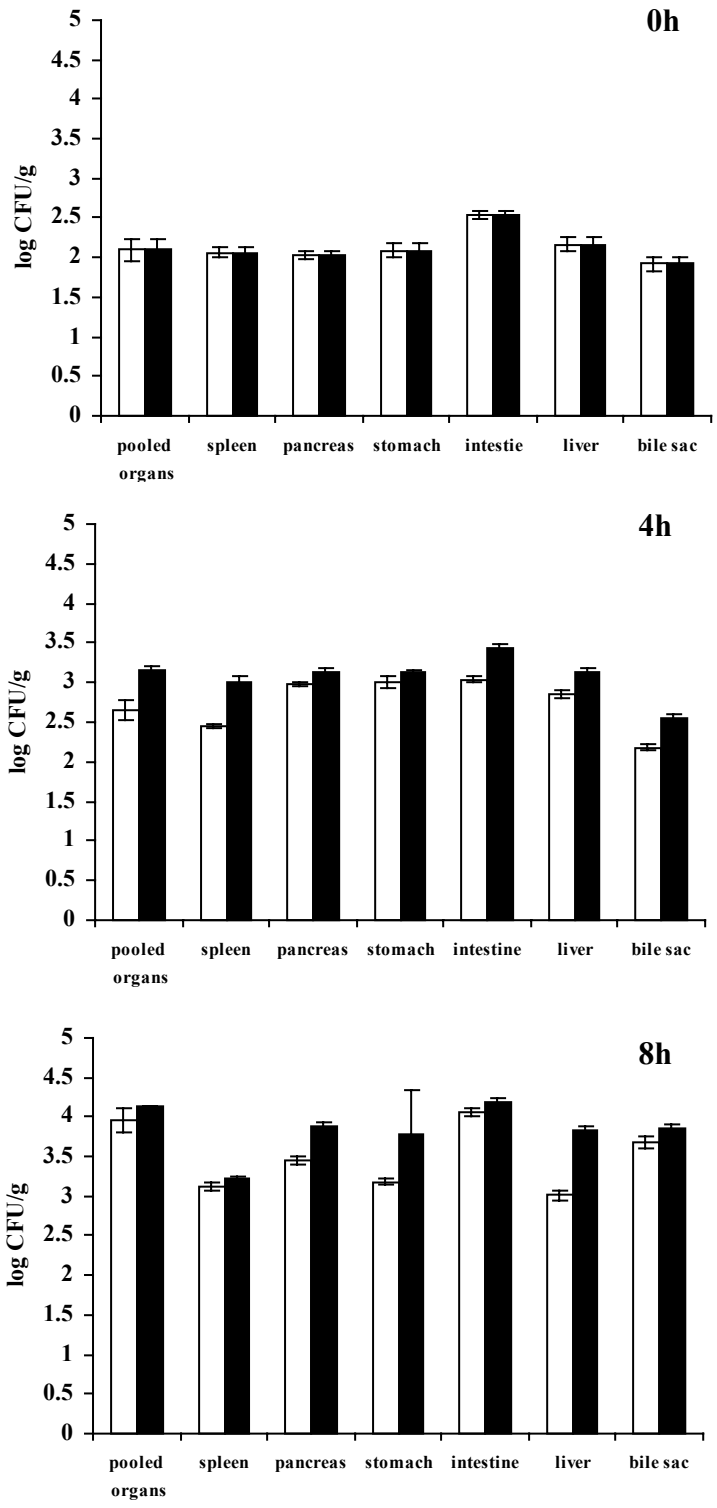

Figure 13 Changes in proteolytic bacteria of individual internal organs during storage for up to 8 h. Bars represent the standard deviation from triplicate determinations. (□) storage in ice, (■) storage at room temperature.

3. Effect of storage condition of tuna internal organs on chemical and biochemical changes of fish sauce during fermentation

3.1 Chemical changes

3.1.1 Changes in pH

The changes in pH of fish sauce during fermentation of 12 months are shown in Figure 14. The pH slightly decreased in all samples during the first month of fermentation and thereafter increased ($p<0.05$). The highest pH was observed at month 4 ($p<0.05$). Subsequently, the decrease was observed up to 6 months. No changes were observed thereafter. The final pH values of fish sauce from all treatments ranged from 5.16 to 5.82. Fish sauce produced from fresh tuna internal organs (the control) was lower than other treatments ($p<0.05$). Ijon and Ohta (1996) reported that pH of bakasang (Indonesian fermented fish sauce) ranged from 5.95 to 6.50, whereas Aquerreta *et al*. (2001) found a pH range of 4.90-5.42 in garum (Greece fermented fish sauce). Lopetcharat and Park (2002) reported that the pH of Pacific whiting fish sauce fermented for 40 days ranged from 6.1 to 6.3. Depending on the degree of protein hydrolysis and organic acid accumulation, the decrease in pH might be associated with amino acids released and organic acids, such as lactic acid, acetic acid produced by some microorganisms. Conversely, the increase in pH might be caused by the formation of basic compounds. The pH of garum increased during the fermentation process, probably as a consequence of the accumulation of basic compounds (Aquerreta *et al*., 2001). Hultmann and Rustad (2004) reported that the increase in pH might be due to the liberation of ammoniacal compounds as a result of endogenous proteolytic activity or the proteolytic microbial flora present in the raw material.

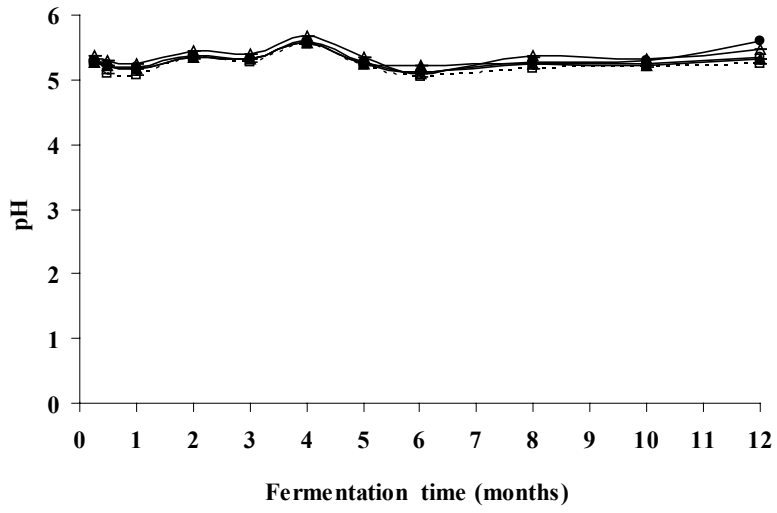

Figure 14 Changes in pH of fish sauce from different quality tuna internal organ during fermentation of 12 months. Bars represent the standard deviation from triplicate determinations. (□) control, (Δ) Ice, 4 h, (▲) RT, 4 h, (○) Ice, 8 h, (●) RT, 8 h.

3.1.2 Changes in TVB and TMA

Changes in TVB and TMA contents of fish sauce during fermentation are present in Figure 15 (a), (b). The values increased as the storage time increased ($p < 0.05$). At all fermentation times studied, TVB and TMA contents of fish sauce produced from fresh tuna internal organs were lower than those of fish sauce produced from tuna internal organs stored at room temperature and in ice ($p<0.05$). During extended storage at higher temperature, the autolysis and microbial spoilage took place and resulted in the increase in TVB and TMA contents. The greater TVB and TMA contents were observed in tuna internal organs stored at room temperature when compared with those of samples stored in ice ($p<0.05$). This was probably owing to the higher growth rate of microorganisms at room temperature. As a result,

TVB and TMA were accumulated to a greater extent prior to fermentation. However, both TVB and TMA were also produced during fermentation as shown by the continuous increase in both values.

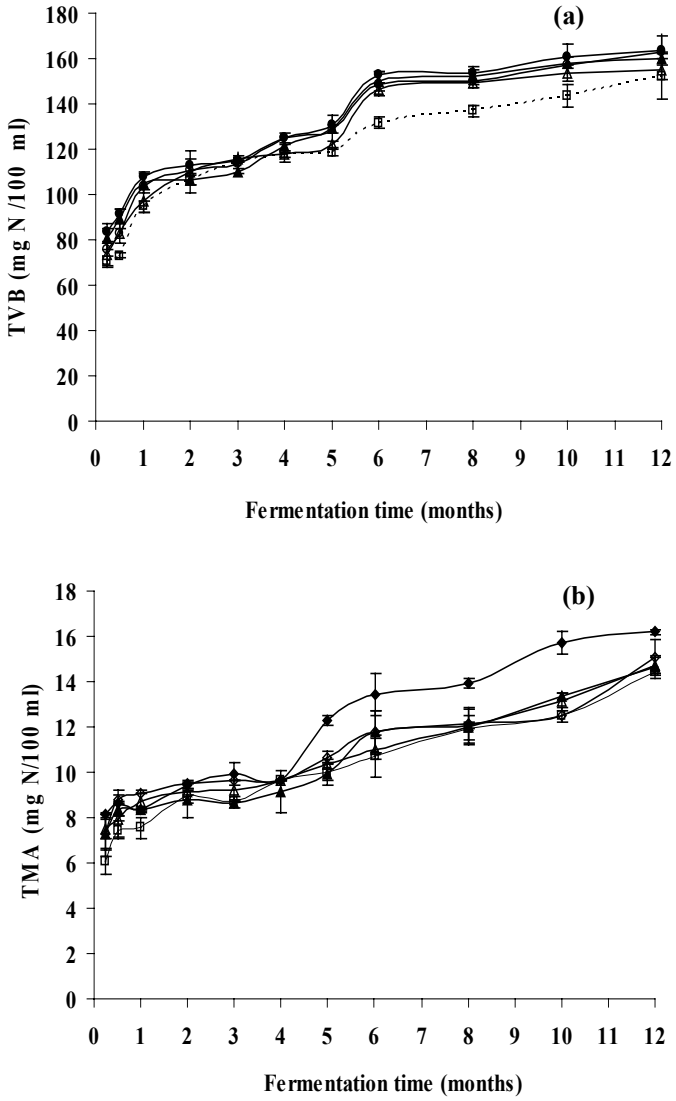

Figure15 Changes in TVB (a) and TMA (b) contents of fish sauce from different quality tuna internal organ during fermentation of 12 months. Bars represent the standard deviation from three determinations. (□) control, (Δ) Ice, 4 h, (▲) RT, 4 h, (○) Ice, 8 h, (●) RT, 8 h.

3.1.3 Changes in total nitrogen

The total nitrogen content in fish sauce during 12 months of fermentation is shown in Figure 16. Total nitrogen content in fish sauce increased continuously during fermentation ($P<0.05$). At early stage of fermentation (0.25-1 months), total nitrogen content of fish sauce produced from tuna internal organs stored at room temperatures for either 4 or 8 h was greater than that of fish sauce produced from tuna internal organs stored in ice ($p<0.05$). Thereafter, no marked differences in total nitrogen content were noticeable among all samples. During fish sauce fermentation, liquid is formed in association with the solubilization of nitrogenous components over a period of fermentation by the activities of proteolytic enzymes. Nitrogenous compound in fish sauce are composed of protein and nonprotein nitrogen (NPN) compounds such as free amino acid, nucleotides, peptide, ammonia, urea and TMAO (Shahidi, 1994; Finne, 1992). Wilaipan (1990) reported that high quality fish sauce must have a total nitrogen content of greater than 16.3 g N/l based on the Kjeldahl method. Total nitrogen content is used as an indicator to determine the grade and price of fish sauce in Thailand. Products containing total nitrogen content over 20 g/L are classified as Grade I and 15 to 20 g/L as Grade II (Thai Industrial Standard, 1983). From the result, total nitrogen content of all samples was higher than 20 g/L after 4 months of fermentation. Regardless of storage condition, fish sauce fermentation from tuna internal organs could be completed within 4 months based on total nitrogen content. Beddows *et al.* (1979a) reported that budu fermentation can be completed within 5 months. Saisithi *et al.* (1966) reported that 12 months were commonly used for fish sauce fermentation. From the result, rate of the increase in total nitrogen content was slower after 4 months of fermentation.

The result was in agreement with Orejana and Liston (1981) who found that the rate of increase in soluble nitrogen decreased after 4 months due to the great reduction of trypsin-like enzyme caused by the accumulation of amino acid and small peptides during the fermentation. Fermentation of fish sauce was most likely due to combination effects of enzymatic activities in fish viscera and from microorganism. Haard (1994) reported that fish viscera are potential source of proteinases.

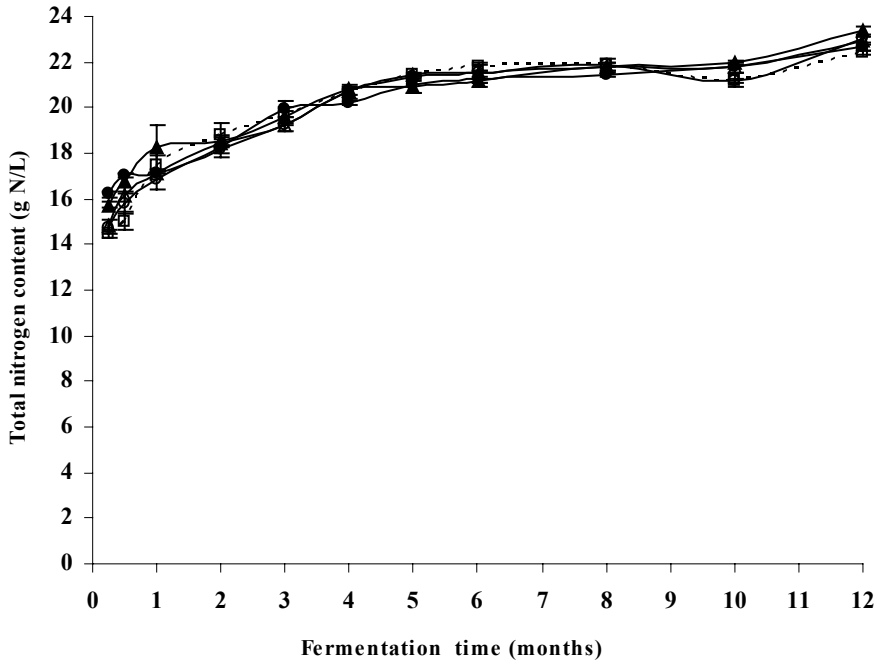

Figure 16 Changes in total nitrogen content of fish sauce from different quality tuna internal organ during fermentation of 12 months. Bars represent the standard deviation from triplicate determinations. (□) control, (Δ) Ice, 4 h, (▲) RT, 4 h, (○) Ice, 8 h, (●) RT, 8 h.

3.1.4 Changes in formol and ammoniacal nitrogen

Changes in formol and ammoniacal nitrogen contents (Figure 17, 18) were similar to those of total nitrogen content throughout the fermentation. Generally, fish sauce produced from fresh tuna internal organs had the lower formol and ammoniacal nitrogen content than those from samples kept at either room temperature or in ice ($p<0.05$). The increase in formol nitrogen content suggested the increased hydrolysis of peptide (Tungkawachara *et al.,* 2003) caused by the endogenous or microbial proteinases. Ammoniacal nitrogen content represents ammonia formed. Thus, not only free amino acid or peptide but also ammonia was produced during the extended fermentation time. Ammonia might be formed by deamination process (Lopetcharat *et al.,* 2002).

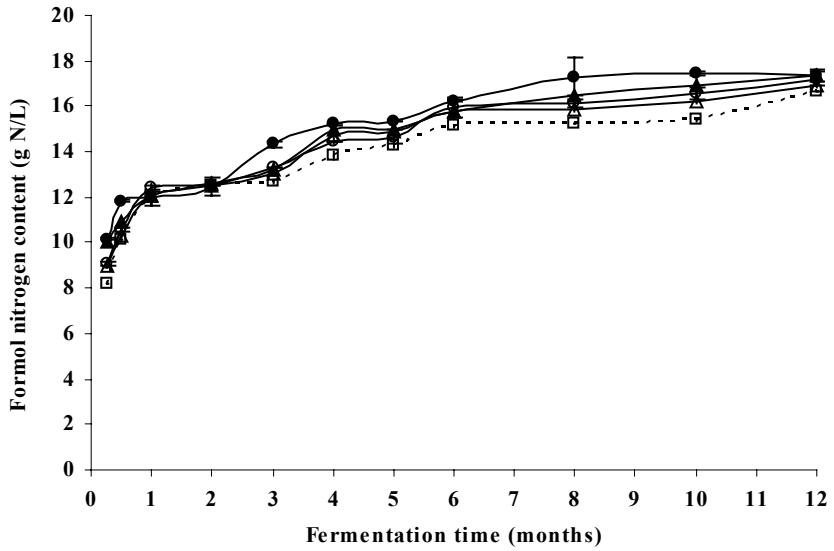

Figure 17 Changes in formol nitrogen content of fish sauce from different quality tuna internal organs during fermentation of 12 months. Bars represent the standard deviation from triplicate determinations. (□) control, (Δ) Ice, 4 h, (▲) RT, 4 h, (○) Ice, 8 h, (●) RT, 8 h.

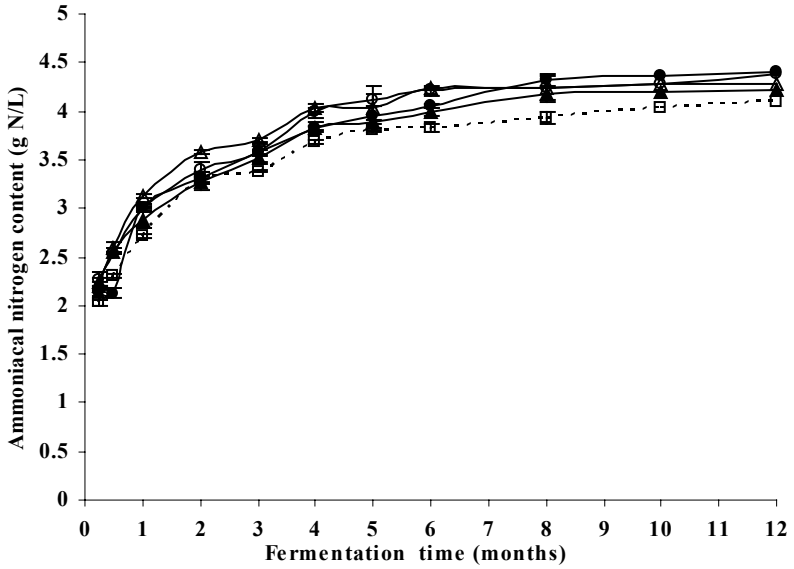

Figure 18 Changes in ammoniacal nitrogen content of fish sauce from different quality tuna internal organ during fermentation of 12 months. Bars represent the standard deviation from triplicate determinations. (□) control, (Δ) Ice, 4 h, (▲) RT, 4 h, (○) Ice, 8 h, (●) RT, 8 h.

3.1.5 Changes in amino nitrogen

Amino nitrogen content of fish sauce during fermentation is depicted in Figure 19. Amino nitrogen content in all samples increased with increasing fermentation time ($p<0.05$). Amino nitrogen is usually used as an indicator for degree of fermentation (Lopetcharat *et al.,* 2002). The amino nitrogen content represents the amount of primary amino groups in fish sauce. An increase in amino nitrogen concentration is related to the degradation of the polypeptide (Tungkawachara *et al.,* 2003). The longer fermentation time allowed more breakdowns of soluble protein and peptide into free amino acids and volatile nitrogen (Chaveesuk *et al.,* 1993). The increased amino nitrogen contents could be due to hydrolytic activity of fish enzymes

that was active during the early storage of fermentation (Beddows *et al.,* 1980). Amino nitrogen contents were approximately 12.55 to 13.20 g N/L in all fish sauce samples after 12 month of fermentation. According to the Thai Industrial Standard, amino nitrogen contents must be more than 10 g/L (Thai Industrial Standard, 1983). The results indicated the fermentation of fish sauce from tuna internal organs could be achieved within 4 months based on amino nitrogen content. From the result, fish sauce produced from the fresh sample tended to have the lowest amino nitrogen contents and fish sauce from the sample stored at room temperature for 8 h had the highest amino nitrogen content. This might be owing to the greater hydrolysis of samples prior to fermentation. As a consequence, the greater amino groups in peptides or free amino acids could be found in fish sauce produced from tuna internal organs stored at room temperature for a longer time (8 h).

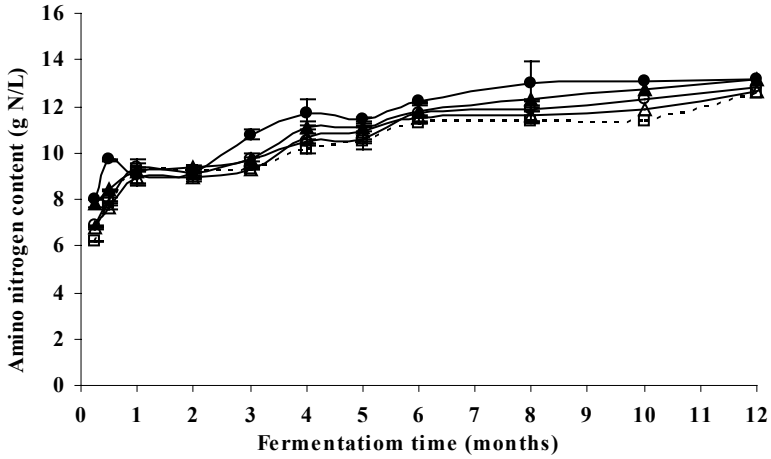

Figure 19 Changes in amino nitrogen content of fish sauce from different quality tuna internal organs during fermentation of 12 months. Bars represent the standard deviation from triplicate determinations. (□) control, (Δ) Ice, 4 h, (▲) RT, 4 h, (○) Ice, 8 h, (●) RT, 8 h.

3.1.6 Changes in salt content

Salt content of fish sauce during 12 months of fermentation period was monitored as shown in Figure 20. Generally, salt content increased at the early stage of fermentation (0.25-2 months) and remained relatively constant at about 28-30% up to 12 months of fermentation. The salt contents in all samples were not different ($p > 0.05$). Lopetcharat and Park. (2002) reported that salt content in the fish sauce from Pacific whiting waste increased at day 5. Thereafter, it remained constant at about 25-30% during 60 days of fermentation. This could be due to the salt concentration having reached equilibrium (Chayovan *et al.,* 1983). Wilaipan (1990) found that salt content in commercial fish sauce produced in Thailand was 25%. The result indicated that the salt content of fish sauce produced from tuna internal organs obtained in this experiment was slightly higher than that of commercial fish sauce.

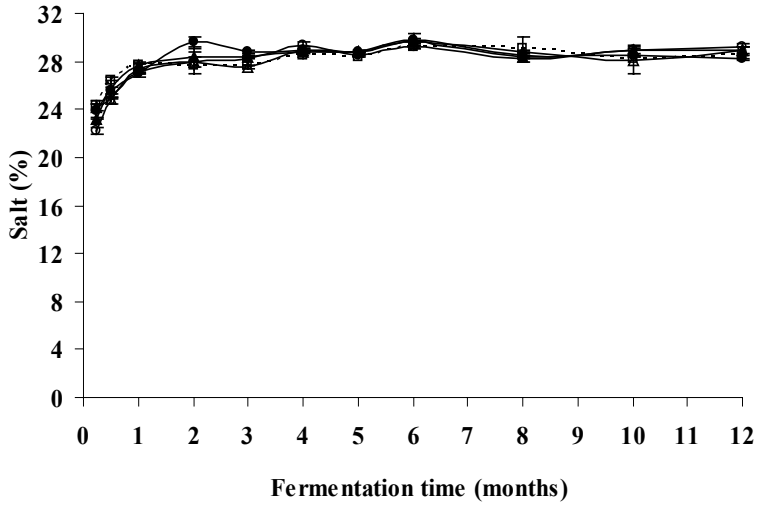

Figure 20 Changes in salt contents of fish sauce from different quality tuna internal organs during fermentation of 12 months. Bars represent the standard deviation from triplicate determinations. (□) control, (Δ) Ice, 4 h, (▲) RT, 4 h, (○) Ice, 8 h, (●) RT, 8 h.

3.1.7 Changes in histamine content

Changes in histamine content of fish sauce during fermentation are shown in Figure 21. In general, fish sauce produced from tuna internal organs kept in ice had the greater histamine content than those produced from tuna internal organs kept at room temperature and from fresh tuna internal organs ($p<0.05$). The highest histamine content was obtained in fish sauce produced from tuna internal organs stored in ice for 4 h, compared with other samples throughout the fermentation of 12 months ($p<0.05$). For fish sauce made from tuna internal organs stored at room temperature, higher histamine contents were obtained in the fish sauce produced from tuna internal organs kept for 8 h, compared with those stored for 4 h ($p<0.05$). The histamine content in fish sauce was coincidental with that found in raw material.

The constant histamine content was found in all samples when the fermentation time increased up to 6 months ($p>0.05$). The activity of histamine forming bacteria might be decreased due to the increase in salt concentration (~25%). As a result, no additional histamine was produced. Chin and Koehler (1983) reported that the very high concentration of salt used in the mixture during fermentation might inhibit the growth of microorganisms that could decarboxylate free histidine to form histamine. Nevertheless, the histamine content tended to be increased after 6 months of fermentation.

Histamine in foods is mainly formed by amino acid decarboxylases of bacteria (Rodtong *et al.,* 2005). During storage, the protein breakdown products, peptides and amino acids, represent precursors for amine formation used by spoilage microorganisms (Straub *et al.,* 1995; Straub *et al.,* 1994). The freshness of fish greatly influenced the formation of histamine during the fermentation process in the

manufacture of fish sauce and the very high concentration of salt also inhibited the growth of microorganisms that could decarboxylate histidine to form histamine (Sanceda, 1999).

Generally, fish sauce produced from high fresh quality raw materials had lower histamine values than that produced from low quality raw materials (Brillantes *et al.,* 2002). Histamine forming bacteria could grow and produce histamine over a wide range of temperatures and their growth was more rapid at high abuse temperatures (Sanceda *et al.,* 1996). Surprisingly, histamine levels of fish sauce produced from tuna internal organs kept in ice were higher than those produced from those kept at room temperature and from fresh viscera.

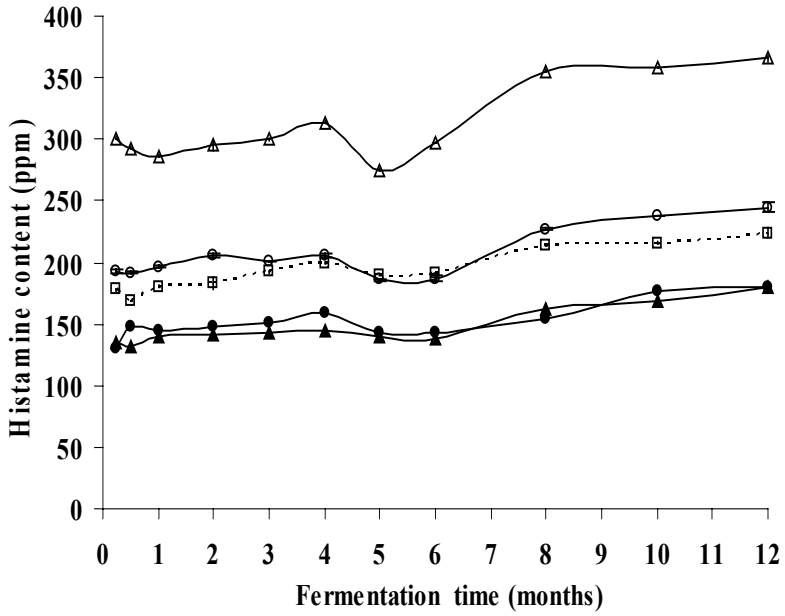

Figure 21 Changes in histamine content of fish sauce from different quality tuna internal organs during fermentation of 12 months. Bars represent the standard deviation from triplicate determinations. (□) control, (Δ) Ice, 4 h, (▲) RT, 4 h, (○) Ice, 8 h, (●) RT, 8 h.

3.1.8 Changes in biogenic amines

Changes of biogenic amines in fish sauce produced from tuna internal organs during fermentation are shown in Table 4 – 9. During the fermentation up to 12 months, b-phenylethylamine, putrescine, cadavarine, histamine, tyramine and spemidine were found in the fish sauce obtained. In general, fish sauce produced from tuna internal organs kept in ice had the greater biogenic amine contents than those produced from tuna internal organs kept at room temperature and from those produced from fresh tuna internal organs ($p<0.05$). At the end of fermentation, b-phenylethylamine decreased from 30-69 ppm to 13-38 ppm and putrescine decreased from 25-48 ppm to 16-38 ppm. Cadavarine, histamine, tyramine and spermidine remained constant during the fermentation, while tryptamine was not detected throughout the fermentation time. For fish sauce produced from tuna internal organs stored at room temperature, the biogenic amines at the higher contents were observed in the fish sauce produced from tuna internal organs stored for 8 h compared with those produced from tuna internal organs stored for 4h ($p<0.05$).

Dapkevicius *et al.* (2000) reported that the decrease in biogenic amines caused by *Lactobacillus sake* strains which can degrade biogenic amines in fermented fish pastes, while Leuschner *et al* (1998) reported that *Micrococcus varians* strains degrade tyramine in fermented sausage. Therefore, the decrease in biogenic amine in fish sauce made from tuna internal organs kept at room temperature or in ice might be owing to microbial degradation as well as the dilution effect caused by the greater liquid formed.

From the result, after 3 month of fermentation ß – phenylethylamine and putrescine decreased. Riaz *et al* (1986) found that proteolysis continued until 160

days (5.3 months) while Beddows *et al*. (1979b) found the volume of liquid produced during fermentation of Malaysian fish sauce, Budu, reached a maximum after 120 to 140 days (4.0 to 4.7 months). The decrease in b–phenylethylamine and putrescine, could also be due to the depletion of phenylethylamine, and ornithine, respectively. However, Sanceda *et al*. (1996) reported that histamine content in fish sauce did not increase even after histidine was added with the purpose of accelerating the fermentation process. The constant contents of cadavarine, histamine, tyramine and spermidine after 3 months indicated that decarboxylase had been inactivated and there was no increase in liquid amount formed.

Table 4 Changes in ß – phenylethylamine content of fish sauce produced from tuna internal organs during fermentation for up to 12 months.

Condition	Fermentation time (months)			
	3	6	9	12
fresh	36 ± 3.98^{aBC}	41 ± 4.30^{aC}	32 ± 1.50^{aC}	13 ± 8.62^{bC}
Ice 4 h	69 ± 1.63^{aA}	49 ± 1.60^{cA}	56 ± 3.62^{bA}	38 ± 1.39^{dA}
Ice 8 h	38 ± 3.96^{aB}	40 ± 4.33^{aB}	29 ± 0.08^{bB}	22 ± 5.86^{bBC}
RT 4 h	$30 \pm 2.^{74aC}$	$35 \pm 1.^{34aBC}$	24 ± 1.07^{aC}	22 ± 2.97^{aAB}
RT 8 h	37 ± 4.71^{aB}	31 ± 4.59^{aC}	30 ± 2.90^{aB}	18 ± 6.90^{bBC}

Mean ± Standard deviation from triplicate determinations.
Different letters in the same row indicate the significant differences ($p< 0.05$).
Different capital letters in the same column indicate the significant differences ($p< 0.05$).

Table 5 Changes in putrescine content of fish sauce produced from tuna internal organs during fermentation for up to 12 months.

Condition	Fermentation time (months)			
	3	6	9	12
fresh	27 ± 1.99^{aBC}	27 ± 3.98^{aB}	23 ± 1.02^{aBC}	16 ± 10.59^{aB}
Ice 4 h	48 ± 1.85^{aA}	43 ± 4.12^{abA}	40 ± 4.13^{bA}	38 ± 1.18^{bA}
Ice 8 h	31 ± 1.80^{aB}	26 ± 3.01^{bB}	25 ± 1.33^{bB}	24 ± 1.57^{bB}
RT 4 h	23 ± 1.39^{aC}	21 ± 1.21^{abB}	20 ± 1.44^{bcC}	18 ± 1.34^{cB}
RT 8 h	25 ± 3.27^{aC}	$23 \pm 2.^{52aB}$	21 ± 1.44^{aBC}	23 ± 0.86^{aB}

Mean ± Standard deviation from triplicate determinations.
Different letters in the same row indicate the significant differences ($p< 0.05$).
Different capital letters in the same column indicate the significant differences ($p< 0.05$).

Table 6 Changes in cadaverine content of fish sauce produced from tuna internal organs during fermentation for up to 12 months.

Condition	Fermentation time (months)			
	3	6	9	12
fresh	56 ± 3.19^{aBC}	56 ± 7.96^{aB}	57 ± 2.78^{aBC}	58 ± 4.81^{aB}
Ice 4 h	100 ± 1.91^{aA}	90 ± 9.05^{aA}	96 ± 9.48^{aA}	95 ± 3.97^{aA}
Ice 8 h	63 ± 1.17^{aB}	57 ± 8.56^{aB}	65 ± 4.33^{aB}	63 ± 3.69^{aB}
RT 4 h	51 ± 4.55^{aC}	45 ± 3.75^{aB}	51 ± 3.54^{aC}	47 ± 1.39^{aC}
RT 8 h	54 ± 6.74^{bC}	53 ± 4.66^{bB}	58 ± 4.05^{abBC}	64 ± 5.23^{aB}

Mean ± Standard deviation from triplicate determinations.
Different letters in the same row indicate the significant differences ($p< 0.05$).
Different capital letters in the same column indicate the significant differences ($p< 0.05$).

Table 7 Changes in histamine content of fish sauce produced from tuna internal organs during fermentation for up to 12 months.

Condition	Fermentation time (months)			
	3	6	9	12
fresh	141 ± 2.39^{aC}	135 ± 1.53^{aB}	138 ± 4.90^{aB}	135 ± 0.89^{aB}
Ice 4 h	247 ± 5.79^{aA}	222 ± 8.98^{bA}	234 ± 12.22^{abA}	233 ± 0.49^{abA}
Ice 8 h	153 ± 6.32^{aB}	141 ± 11.09^{aB}	148 ± 8.71^{aB}	147 ± 0.45^{aB}
RT 4 h	109 ± 3.26^{aD}	100 ± 4.03^{aC}	107 ± 0.98^{aC}	109 ± 0.15^{aC}
RT 8 h	109 ± 2.09^{aD}	99 ± 5.31^{bC}	111 ± 3.44^{aC}	108 ± 0.50^{aB}

Mean ± Standard deviation from triplicate determinations.
Different letters in the same row indicate the significant differences ($p < 0.05$).
Different capital letters in the same column indicate the significant differences ($p < 0.05$).

Table 8 Changes in tyramine content of fish sauce produced from tuna internal organs during fermentation for up to 12 months.

Condition	Fermentation time (months)			
	3	6	9	12
fresh	5 ± 0.22^{aB}	5 ± 0.25^{aB}	5 ± 1.03^{aBC}	5 ± 0.89^{aB}
Ice 4 h	10 ± 0.57^{aA}	10 ± 1.00^{abA}	9 ± 0.30^{bA}	9 ± 0.49^{bA}
Ice 8 h	6 ± 1.20^{aB}	5 ± 0.98^{aBC}	6 ± 0.20^{aB}	5 ± 0.45^{aB}
RT 4 h	5 ± 1.26^{aB}	4 ± 0.35^{aC}	4 ± 1.22^{aC}	3 ± 0.15^{aC}
RT 8 h	5 ± 1.18^{aB}	5 ± 0.82^{aBC}	6 ± 0.44^{aB}	5 ± 0.50^{aB}

Mean ± Standard deviation from triplicate determinations.
Different letters in the same row indicate the significant differences ($p < 0.05$).
Different capital letters in the same column indicate the significant differences ($p < 0.05$).

Table 9 Changes in spermidine content of fish sauce produced from tuna internal organs during fermentation for up to 12 months.

Condition	Fermentation time (months)			
	3	6	9	12
fresh	4 ± 0.66^{aA}	5 ± 0.96^{aA}	4 ± 0.28^{aA}	4 ± 0.44^{aA}
Ice 4 h	4 ± 0.26^{aA}	4 ± 0.70^{abA}	4 ± 0.47^{abA}	3 ± 0.30^{bA}
Ice 8 h	5 ± 0.94^{aA}	4 ± 0.76^{aA}	4 ± 0.46^{aA}	3 ± 0.39^{aA}
RT 4 h	5 ± 1.07^{aA}	4 ± 0.23^{abA}	4 ± 1.20^{abA}	4 ± 0.33^{bA}
RT 8 h	5 ± 6.00^{aA}	4 ± 1.00^{aA}	4 ± 0.31^{aA}	4 ± 0.26^{aA}

Mean ± Standard deviation from triplicate determinations.
Different letters in the same row indicate the significant differences ($p< 0.05$).
Different capital letters in the same column indicate the significant differences ($p< 0.05$).

3.2 Physical changes

3.2.1 Changes in fluorescence intensity

The fluorescence intensity of all samples reached a maximal value at 4-5 months of fermentation. After 5 months, fluorescence intensity decreased sharply up to 12 months of fermentation (Figure 22) ($p<0.05$). Benjakul *et al*. (2005a) used the fluorescence intensity to monitor the intermediate product of Maillard reaction in PPP-glucose model system heated at 100°C. Nonenzymatic interaction between a reducing sugar and an amino acid, peptide or protein has been known as the Maillard reaction. The Maillard reaction produced a variety of intermediate products and brown pigments (melanoidins) are finally formed (Van Boekel, 1998). The decrease in fluorescence intensity was observed with increasing fermentation time, suggesting

the conversion of those intermediates to the final brown products. Development of fluorescent compounds occurs in the Maillard reaction prior to the generation of brown pigments (Jing and Kitts, 2002; Morales *et al.,* 1996). Fluorescent compounds are possible precursors of brown pigments (Labuza and Baisier, 1992). The decrease in fluorescence intensity was observed as fermentation time increased, presumably due to the decrease in precursor, especially reducing sugar as well as the development of browning from those fluorescent compounds.

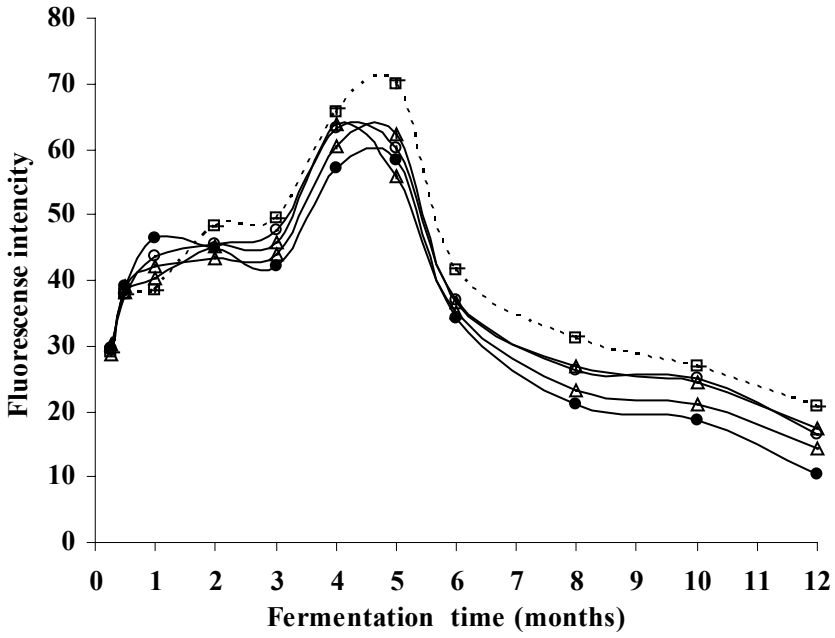

Figure 22 Changes in fluorescence intensity of fish sauce from different quality tuna internal organs during fermentation of 12 months. Bars represent the standard deviation from triplicate determinations. (□) control, (Δ) Ice, 4 h, (▲) RT, 4 h, (○) Ice, 8 h, (●) RT, 8 h.

3.3.2 Changes in A_{294}

Continuous increase in A_{294} in all samples was observed as the fermentation time increased (Figure 23) ($p<0.05$). However, the decrease in A_{294} was noticeably after 6 months of fermentation, indicating the conversion of those intermediates to brown products. Fish sauce produced from tuna internal organs stored for 8 h at room temperature tended to have the highest A_{294}. Fish sauce produced from fresh tuna internal organs had a lower A_{294} than other samples when the fermentation was higher than 3 months. A_{294} was used to determine the non-fluorescent intermediate compounds of Maillard reaction (Benjakul *et al.,* 2005a; Ajandouz *et al,* 2001; Lerici *et al.,* 1990). The differences in A_{294} among the samples might be owing to the differences in reaction determined by different amount and reactivity of reactants.

The differences in changing pattern between fluorescence intensity and A_{294} of fish sauces during fermentation suggested that different types of intermediate products, either fluorescent or non-fluorescent compounds, were formed and underwent the final stage of reaction with different rates.

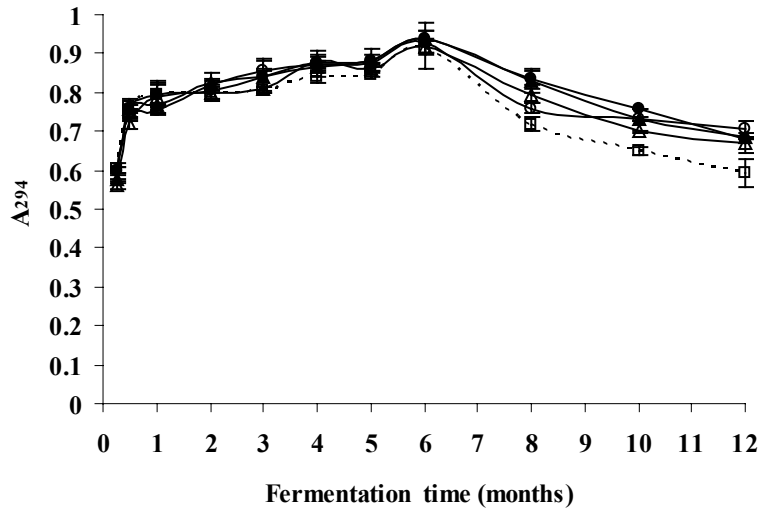

Figure 23 Changes in A_{294} of fish sauce from different quality tuna internal organs during fermentation of 12 months. Bars represent the standard deviation from triplicate determinations. (□) control, (Δ) Ice, 4 h, (▲) RT, 4 h, (○) Ice, 8 h, (●) RT, 8 h.

3.2.3 Changes in A_{420}

An increase in browning of fish sauce, as measured by A_{420}, was observed as the fermentation time increased ($p<0.05$) (Figure 24). Fish sauce produced from tuna internal organs stored for 8 h at room temperature showed a greater increase in browning intensity than that produced from fresh tuna internal organs as well as from samples kept in ice. Lopetcharat *et al.* (2002) reported that A_{420} of commercial fish sauce, which was ripened with additional processes for color and flavor development, varied from 1.1 to 4.2. The increase in A_{420} throughout the fermentation was coincidental with the increase in the total nitrogen and amino nitrogen contents. It was noted that fish sauce made from tuna internal organs stored

at room temperature for 8 h containing a greater amino nitrogen content (Figure 14) showed the highest A_{420}. Most of the nitrogenous compounds in fish sauce are free amino acids and small peptides, which contribute to brown color development via Maillard reaction. Even though reducing sugar content in fish sauce is low, carbohydrate derivative, such as glucose-6-phosphate and other substances presenting in the metabolic pathways, can also act as reactants to initiate the Maillard reaction (Kawashima and Yamanaka, 1996). The increase in A_{420} was used as an indicator for browning development in the final stage of the browning reaction (Ajandouz *et al.,* 2001; Morales *et al*., 2001).

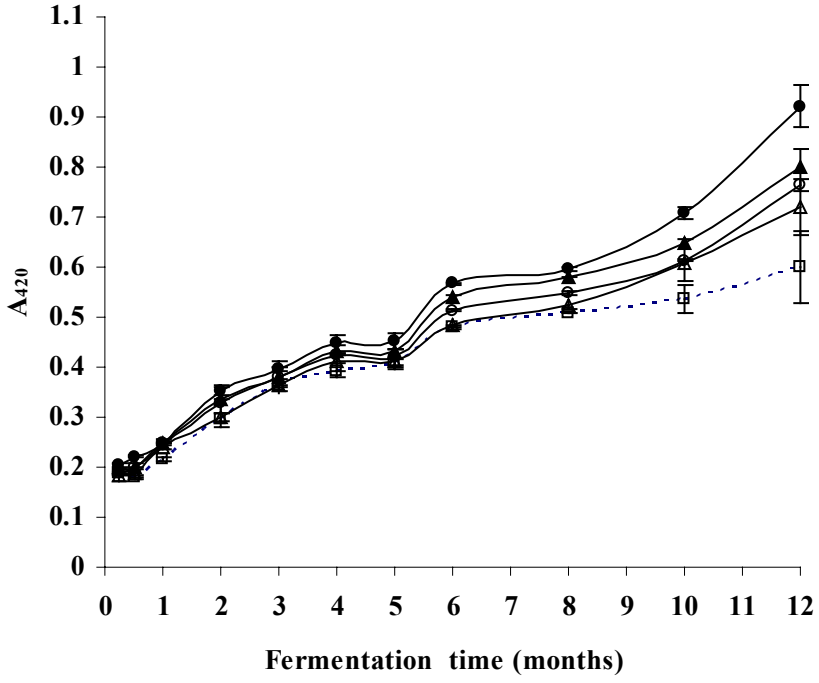

Figure 24 Changes in A_{420} of fish sauce from different quality tuna internal organs during fermentation of 12 months. Bars represent the standard deviation from triplicate determinations. (□) control, (Δ) Ice, 4 h, (▲) RT, 4 h, (○) Ice, 8 h, (●) RT, 8 h.

3.2.4 Changes in color L*, a* and b* value

Color values of fish sauces during fermentation are shown in Figure 25. The results indicated a decrease in L*-value (lightness) during the first 6 months of fermentation ($p<0.05$). The a*-value of all samples increase up to 4 months of fermentation ($p<0.05$). Thereafter, the constant a*-value was obtained. For b*-value, it decreased and reached the minimal at month 5 with the subsequent increase up to 12 months ($p<0.05$). This suggested the development of yellowness of fish sauce with increasing fermentation time. The decreases in L*-value and increases in a*-value were accordance with brown color development. Lee *et al*. (1997) reported that fish and soy sauce became darker with melanoidine produced by the Maillard reaction during storage. Kim *et al*. (2004) found that the color value (L*, a*, and b*) of salted and fermented anchovy sauce decreased during storage.

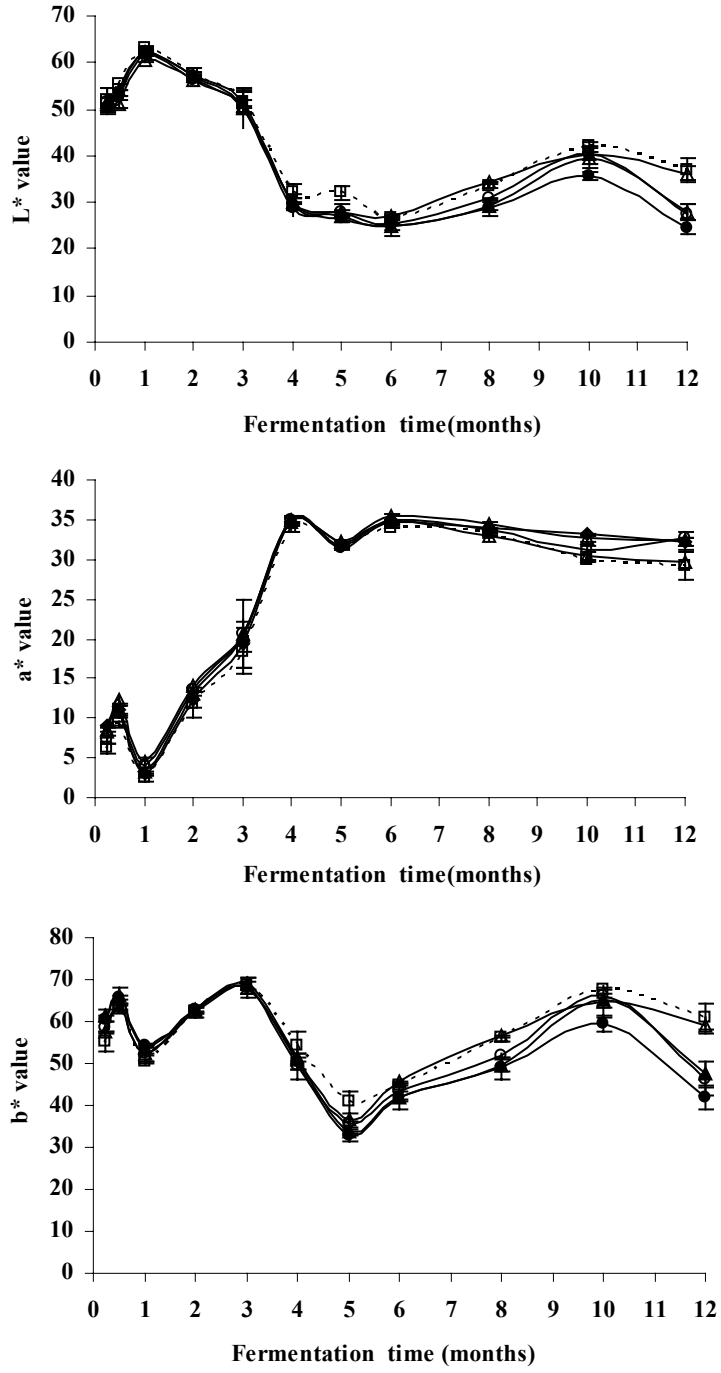

Figure 25 Changes in color values of fish sauce from different quality tuna internal organs during fermentation of 12 months. Bars represent the standard deviation from triplicate determinations. (□) control, (Δ) Ice, 4 h, (▲) RT, 4 h, (○) Ice, 8 h, (●) RT, 8 h.

3.3 Microbiological changes

Total viable count (TVC), proteolytic bacteria and halophilic bacteria in all samples steadily decreased during the extended fermentation (Figure 26) ($p < 0.05$). Fish sauce produced from tuna internal organs kept at room temperature had a greater number of all microorganisms tested than those produced from samples kept in ice throughout the fermentation ($p<0.05$). Samples stored either in ice or at room temperature for a longer time gave the fish sauce with the higher TVC, proteolytic bacteria and halophilic bacteria ($p<0.05$). During iced storage, the growth rate of microorganisms could be retarded, resulting in the lower microbial counts. Jeyasekaran *et al*. (2004) found that fresh fish is susceptible to rapid spoilage at the ambient temperature. The microbial load found in the raw material directly affected the count obtained in the fish sauce. Microbial counts decreased continuously when the fermentation time increased, possibly caused by high concentrations of salt. Lopetcharat *et al.* (2002) reported that an increase in the number of halophilic microorganisms was observed at day 10 during Pacific whiting fish sauce fermentation, and then decreased rapidly to an undetectable level at day 20. TVC of all sample decreased to less than 1 log CFU/ml after 4 months of fermentation, except that TVC of fish sauce made from internal organs stored at room temperature for 8 h was lower than 1 log CFU/ml after 5 months ($p<0.05$). Halophilic bacteria and proteolytic bacteria counts decreased gradually during fermentation and were lower than 1 log CFU/ml after 4 and 5 months, respectively ($p<0.05$). Those microorganisms might contribute to the hydrolysis of proteins as well as the flavor development of fish sauce obtained.

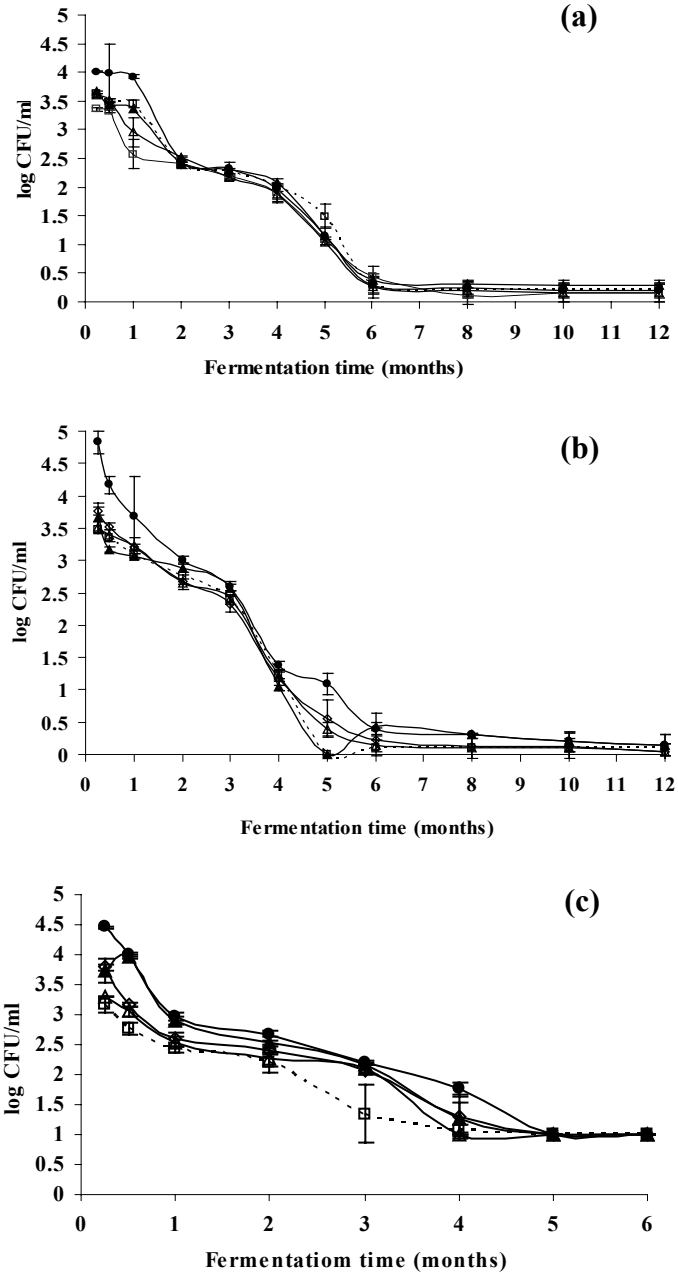

Figure 26 Total viable counts (a), halophilic bacteria (b) and proteolytic bacteria count (c) of fish sauce from different quality tuna internal organs during fermentation of 12 months. Bars represent the standard deviation from triplicate determinations. (□) control, (Δ) Ice, 4 h, (▲) RT, 4 h, (○) Ice, 8 h, (●) RT, 8 h.

4. Effect of calcium chloride and pH on proteolytic activity and changes of fish sauce from tuna internal organs with different salt levels during fermentation.

4.1 Proteolytic activity

The effect of calcium chloride ($CaCl_2$) and pH on the proteolytic activity in the fish sauce produced from tuna internal organs with different salt levels is presented in Figure 27. The activity of all treatments decreased continuously with increasing fermentation time ($p<0.05$) (Figure 27). The sharp decrease was found after 2 months of fermentation. Generally, the rate of changes varied, depending on the salt concentration, pH and $CaCl_2$ concentration. The decrease in activity during fermentation might be due to the denaturation of enzymes, particularly in the presence of high salt content (Hernàndez-Herrero *et al.,* 1999).

At the early stage of fermentation, the proteolytic activity of samples with pH adjusted to 9 was higher than those of sample without pH adjustment. However, no marked differences were found with sample containing $CaCl_2$ at 0 and 1.5% when the fermentation time increased. It was noted that pH adjustment to 9 resulted in the substantial increase in proteolytic activity of sample containing 3% $CaCl_2$ at both levels of salt. Proteinase found in the intestine including trypsin, chymotrypsin, collagenase, and elastase are normally secreted from the pyloric caeca and pancreas (Haard and Simpson. 1994). Trypsin is concentrated in pyloric caeca and active at neutral and alkaline condition (Martinez *et al.,* 1988; Martinez and Serra, 1989). Fish trypsin is generally stable at alkaline pH. Purified trypsin from hybrid tilapia (*Tilapia nilotica*) intestines showed an optimum at pH 9 (Shemy and Levin,

1997). Two trypsin like enzymes isolated from gut of capelin showed optimum pH at 8-9. Gildberg (2001) reported that the addition of 5 to 10% enzyme-rich (trypsin and chymotrypsin) cod intestines into minced capelin could reduce the fermentation of fish sauce to 6 months.

Moderate initial alkalification accelerates the tissue solubilization, without affecting the pH of the final product (Gildberg 2001). The fish sauce with15% salt showed the greater activity than that with 25% salt, particularly for sample containing 3% $CaCl_2$. From the result, fish sauce without $CaCl_2$ had the highest proteolytic activity, followed by those with 1.5% and 3.0%, respectively. The fish sauce containing 15% salt and adjusted to pH 9 without $CaCl_2$ showed the highest activity than other treatments. High salt concentration (25%) prolonged fish sauce shelf-life but it inhibited peptidase activity and hence retarded protein hydrolysis (Gildberg, 1989). Salt reduction from 25% to 5-15% accelerated autolysis during fish sauce fermentation (Sikorski *et al.,* 1995). Tongkawachara *et al*. (2003) and Gildberg (1992) reported that increased ionic strength caused by high salt along with the extended incubation time at high temperature (37°C), possibly resulted in the increased denaturation and loss in enzyme activity during fermentation. The activity of trypsin-like enzyme in protein hydrolysate made from 75% fish viscera and 25% salt at 27°C was only 10% after 50 days (Gildberg, 1992). From the result, addition of $CaCl_2$ caused the decrease in proteolytic activity, especially at the higher level used. However, Gildberg (2001) reported that addition of calcium (2 mM) apparently had no effect on either protease stability or activity. Proteolytic activity of crude extracts from the brown shimp was affected by the addition of divalent cations (Vega-Villasante *et al*, 1995). El-beltagy *et al*. (2004) reported that the activation percentage

was increased with increase of activator ($CaCl_2$) and reached 56.1% when 50 mM $CaCl_2$ were used. In the present experiment, addition of calcium chloride (1.5 and 3.0%) apparently had no effect on activation. Ionic effects vary from one enzyme to another, such that an ion could be an activator to one enzyme but an inhibitor to another. Furthermore, the concentration of the ion may have an effect on the response in which it could activate the enzyme at one concentration and inhibit at another (Simpson, 2000). Yoshinaka *et al.* (1983) found that calcium both increases and stabilized the protease activity of fish intestines. Therefore, $CaCl_2$ levels used might be excessive for trypsin activation and turned to function as an inhibitor probably by the "salting out" effect.

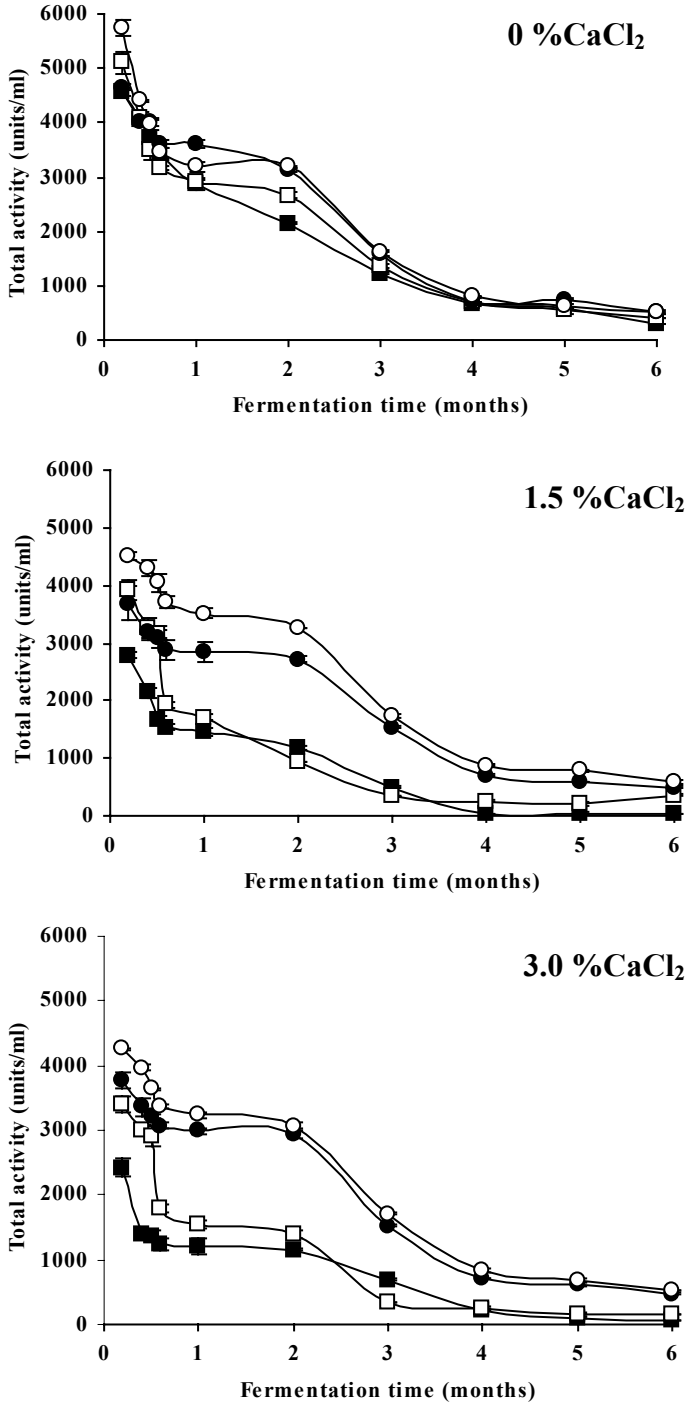

Figure 27 Changes in proteolytic activity of fish sauce from tuna internal organs as affected by salt, $CaCl_2$ and pH adjustment during fermentation. Bars represent the standard deviation from triplicate determinations. (●) 15% salt, (○) 15% salt pH 9, (■) 25% salt, (□) 25% salt pH 9

4.2 Chemical changes

4.2.1 Total nitrogen content

The changes in total nitrogen content of fish sauce produced from tuna internal organs with different treatments throughout the fermentation period are depicted in Figure 28. Total nitrogen content of all treatments increased with increasing fermentation time ($p<0.05$). At early stage of fermentation (0.2-0.6 months), total nitrogen content of fish sauce produced from tuna internal organs with 15% salt was greater than that of fish sauce produced from internal organs with 25% NaCl. Total nitrogen content of samples with pH adjustment to 9 was higher than those of samples without pH adjustment. However, no substantial differences in total nitrogen content of fish sauce added with different $CaCl_2$ levels were observed. From the result, total nitrogen content of fish sauce obtained at month 0.2 of each treatment was similar regardless of $CaCl_2$ concentration. Though it was found that $CaCl_2$ at 3.0% showed the inhibitory effect on proteolytic acivity, total nitrogen content seemed to be similar. This was probably due to the hydrolytic activity was considerably high prior to NaCl or $CaCl_2$ could penetrate into organ tissue. This led to the similar hydrolysis after 0.2 months of fermentation. For the sample added with 15% NaCl, the salt content was adjusted to 25% after 0.6 months (18 days) when the total nitrogen content reached the constant level. Total nitrogen content in fish sauce is mainly from protein nitrogen and non protein nitrogen compounds such as free amino acids, nucleotide, peptide, ammonia, urea and TMAO. These components contribute to the specific aroma and flavor (Finne, 1992; Shahidi, 1994). Addition of the higher amount of salt caused the reduction of the breakdown of the fish meat by autolysis or microbial activities (Gildberg, 1989). Lopetcharat and Park (2002) found

that the release of water-soluble proteins from cell by osmotic pressure resulted in an increased total nitrogen content. Brillantes *et al*. (2002) reported that the high salt content at 25 to 30 % could slow down the rate of protein hydrolysis by both fish and bacterial enzymes. Addition of sodium hydroxide at a moderate salt concentration resulted in the maximization of alkaline digestive proteases, whereas endogenous trypsin and chymotrypsin inhibitors were denatured. These conditions shortened the process to 2 months (Gilberg, 1989).

From the results, the rate of increase in soluble nitrogen content decreased after 0.5 months (15 days). The great reduction of trypsin-like enzyme caused by the fermentation was reported (Orejana and Liston 1981). Total nitrogen content is an objective index used to classify the quality of nampla, Thai fish sauce (Lopetcharat *et al.,* 2002). High quality nampla must have a total nitrogen content of 20 g N/L based on the Kjedahl method (Thai Industrial Standard, 1983). The total nitrogen content, equivalent to higher than 20 g N/L, was obtained from fish sauce produced from internal organs with 15% NaCl regardless of $CaCl_2$ concentrations after 1 month (30 days). Normally, 12 months of fermentation are enough for nampla production (Saisithi *et al.,* 1966) and 154 days are for budu (Beddows *et al.,* 1979). Faster fermentation for fish sauce made from tuna internal organs was most likely due to combined effects of high enzymatic activity and low salt concentration, especially at the early stage of fermentation.

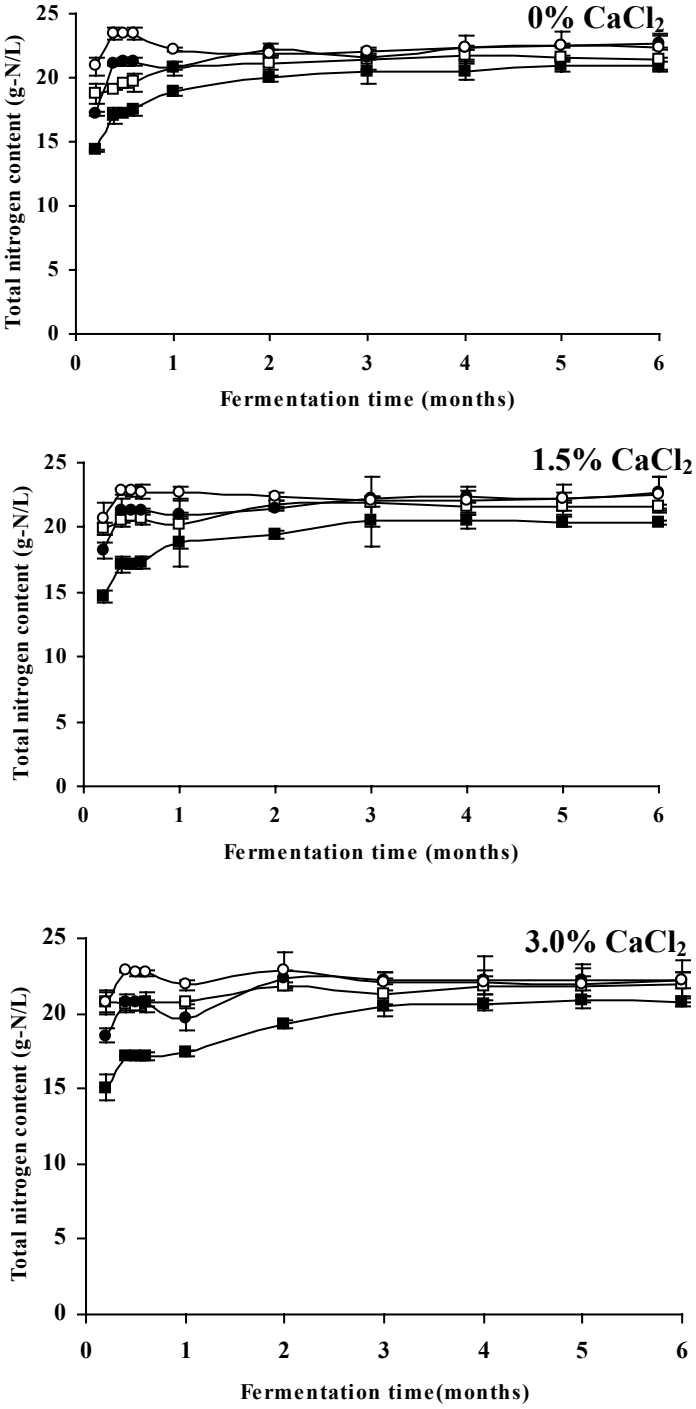

Figure 28 Changes in total nitrogen content of fish sauce from tuna internal organs as affected by salt, $CaCl_2$ and pH adjustment during fermentation. Bars represent the standard deviation from triplicate determinations. (●) 15% salt, (○) 15% salt pH 9, (■) 25% salt, (□) 25% salt pH 9

4.2.2 Changes in formol nitrogen content

Formol nitrogen content of fish sauce with different treatment during fermentation is depicted in Figure 29. Formol nitrogen content in all samples increased with increasing fermentation time ($p<0.05$). Formaldehyde nitrogen content is used as a convenient index of the degree of protein hydrolysis (Chaveesuk *et al.,* 1993). From the result, sample with lower salt concentration and pH adjustment to 9 regardless of $CaCl_2$, contained greater formol nitrogen content, compared with the samples without pH adjustment or higher salt content. From the result, the samples with different $CaCl_2$ concentration generally had no differences in formol nitrogen content throughout the fermentation of 6 months ($p<0.05$). The formol nitrogen content of fish sauce added with 25% salt and 3.0 % $CaCl_2$ without pH adjustment had the lowest formol nitrogen content. This suggested the lower hydrolysis of proteins which was coincidental with the lower proteolytic activity of this sample (Figure 27). With 15% salt, formol nitrogen content of sample was greater than sample containing 25% salt. Additionally, the higher formol nitrogen content was found with samples subjected to pH adjustment. This result indicated that endogenous proteinase in tuna internal organs directly directly in liquefaction at low salt concentration. The pH adjustment to alkaline pH also increased the conversion of insoluble proteins to soluble peptides.

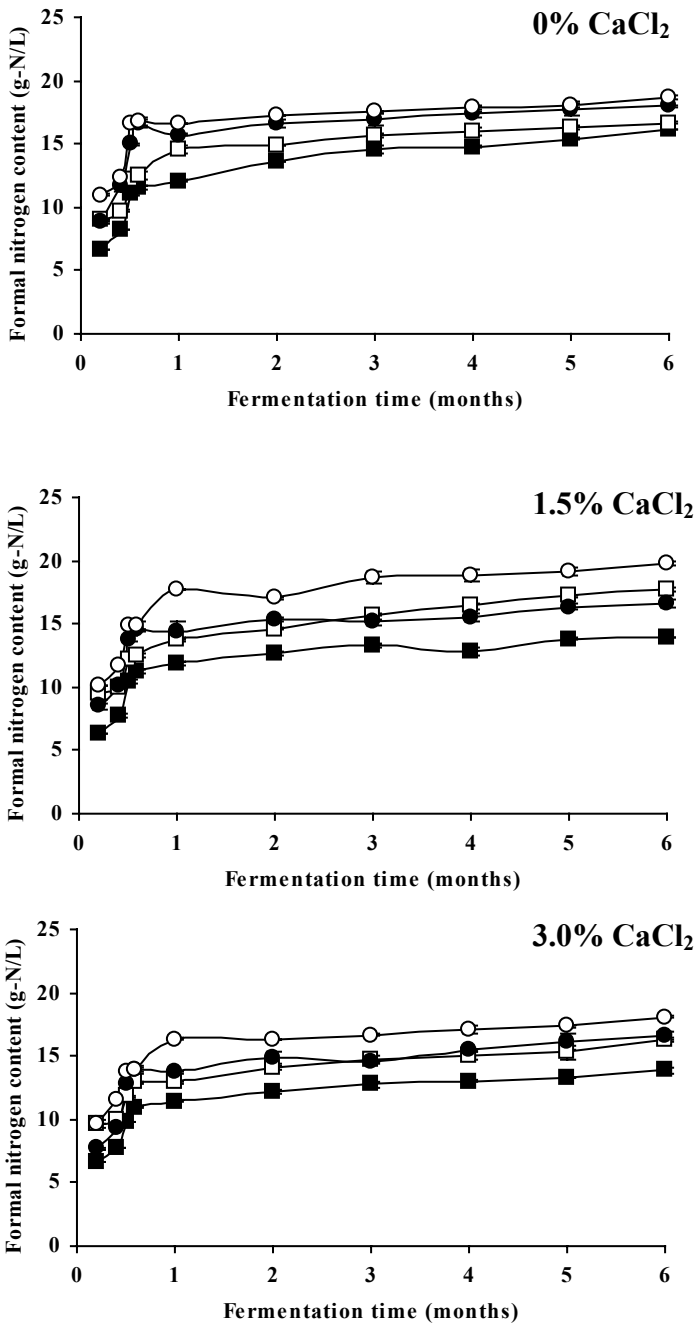

Figure 29 Changes in formol nitrogen content of fish sauce from tuna internal organs as affected by salt, $CaCl_2$ and pH adjustment during fermentation. Bars represent the standard deviation from triplicate determinations. (●) 15% salt, (○) 15% salt pH 9, (■) 25% salt, (□) 25% salt pH 9

4.2.3 Changes in ammonia nitrogen content

Changes in ammonia nitrogen content of fish sauce sample during fermentation of 6 months are depicted in Figure 30. The ammonia nitrogen content of all samples increased as fermentation time increase ($p<0.05$). The ammonia nitrogen content indicates the breakdown of soluble protein and peptides into free amino acid and volatile nitrogen (Lopetcharat *et al.,* 2002; Chaveesuk *et al.,* 1993). The increased ammonia nitrogen content could be due to fish enzymes that were active during early days of fermentation (Beddows *et al.,* 1980). The higher ammonia nitrogen content was observed in samples with lower salt concentration ($p<0.05$). Regardless of $CaCl_2$ addition, the ammonia nitrogen content of fish sauce added with 25% salt without pH adjustment had the lowest ammonia nitrogen content, when compared with sample with lower salt content and pH adjustment. This result suggested that the ammonia or volatile compounds generated by spoilage microorganism might be reduced in the presence of high salt. At low salt concentration, the spoilage might take place, particularly with increasing fermentation time (Beddows and Ardeshir 1979a,b). Salt inhibits microbial activity, including pathogenic and spoilage bacteria. It seemed that the lower amounts of volatile acids produced in the fish added with 25% salt might be due to the inhibited microbial activity brought about by addition of salt (Sanceda *et al.,* 2001). During the anaerobic digestion process, organic nitrogen compounds are transformed to ammonia nitrogen, which partially remains in solution. Lopetcharat *et al.* (2001) reported that during fermentation, the fish protein is hydrolyzed by endogenous enzymes and microorganism into small peptides and amino acids. Ammonia and trimethylamine are formed and are responsible for the ammoniacal note of fish sauce. Since amines could be produced during fermentation, those

compounds might undergo Maillard reaction, leading to flavor as well as color development of fish sauce. The reaction from Schiff base between amine and aldehyde or ketone, which is well known as the Maillard reaction, is believed to play an important role in flavor and color development of fish sauce during the ripening step (Lopetcharat and Park, 2002).

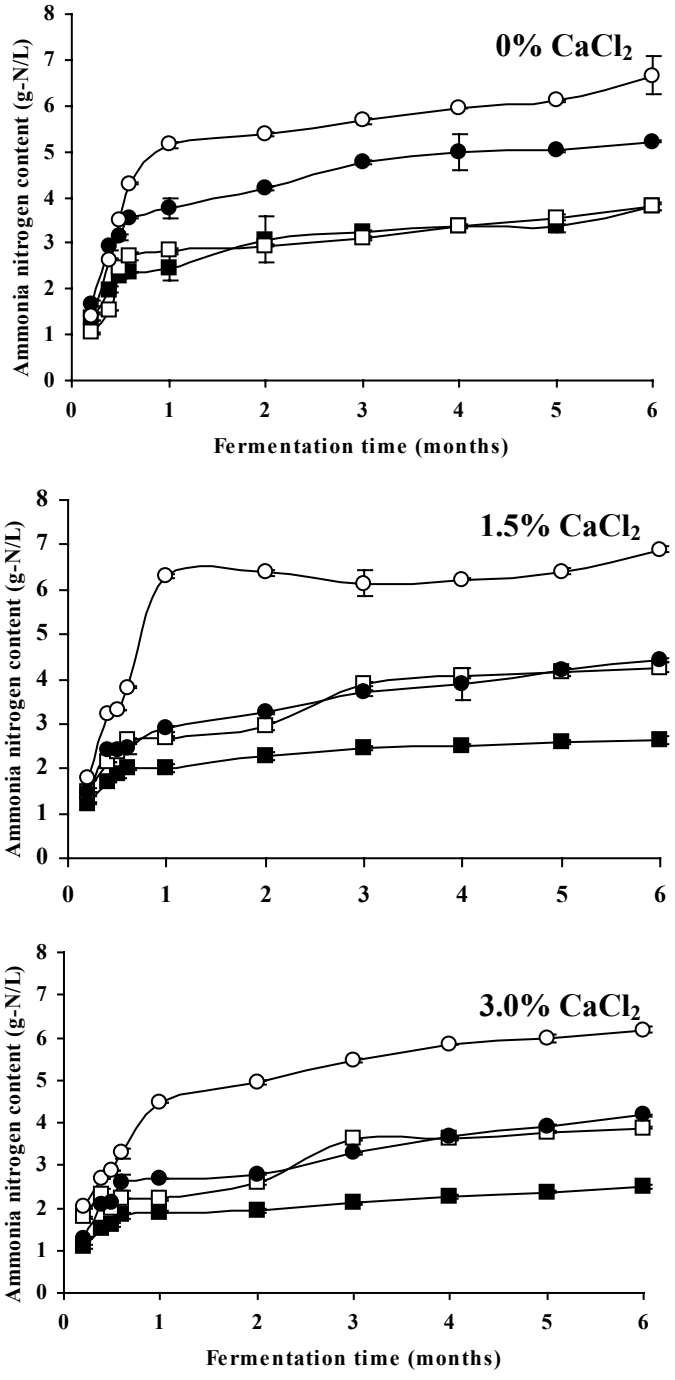

Figure 30 Changes in ammonia nitrogen content of fish sauce from tuna internal organs as affected by salt, $CaCl_2$ and pH adjustment during fermentation. Bars represent the standard deviation from triplicate determinations. (●) 15% salt, (○) 15% salt pH 9, (■) 25% salt, (□) 25% salt pH 9

4.2.3 Changes in amino nitrogen content

Changes in amino nitrogen content of fish sauce from different treatments during 6 months of fermentation are depicted in Figure 31. Amino nitrogen content in all samples increased with increasing fermentation time ($p<0.05$). The amino nitrogen concentration represents the amount of primary amino groups in fish sauce. An increase in amino nitrogen concentration is related to the degradation of polypeptide (Lopetcharat *et al.,* 2002). From the result, fish sauce produced from tuna internal organs with lower salt concentration and pH adjustment contained greater amino nitrogen content, compared with the sample without pH adjustment and higher salt content. Higher salt concentration could retard the hydrolysis of proteins caused by autolysis (Klomklao *et al.,* 2004). With addition of $CaCl_2$ at level of 3.0%, amino nitrogen content was found to be lowered. This result was in accordance with the lower total nitrogen content (Figure 26) and formol nitrogen content (Figure 27) in sample containing 3.0% $CaCl_2$. Amino nitrogen contents were approximately more than 10 g N/L in sample with 15% salt added and different $CaCl_2$ levels after 0.6 months (18 days) of fermentation. According to the Thai Industrial Standard, amino nitrogen contents must be more than 10 g N/L (TISI, 1983). The results indicated that fermentation of fish sauce from tuna internal organs with 15% salt concentration could be achieved within 0.6 months based on amino nitrogen content.

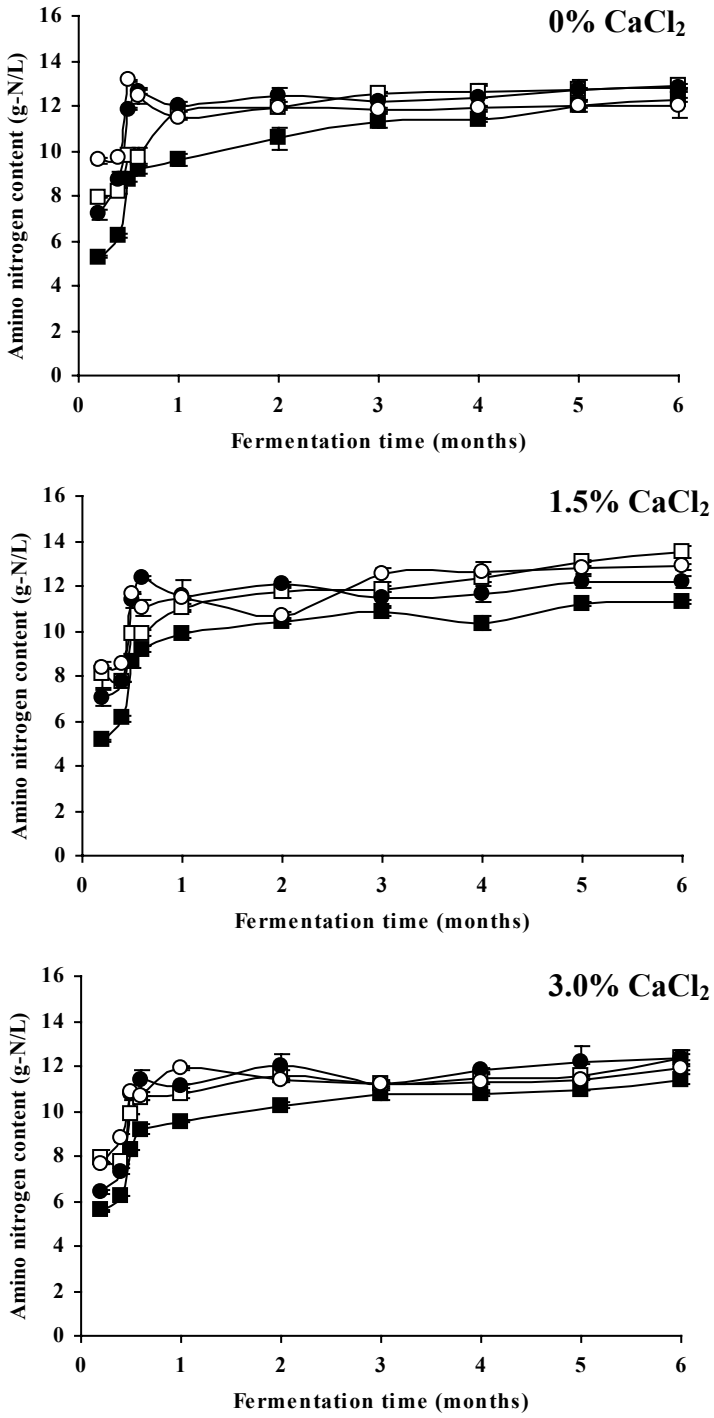

Figure 31 Changes in amino nitrogen content of fish sauce from tuna internal organs as affected by salt, $CaCl_2$ and pH adjustment during fermentation. Bars represent the standard deviation from triplicate determinations. (●)15%salt, (○)15% salt pH 9, (■) 25% salt, (□) 25% salt pH 9

4.2.4 Changes in pH

The changes in pH of fish sauce sample during fermentation of 6 months are depicted in Figure 32. The pH gradually decreased in all sample during the first month of fermentation. Thereafter, it increased after 1 month of fermentation. The final pH value of fish sauce from all treatments ranged from 4.31 to 7.32, depending on the initial pH. The pH value of fish sauce decreased probably due to the dissociation of amino acid and small peptides in the presence of salt (Lopetcharat and Park 2002). Tungkawachara *et al.* (2003) reported that the differences in pH of fish sauce probably resulted from the different free hydrogen ions, free amino acids, and amino acid of oligopeptides. Additionally, fermentation products containing organic acids, such as lactic acid, and acetic acid, also tend to lower the pH of fish sauce (Ijoh *et al*., 1996). Fish sauce produced from tuna internal organs with pH adjusted to 9 was higher than those without pH adjustment. Fish sauce without $CaCl_2$ addition had the higher pH than those added with 1.5% and 3.0% $CaCl_2$.

For the samples added with 15% NaCl, the salt content was adjusted to 25% after 0.6 months (18 days) when the total nitrogen content reached the constant level. From the results after the salt content was adjusted to 25% (after 18 days), the pH gradually increased with increasing fermentation time. The increase in pH throughout the fermentation time was presumably due to the production of basic amines during the fermentation. (Debevere and Boskou, 1996, Pastoriza *et al.,* 1996) For the sample with pH adjustment, these added with 25% salt showed the high pH value, compared with those containing 15% salt, especially when the fermentation time increased.

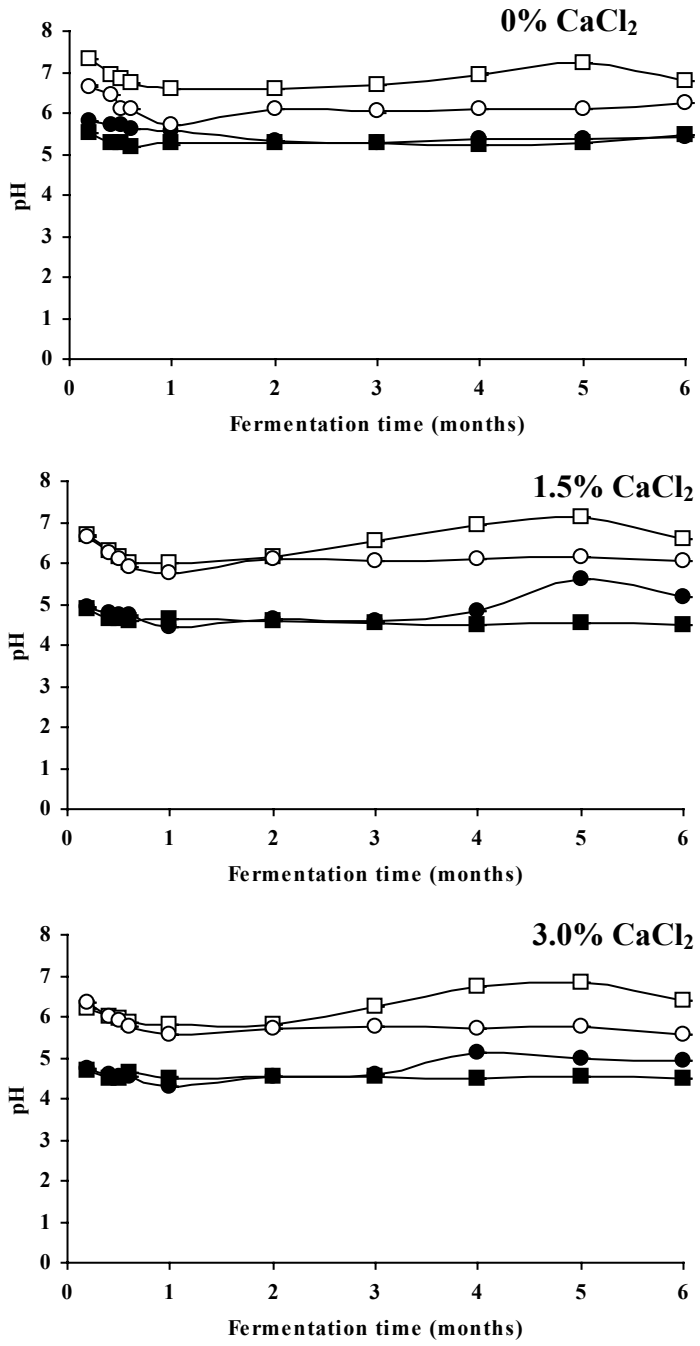

Figure 32 Changes in pH of fish sauce from tuna internal organs as affected by salt, $CaCl_2$ and pH adjustment during fermentation. Bars represent the standard deviation from triplicate determination. (●) 15% salt, (○) 15% salt pH 9, (■) 25% salt, (□) 25% salt pH 9

4.2.5 Changes in TVB and TMA

Total volatile base (TVB) and trimethylamine (TMA) contents of fish sauce produced from tuna internal organs with different treatments throughout the fermentation are depicted in Figure 33 and 34, respectively. TVB and TMA contents of all samples increased with increasing fermentation time ($p<0.05$). From the results, fish sauce with 15% NaCl showed the greater TVB and TMA contents than those with 25% NaCl. This was probably due to the inhibition of microorganisms at high concentration of salt. Jay (1996) also reported that the decreased numbers of microorganisms which caused protein decomposition during fermentation are due to high concentration of salt. Sample with pH adjusted to 9 had the higher TVB and TMA contents compared with samples without pH adjustment, especially after 3 months of fermentation ($p<0.05$). At the end of the fermentation period, the highest TVB content was observed with the samples containing 15% NaCl and 3.0% $CaCl_2$ (149.94mg/100 ml).

TVB and TMA formation from degraded proteins and non-protein nitrogenous compounds is mainly as a result of microbial activity (Connell, 1975). Total volatile bases and trimethylamine are used as an index to assess the quality of seafood products (Lannelogue *et al*., 1982). This result suggested that the spoilage caused by bacteria still occurred, particularly when storage time increased. The increases in both TVB and TMA contents were in accordance with the increase in pH (Figure 32). Those volatile compounds are known to cause the offensive odor in the fish.

For TMA, the pattern of changes was similar to that of TVB. Fish sauce produced from tuna internal organ with 15% NaCl had greater increase in

TMA, compared with fish sauce produced from tuna internal organ containing 25% NaCl regardless of $CaCl_2$ addition. Hansen *et al* (1995) found that the final concentration and the rate of development of TMA depended upon the salt level; decreasing salt resulted in higher concentration of TMA. Connel (1975) reported a maximum limit of 200 mg/100 g sample for TVB content in fish processed with salt. From the result, even though fish sauce produced from tuna internal organ with pH adjustment and low salt (15% NaCl) had the high TVB and TMA contents, those values were not above the limit. The results indicated that fish sauce produced from tuna internal organs with all conditions used could be accepted based on TVB content.

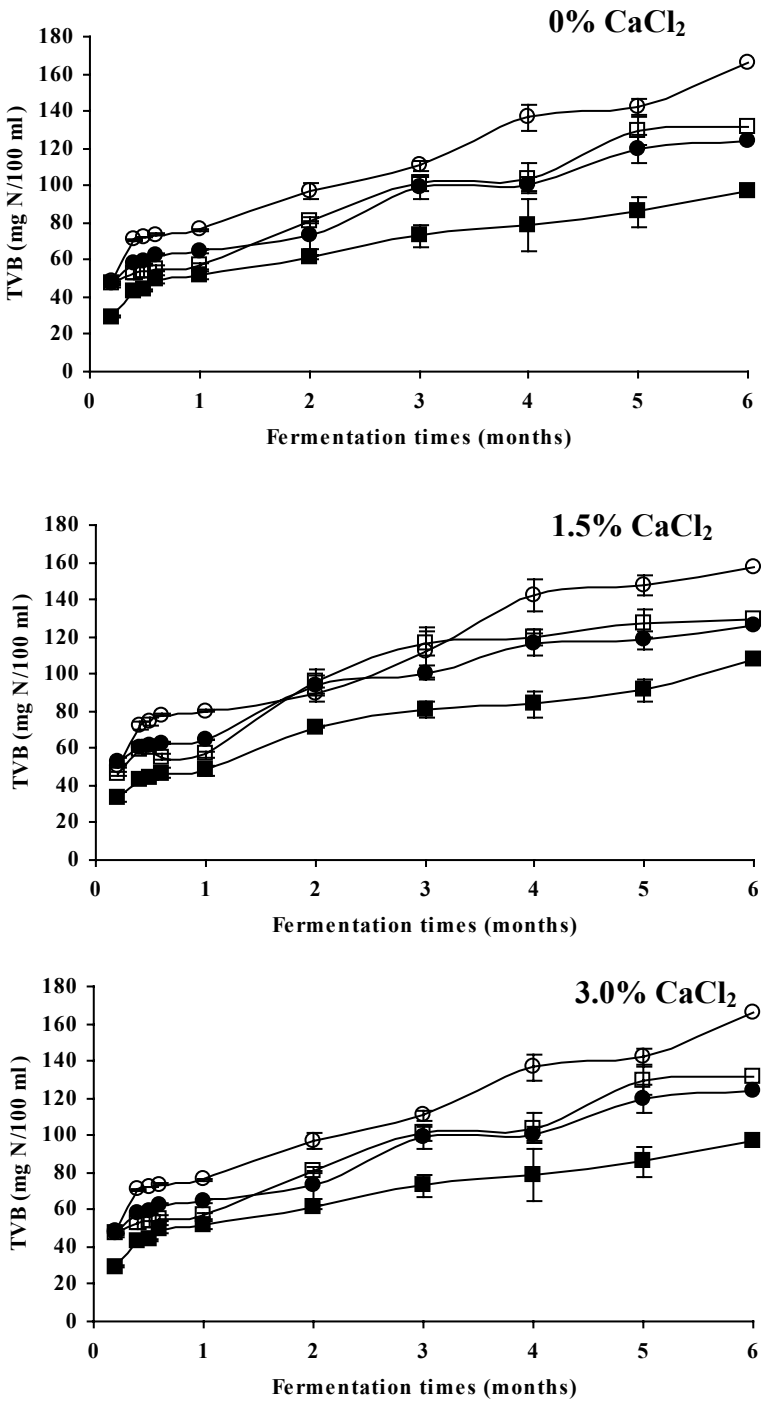

Figure 33 Changes in TVB content of fish sauce from tuna internal organs as affected by salt, $CaCl_2$ and pH adjustment during fermentation. Bars represent the standard deviation from triplicate determinations. (●) 15% salt, (○) 15% salt pH 9, (■) 25% salt, (□) 25% salt pH 9

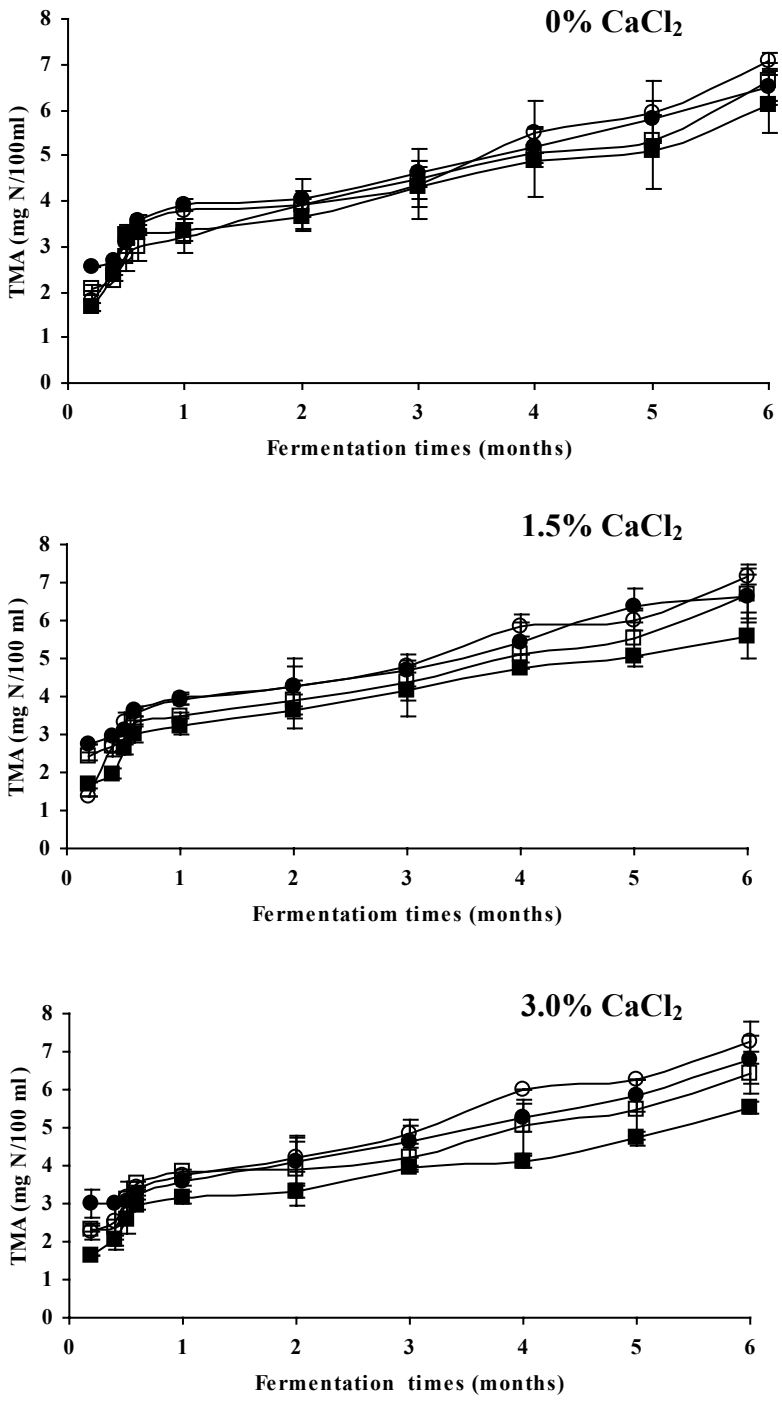

Figure 34 Changes in TMA content of fish sauce from tuna internal organs as affected by salt, $CaCl_2$ and pH adjustment during fermentation. Bars represent the standard deviation from triplicate determinations. (●) 15% salt, (○) 15% salt pH 9, (■) 25% salt, (□) 25% salt pH 9

4.3 Physical changes

4.3.1 Changes in A_{294}

A_{294} was used to determine the intermediate compounds of Maillard reaction (Lerici *et al.,* 1990; Ajandouz *et al.,* 2001). Continuous increase in A_{294} in all samples was observed as the fermentation time increased up to 1 month of fermentation. (Figure 35). The increase in A_{294} suggested the formation of an uncolored compound, which could be the precursor of the Maillard reaction (Ajandouz *et al.,* 2001; Lerici *et al.,* 1990). Thereafter the gradual decrease in A_{294} was noticeable, suggesting the conversation of those intermediates to brown products. The decrease in A_{294} may result from the transformation of some intermediate products into brown polymers (Ajandouz *et al.,* 2001). Fish sauce produced from tuna internal organ with pH adjustment and added with 15% NaCl without $CaCl_2$ tended to had the highest A_{294}. At lower salt and higher pH, the hydrolysis would occur to a greater extent and more free amino group was released for Maillard reaction. At the same levels of $CaCl_2$ addition, samples with pH adjusted to 9 showed the greater A_{294}. Fish sauce produced with low salt concentration showed the highest A_{294}. The higher concentration of $CaCl_2$ used, the lower the increase in A_{294} was found.

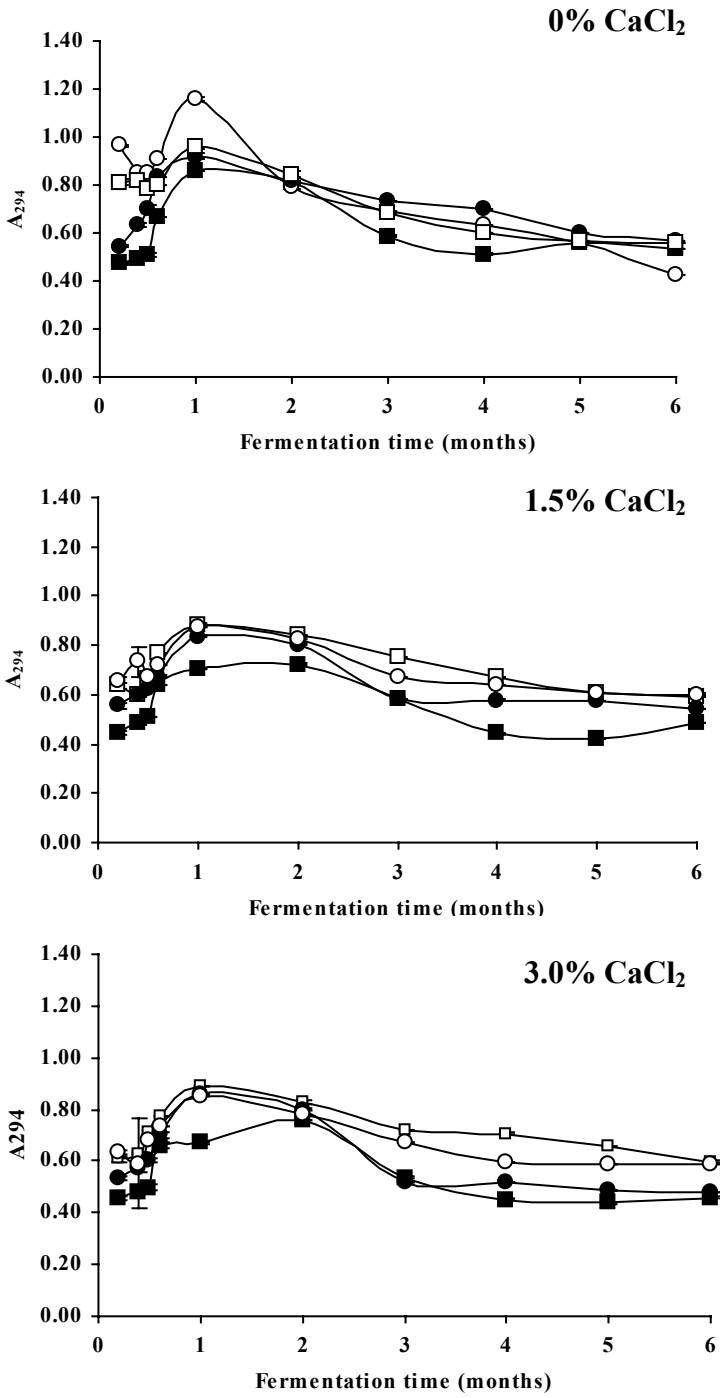

Figure 35 Changes in A_{294} of fish sauce from tuna internal organs as affected by salt, $CaCl_2$ and pH adjustment during fermentation. Bars represent the standard deviation from triplicate determinations. (●) 15% salt, (○) 15% salt pH 9, (■) 25% salt, (□) 25% salt pH 9

4.3.2 Changes in fluorescence intensity

The fluorescence intensity of all samples, except sample produced from tuna internal organ without $CaCl_2$, reached a maximal value at 4-5 months of fermentation. Thereafter, fluorescence intensity decreased sharply up to 6 months of fermentation (Figure 36). Decrease in fluorescence intensity was generally observed with the increasing fermentation time. Development of fluorescent compounds occurs in the Maillard reaction prior to the generation of brown pigments (Morales *et al*., 1997; Jing and Kitts, 2002). Fluorescent compounds are possible precursors of brown pigments (Labuza and Baisier, 1992). Therefore, the decrease in fluorescence intensity was presumably due to the formation of brown pigment from those fluorescent precursors. At the same levels of $CaCl_2$ addition, samples without pH adjustment and add 25% NaCl showed the highest fluorescence intensity. From the results, the difference in fluorescence intensity was probably due to the differences in peptide amount and patterns. This might be associated with the varying reaction rate and the formation of fluorescent compounds.

From the results, fluorescence intensity of fish sauce produced with pH adjustment to 9 and added with 25% NaCl in the absence of $CaCl_2$ showed the lower fluorescence intensity at 6 months of fermentation. Martins *et al*. (2003) reported that the increase in pH of the system influenced the Maillard reaction rate. The lowest fluorescence intensity in fish sauce produced with pH adjustment to 9 and the addition of 25 % NaCl was probably caused by the rapid transformation of the intermediates to brown compounds.

The lowest A_{294} in fish sauce produced from tuna internal organs with 25 % NaCl without pH adjustment was observed in the early stage of fermentation.

However, A_{294} was increased continuously up to 5 months of fermentation with the slight decrease at month 6. Different intermediate products might be different in term of reactivity to form brown pigment. From the result, it was postulated that colorless intermediate (A_{294}) might undergo the polymerization to form brown pigment much faster than fluorescent compound.

The difference in pattern between fluorescence intensity and absorbance (A_{294}) (Figure 34) of fish sauce suggested that different types of intermediate products, either fluorescent or non fluorescent compounds, were formed and underwent the final stage of reaction at different rates. The intermediate products occurred in the advanced stage have the UV absorbance at 294 nm. Therefore, UV absorbance can be used as the method to detect Maillard intermediate products (Ajandouz *et al.,* 2001). Fluorescent compounds are also generated in the advanced stage of Maillard reaction prior to the generation of brown pigment (Jing and Kitts, 2004; 2002). The fluorescent intermediates were possibly reactive in formation of brown product (Benjakul *et al.,* 2005a,b; Morales and Van Boekel, 1997).

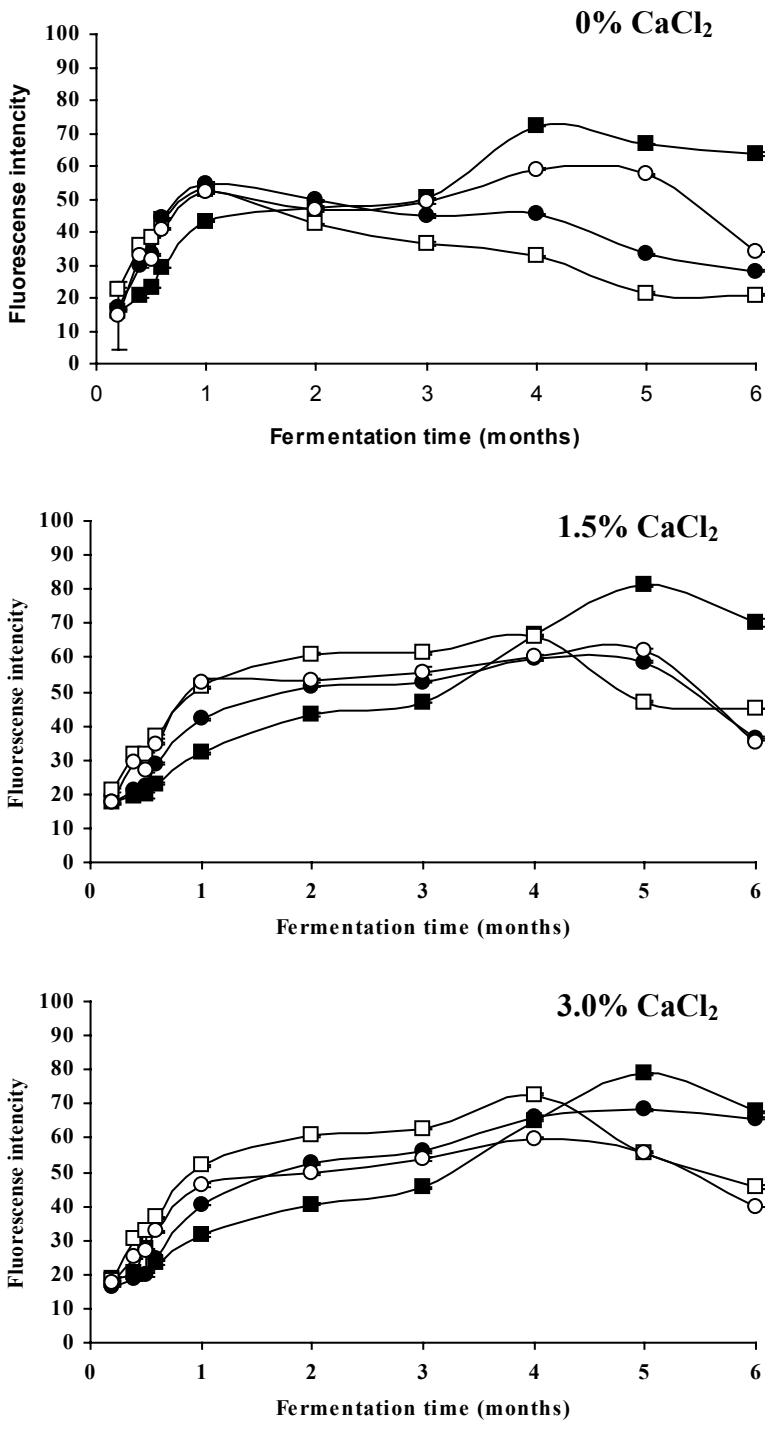

Figure 36 Changes in fluorescence intensity of fish sauce from tuna internal organs as affected by salt, $CaCl_2$ and pH adjustment during fermentation. Bars represent the standard deviation from triplicate determinations. (●) 15% salt, (○) 15% salt pH 9, (■) 25% salt, (□) 25% salt pH 9

4.3.3 Changes in A_{420}

Brown pigments increase as the browning and caramelization reactions progress (Moss and Otten, 1989). The increase in A_{420} was used as an indicator for browning development in the final stage of the browning reaction (Ajandouz *et al.,* 2001; Morales and Jimenez-Perez, 2001). Increase in browning was observed in all samples during fermentation (Figure 37). Browning of all samples was highest at month 6. However, degree of browning development varied with treatments. After 6 months of fermentation, greater browning was found in the samples containing 25% salt with pH adjustment and without $CaCl_2$. The increase in browning was found to depend on salt concentration. When the higher concentration of salt was used, the lower increase in browning was found. Conversely, at pH 9, the browning was mush increased in the presence of 25% salt. Thus, browning reaction might be favored at alkaline pH. Ajandouz *et al.* (2001) reported that the browning was more developed under alkaline pH in fructose-lysine model system. The brown color in fish sauce was caused by non-enzymatic browning reaction such as Maillard reaction (Lopetenarat *et al.,* 2002). The higher $CaCl_2$ concentration resulted in the lower browning intensity. Balakrishnan *et al.* (2001) found that calcium chloride works as a chelating agent or metal scavenger since it combines with metals such as iron and copper which can catalyze oxidation and also cause discoloration. According to De Poix Rouet-Mayer and Philippon (1980), the inhibitory effect on enzymatic browning would be due to chloride ions.

From the result, the browning development was more intense as the fermentation time increased. This was coincidental with the increase in formol nitrogen content and TVB and TMA contents.

Kawashima and Yamanaka (1996) reported that the nitrogenous compounds in fish sauce are free amino acids and small peptides, which contribute to brown color development. Even though reducing sugar content in fish is low, carbohydrate derivatives, such as glucose-6-phosphate and other substances present in the metabolic pathways, can also act as reactants to initiate the Maillard reaction. Therefore, the addition of $CaCl_2$ and pH adjustment not only affected the hydrolysis, but also influenced the color development in fish sauce.

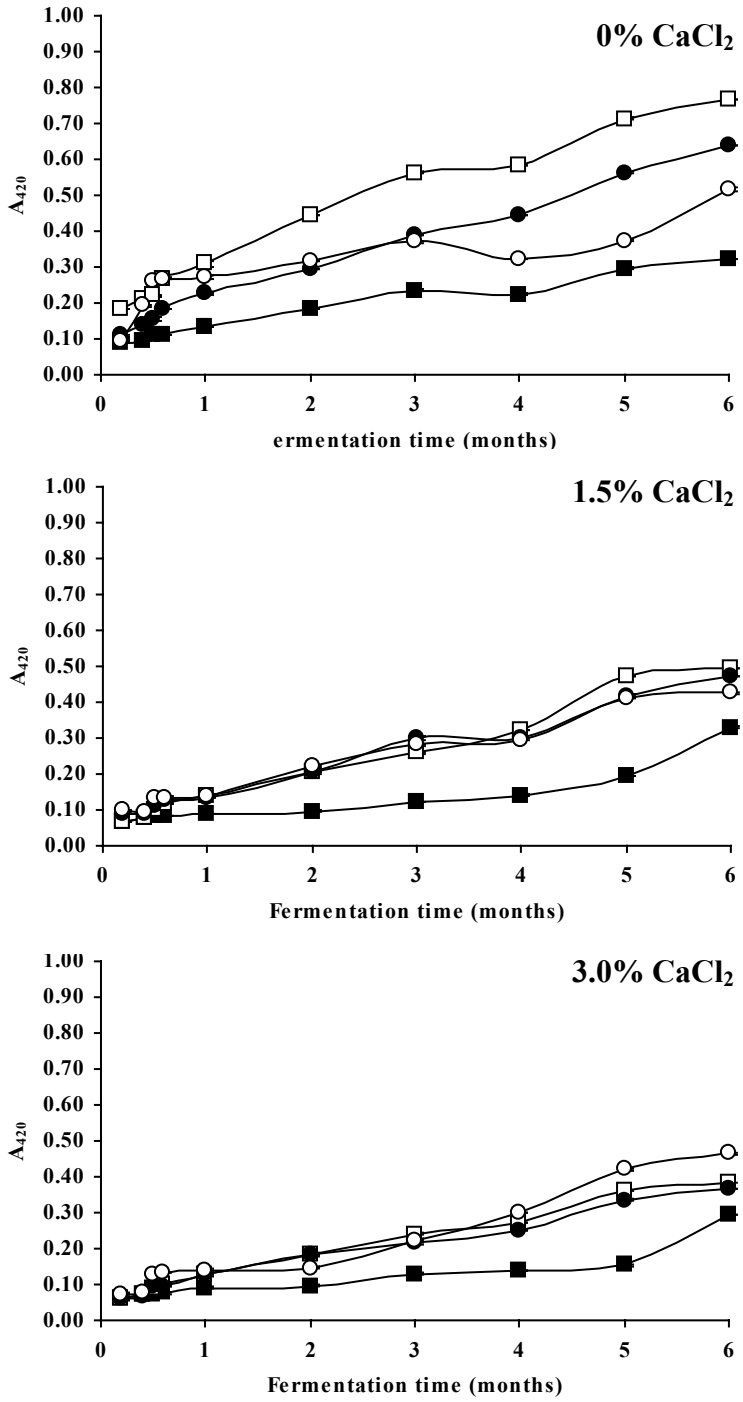

Figure 37 Changes in A_{420} of fish sauce from tuna internal organs as affected by salt, $CaCl_2$ and pH adjustment during fermentation. Bars represent the standard deviation from triplicate determinations. (●) 15% salt, (○) 15% salt pH 9, (■) 25% salt, (□) 25% salt pH 9

4.3.4 Changes in the Hunter L*, a* and b*

Color is one of the most important appearance attributes of food materials since it fluences consumer acceptability (Makan, 2001). Apart from consumer acceptability, color is also used for process control. The changes in the Hunter L*, a* and b* of fish sauce samples obtained from different treatments varied (Figure 38, 39 and 40). The color of fish sauce samples was developed gradually as the fermentation time increased. Generally, samples with high salt (25%) regardless of $CaCl_2$ addition had the lowest color development as indicated by the lowest increase in a* and b* values and the lowest decrease in L*values when the fermentation time increased ($p<0.05$). The L*-value shows lightness, where as a* and b*- values indicated redness and yellowness of product, respectively. The b*-value increased with increasing fermentation time, especially, within the first month.

The higher $CaCl_2$ concentration resulted in the lower color intensity. At the same levels of $CaCl_2$, samples with pH adjustment and 15% NaCl showed the greater color intensity. From the result, the sample containing low salt with pH adjustment and without $CaCl_2$ addition exhibited more redness as shown by the greater a*-value. The development of red color was coincidental with decrease in lightness (L*-value), particularly when the fermentation time increased. In general, the decreases in L*-value indicate darker color and a shift in color from yellow-brown to orange-brown, respectively (Bates *et al.,* 1998; Nicoli *et al.,* 1997). Calligaris *et al.* (2004) used the b*-value to indicate the browning development in heated milk. From the result, the decrease in L*-value and increase in a*-value were in the accordance with brown color development as monitored by A_{420} (Figure 37). Sanceda *et al.* (1996) reported that the color of fish sauce produced from sardine (*Sardinops melanostictus*) tended to

be darker as fermentation progressed. Darker color of the fish sauce could be attributed to the longer fermentation period of the fish sauce production (Chaveesuk *et al.,* 1993). Kahyaoglu and Kaya (2005) reported that the increasing in the a-value was due to formation of brown pigments through the non-enzymatic browning. Increase in the a*-value during fermentation was correlated with decrease in the L*-value.

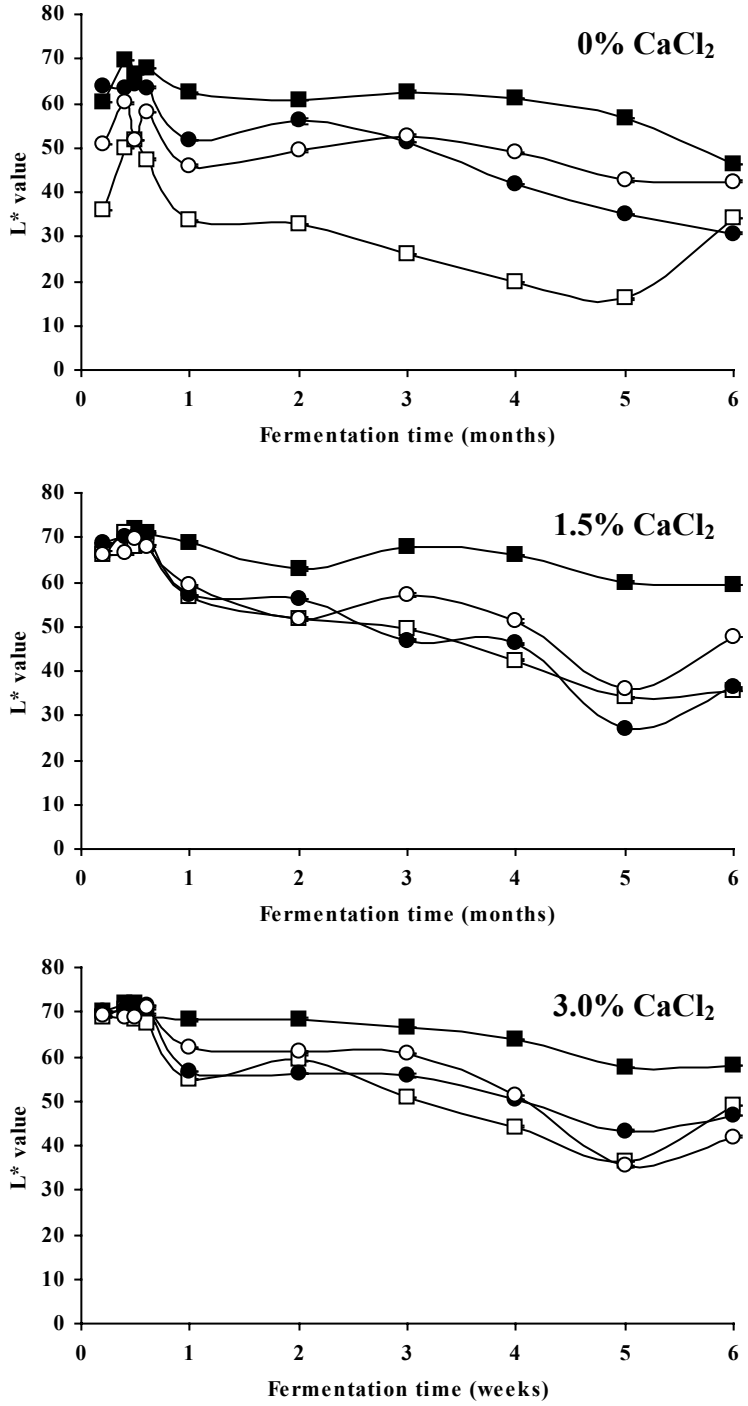

Figure 38 Changes in L*- value of fish sauce from tuna internal organs as affected by salt, $CaCl_2$ and pH adjustment during fermentation. Bars represent standard deviation from triplicate determinations. (●) 15% salt, (○) 15% salt pH 9, (■) 25% salt, (□) 25% salt pH 9

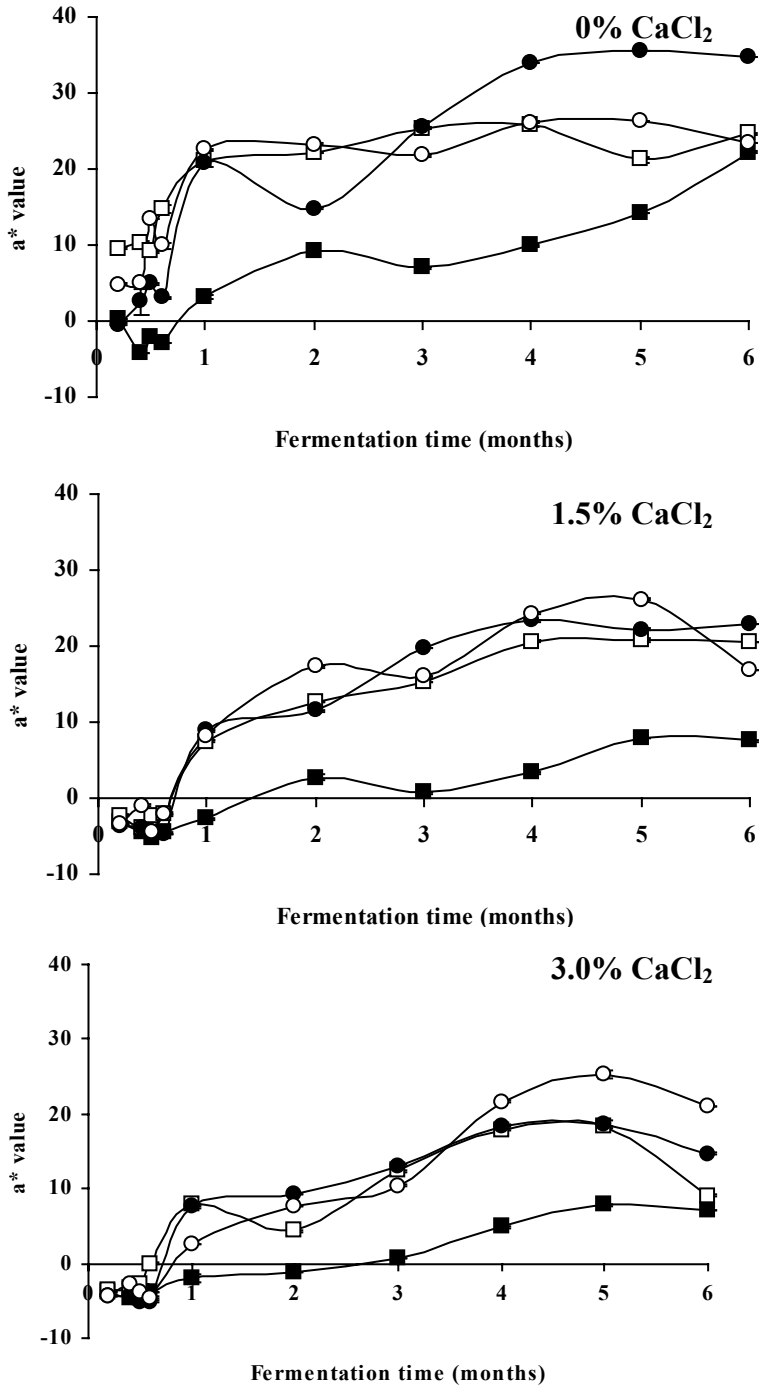

Figure 39 Changes in a*- value of fish sauce from tuna internal organs as affected by salt, $CaCl_2$ and pH adjustment during fermentation. Bars represent the standard deviation from triplicate determinations. (●) 15% salt, (○) 15% salt pH 9, (■) 25%salt, (□) 25% salt pH 9

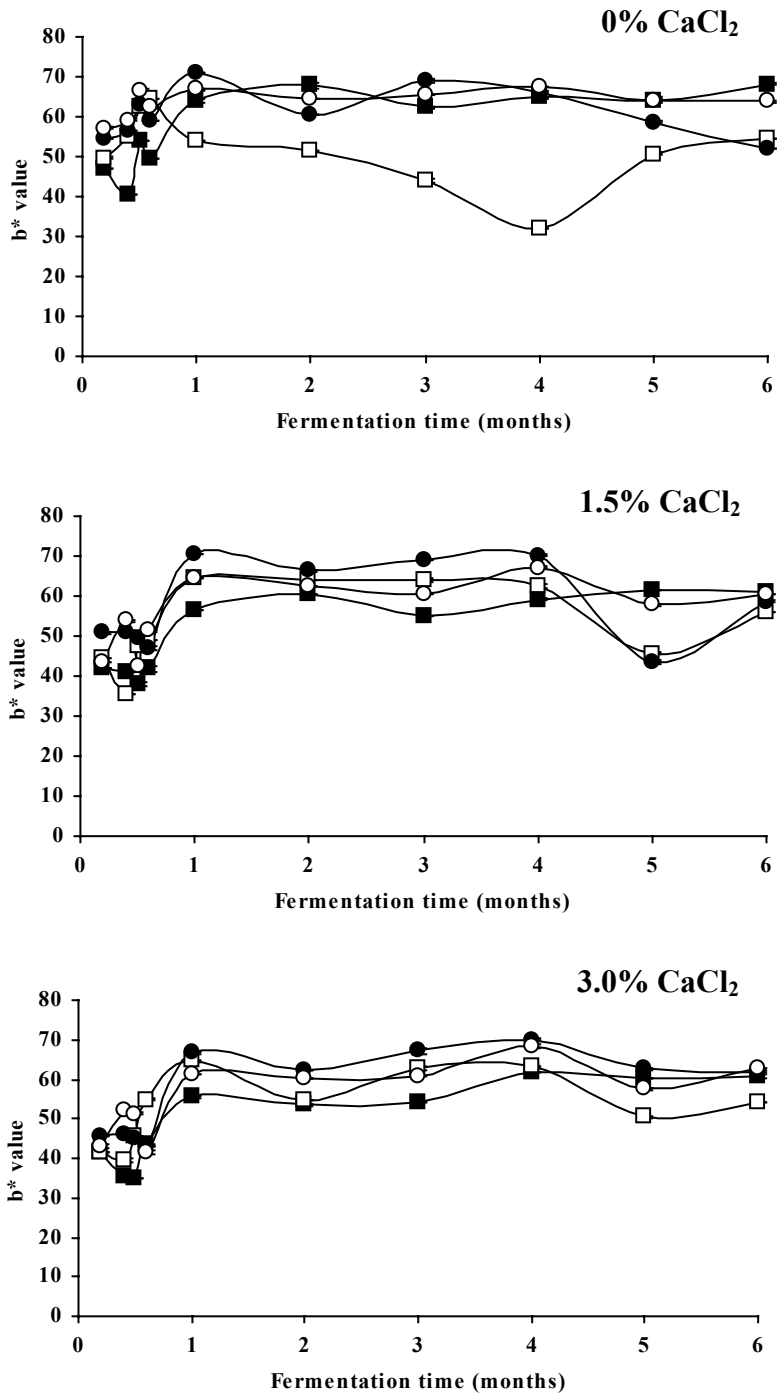

Figure 40 Changes in b*- value of fish sauce from tuna internal organs as affected by salt, $CaCl_2$ and pH adjustment during fermentation. Bars represent the standard deviation from triplicate determinations. (●) 15% salt, (○) 15% salt pH 9, (■) 25% salt, (□) 25% salt pH 9