

Histamine Formation in Fish Sauce Production

S. BRILLANTES, S. PAKNOI, AND A. TOTAKIEN

ABSTRACT: The formation of histamine in fish sauce made from *Stolephorus sp.* was studied. In the first experiment, fish were iced on board and mixed with salt at the factory, while in the second experiment fish were mixed with salt on board collection vessels. Eight batches were fermented for 12 mo. Histamine levels increased during fermentation to 22 to 159 and 589 to 686 ppm in the first and second experiments respectively. Good correlation between histamine levels in raw material and final products was found. It was concluded that histamine was formed both in the raw material and during fermentation. It was speculated that histidine decarboxylase enzymes formed prior to fermentation produced histamine during fermentation.

Key words: fish sauce, histamine, histidine decarboxylase, halophilic bacteria, *Stolephorus sp.*

Introduction

THAI FISH SAUCE, *NAMPLA*, IS A FERMENTED PRODUCT THAT IS produced from small pelagic fish mixed with salt and stored in tanks at ambient temperatures for 6 to 12 mo. It is similar in organoleptic characteristics and method of production with other fish sauces from Cambodia, Indonesia, Malaysia, Myanmar, Philippines, Vietnam, and Japan. It is an important ingredient in most Thai dishes and it is a major export item for the country. Two main methods of production are employed. Small-scale plants with less than 10000 tons capacity use fresh fish, which are caught overnight and kept in ice. The fish are then mixed with salt at the factories with the use of rotary mechanical mixers, transferred to fermentation tanks, and topped up with a bed of salt. Large-scale plants on the other hand use fish collected over several days. Fish are not treated with ice or salt until they are transferred to the collection vessels' holds where they are layered alternately with salt. At the plants, the fish-salt mixtures are put in fermentation tanks, topped with a bed of salt and further added with saturated brine.

The main species used in fish sauce production are *Stolephorus*, *Sardinella*, and *Cirrhinus*. Since these species are known to produce histamine when they are temperature-abused (Sakaguchi and others 1984; Russell and Maretic 1986; Taylor 1989), histamine is thus identified as a hazard in fish sauce. Histamine is a chemical that causes severe illness and is regulated in fish sauce at a maximum of 200 ppm by Canada and 500 ppm by the USA. Many works (Sato and others 1995; Sanceda and others 1996; Brillantes and Samosorn 2001) have reported that fish sauces from Asia contain high levels of histamine but nobody had investigated how and when histamine is formed in fish sauce. Total nitrogen (total N) content is used as an indicator to determine the grade and price of fish sauce in Thailand, with products containing over 20 g/L total N classified as Grade I and 15 to 20 g/L total N as Grade II (TISI, 1983). Brillantes and Samosorn (2001) examined the levels of histamine in relation to total N contents in 549 commercial samples of *nampla* and found histamine contents varied widely from 100 to 1000 ppm and also noted that products from small-scale factories had relatively lower histamine contents than those from large-scale operations. However, they did not find any relationship between the amounts of histamine and total N.

The purpose of this study is thus to investigate the formation

of histamine in fish sauce in order to find ways to control this hazard at levels below the limits allowed by the importing countries. The effects of the 2 methods of production on histamine contents in the final products will also be examined.

Materials and Methods

IN THE FIRST EXPERIMENT, THE PRODUCTION OF COMMERCIAL batches of fish sauce was monitored in a small-scale factory in Rayong province. *Stolephorus sp.* anchovies, caught overnight by 4 boats in the Gulf of Thailand, were iced and stored in the fishing boats' holds and delivered to the fish sauce factory the next morning. The fish were off loaded onto plastic baskets and transported on uncovered trucks to the factory about 5 min away. When fish arrived at the plant, they were in good and fresh condition with temperatures at 5 to 15 °C in the center of the fish containers. The whole fish, about 6 to 9 cm long, were immediately mixed with salt in the ratio 3:2 (fish:salt) with the use of a mechanical rotary mixer. About 4 tons of fish from each of the 4 boats were used and marked as batches A, B, C, and D. Each batch was poured into separate fermentation tanks, topped up with a bed of salt, then covered with a sheet of fiberglass and left to ferment for 12 mo.

In the second experiment, the production of commercial batches of fish sauce was monitored in a large-scale factory in Chonburi province. Anchovies gathered in 4 trips of a collection vessel owned by the fish sauce factory were used. In each trip, the collection vessel collected fish from various fishing boats for about 2 to 4 d. Fish were not treated with ice or salt until they were transferred to the collection vessels where they were immediately shoveled into the holds and layered alternately with salt at the approximate ratio of 1:1. At the fish landing, the fish were bailed out into plastic baskets then loaded onto covered trucks and driven to the plant about 10 min away. At this point, the fish were already soft and liquid produced from the initial stages of fermentation dripped from the baskets. Temperatures in the center of the fish containers were at 32 to 35 °C. About 12 tons of fish-salt mixtures from each trip of the collection vessel were used for the experiment and marked as batches E, F, G, and H. Each batch was poured into separate tanks, topped with a bed of salt, left to settle for a few hours, and then filled further with saturated brine, about 3000 to 4000 liters, or about 19 to 25% of the total volume. The added brine replaces the salt brine lost on the

Table 1—TMA (mg%), TVB (mg%), and histamine (ppm) contents of raw material in batches A to D (n = 2).

Batch	TMA	TVB	Histamine
A	0.5 ^a	18.9 ^a	2 ^a
B	0.5 ^a	19.2 ^a	5 ^a
C	2.7 ^b	25.8 ^b	16 ^a
D	5.7 ^c	26.7 ^b	78 ^b

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

boats and during transport, and helps to influence the osmotic diffusion between the water in fish tissues and salt molecules from the surrounding brine (Voskresenski 1965). Finally, the tanks were covered with sheets of fiberglass and left to ferment for 12 mo.

Samples of fish raw material (1 kg/batch) from batches A to D were taken for histamine, trimethylamine (TMA), and total volatile base (TVB) analyses. Raw materials from batches E to H were not tested because the fish were already mixed with salt for several d when they arrived at the factory; instead, 5 kg of fresh anchovies were left at ambient temperature, 32 to 38 °C, for 12 h and the deterioration in quality was monitored. Samples (200 g) were taken every 2 h for the determination of TMA, TVB, and histamine contents. Finally, the liquids produced during fermentation that were drawn off from the bottom of each tank after 1 week, 1 mo, 2 mo, and every 2 mo thereafter were tested for histamine, total N, pH, salt, and total halophilic bacteria.

Chemical analysis

Histamine determinations on fish sauce were carried out by HPLC method using solid-phase extraction (Brillantes and Samosorn 2001). One ml of fish sauce sample was shaken with 40 ml of extraction solution of methanol for 1 min then sample extracts were adjusted to 50 ml and applied onto Bond Elut SCX. The histamine residues were eluted with 5 ml of mixed solution of 0.5M KH_2PO_4 (pH 6.5) – methanol (1:1, v/v). The eluents were collected in 5 ml volumetric flasks, then injected to HPLC. Conditions in histamine determination consisted of a mobile phase of 0.1M KH_2PO_4 (pH 5.9): methanol (7:3, v/v) and a post column reagent of 0.1% *O*-phthalaldehyde, 10% mercaptoethanol, and 0.05M sodium tetraborate (pH 10) in the ratio of 5:1:94. The flow rate of both reagents was at 1.2 ml/min. Histamine was detected by fluorescence detector at 350 nm excitation wavelength and 444 nm emission wavelength with the use of column Zorbax SCX (25 cm x 4.6 mm id), 5 mm. Analysis of histamine in fish raw material was conducted by homogenizing 5 g of blended meat with 40 mL of extraction solution of methanol:HCl = 1:1(v/v). The final volume was adjusted to 50 ml prior to injecting to HPLC following Walters (1984).

Total N and salt contents in each sample were determined according to AOAC (1995). TMA and TVB were determined by the micro-diffusion technique (Beatty and Gibbons 1936; Conway and Byrne 1936). Every test was done in duplicate.

Microbiological analysis

Fish sauces were analysed for halophilic bacteria using a pour plate count technique. Trypticase soy agar (TSA) containing 10% NaCl (w/v) was used as the growth media. Serial sample dilutions were prepared in 0.1% peptone water (Difco Laboratory, Detroit, Mich., U.S.A.); the plates were incubated aerobically at 37 °C for 72 h (Chaveesuk and others 1993). Duplicate plates were obtained for each dilution; thus the data presented are the

Table 2—Halophilic bacteria in all batches of fish sauce samples during fermentation for 12 mo

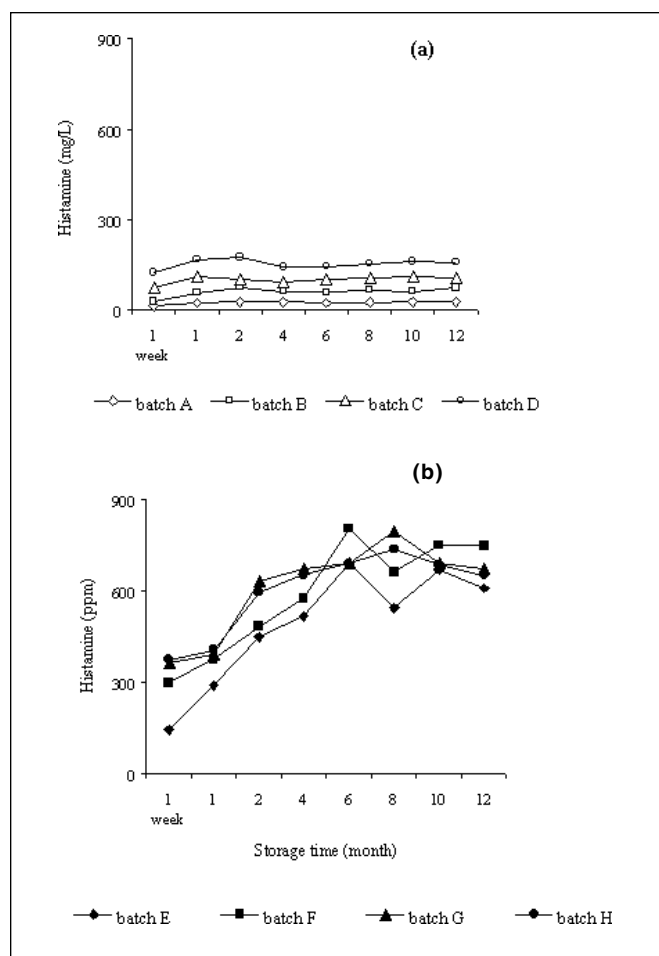
Batch (mo)	Halophilic bacteria (cfu/ml) ^a /fermentation time			
	First week	1	6	12
A	3.5×10^3	$< 10^2$	Nd	Nd
B	2.2×10^2	$< 10^2$	Nd	Nd
C	4.1×10^2	$< 10^2$	$< 10^2$	$< 10^2$
D	3.7×10^3	$< 10^2$	$< 10^2$	$< 10^2$
E	4.2×10^2	$< 10^2$	$< 10^2$	$< 10^2$
F	2.7×10^2	$< 10^2$	$< 10^2$	$< 10^2$
G	3.8×10^3	$< 10^2$	$< 10^2$	$< 10^2$
H	1.4×10^3	3.1×10^2	$< 10^2$	$< 10^2$

^aNd: not detected

average of 2 plate counts. In order to identify the presence of histamine-forming bacteria, the surviving bacteria were tested following the method of Taylor and Woychik (1982).

Statistical analysis

Tests of significance for the analytical study were done using analysis of variance (ANOVA) and multiple Duncan's range test. Regression and correlation were carried out as described by Laitinen and Harris (1975).

**Figure 1—Histamine levels in fish sauce (a) batches A-D and (b) batches E-H during fermentation for 12 months**

Results

TABLE 1 SHOWS THE DEGREE OF FRESHNESS OF THE VARIOUS batches of raw materials, with the use of TMA, TVB, and histamine values as indicators. Histamine contents in batches A to C were not significantly different from each other while they were significantly lower than batch D. Similarly, TMA values in batches A to C were significantly lower ($P < 0.05$) than in batch D. Lastly, TVB values in batch D were significantly different ($P < 0.05$) from batches A and B but not significantly different ($P > 0.05$) from batch C.

Figure 1 shows the levels of histamine in the liquids of batches A to D and E to H during 12 mo of fermentation. In batches A to D histamine levels were at 13 to 124 ppm in the first-week liquids then increased to 22 to 159 ppm after 12 mo, whereas in batches E to H they were at 145 to 374 ppm in the first week and increased to much higher levels, reaching 589 to 686 ppm at the end of 12 mo. From the first week of fermentation histamine in batches A to D increased gradually, peaked on the first or second month ($P < 0.05$), then dropped and remained relatively stable until the end of 12 mo ($P > 0.05$). Peak histamine levels in batches E to H were reached on the 6th to 10th mo ($P < 0.05$), after which they remained relatively stable ($P > 0.05$). During the 12 mo, histamine levels in batches A to D were significantly different ($P < 0.05$) between each other, with the highest levels found in batch D. On the other hand, histamine levels in batch E were significantly lower ($P < 0.05$) than batches F to H but there were no significant differences ($P > 0.05$) between F to H. When histamine contents in the raw materials of batches A to D were plotted against those in the first-week liquids and in the final products, the correlation coefficients (r) were 0.9311 and 0.8783 respectively. On the other hand, histamine contents in the first-week liquids of batches E to H against those in the final products showed a correlation coefficient of 0.9136.

Figure 2 shows the changes in the amounts of TMA, TVB, and histamine in *Stolephorus sp.* at every 2 h intervals when fish were left at ambient temperature (32 to 38 °C) for 12 h. All freshness

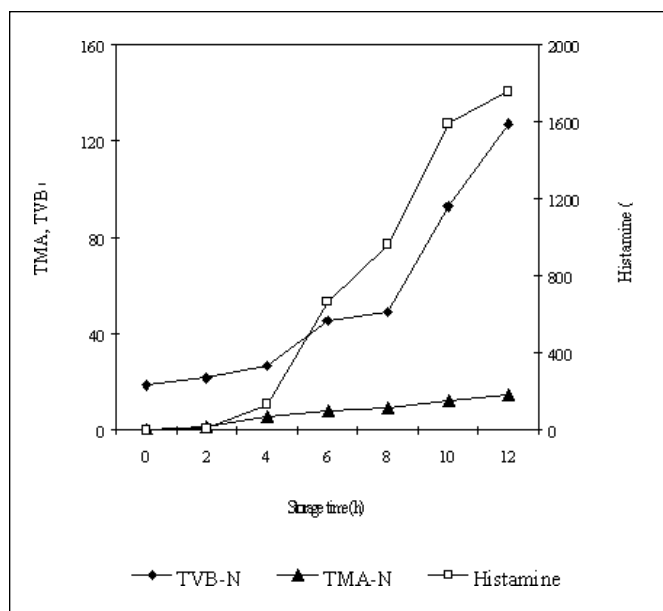


Figure 2—Changes of TMA, TVB and Histamine contents of *Stolephorus sp.* left at ambient temperature for 12 h.

indices were very low at 0 to 2 h, started to increase significantly ($P < 0.05$) at 4 h with TMA values at 5.6 mg%, TVB at 26.7 mg%, and histamine at 132.7 ppm, then increased exponentially thereafter. Final values after 12 h were 14.8 mg% for TMA, 126.8 mg% for TVB, and 1754 ppm for histamine.

Figure 3 shows the levels of total N in the liquids of batches A to D and E to H during 12 mo of fermentation. Initial levels of total N in batches A to D ranged from 3.0 to 4.9 g/L, increased dramatically ($P < 0.05$) during the fermentation period especially in the first 4 mo, then remained relatively stable ($P > 0.05$) after 6 mo. In batches E to H total N levels started high at 4 to 9 g/L in the first-week liquids, increased significantly ($P < 0.05$) until 7 mo then remain relatively stable ($P > 0.05$) with final values of 18 to 22 g/L at the end of 12 mo. During 12 mo of fermentation, total N in batch A to D were not significantly different ($P > 0.05$) from each other, while batch F and H were significantly higher ($P < 0.05$) than batch E and G.

Table 2 shows the number of halophilic bacteria in all batches of fish sauce during fermentation. In all batches, halophilic bacteria decreased from 10^2 to 10^3 /ml on the first week to less than 10^2 /ml after 1 mo and further decreased to not detected levels in batch A to B after 6 mo.

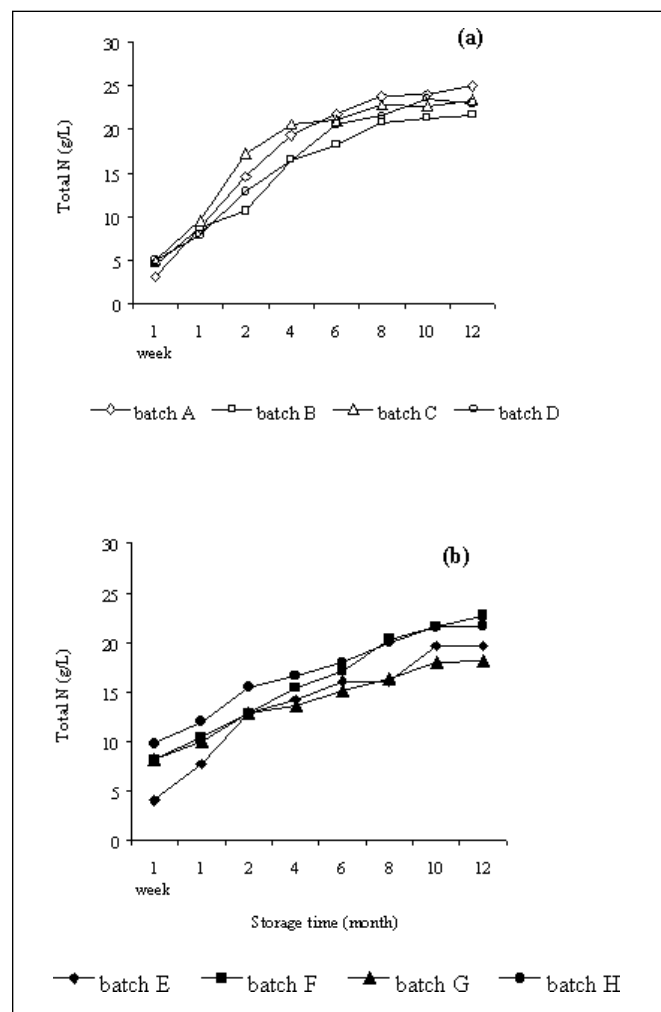


Figure 3—Total N contents of fish sauce in (a) batches A-D and (b) E-H during fermentation for 12 months.

Discussion

HISTAMINE LEVELS IN TUNA THAT ARE JUDGED TO BE OF AN ACCEPTABLE quality based on organoleptic and physical analysis are in the order of 10 to 20 ppm according to USFDA Compliance Policy Guide, CPG 7108.24. The histamine contents below 20 ppm in the raw materials of batches A to C and above 20 ppm in batch D reflected the organoleptic qualities observed. Fish in batches A to C exhibited very fresh qualities while in batch D showed some signs of deterioration. TMA and TVB values also indicated the same characteristics. Immediate icing of the fish after catching and keeping them in chilled condition until delivery to the processing plant could have restricted or minimized the growth of histamine-forming bacteria in batches A to C. Several authors (Hardy and Smith 1976; Baldarati and others 1980; Klausen and Lund 1986) have reported that there was little or no formation of histamine when temperature of fish was below 10 °C. Delays and improper or insufficient icing could have caused the lower quality in batch D. These conditions could have also allowed the growth of histamine-forming bacteria causing higher histamine contents as reported by Ababouch and others (1986) and as confirmed by the sharp increase in histamine after 4 h when anchovies were left at ambient temperature in this study.

Histamine contents were also relatively low in the first-week liquids of batches A to D with only batch D exceeding 100 ppm. The immediate mixing with salt upon arrival at the factory while the fish were still at low temperatures may have helped to stop bacterial processes (Voskresensky 1965). The works of Taylor and Woychik (1982) and also Taylor and Speckhard (1984) reported that levels of 3.5 to 5.5% salt could inhibit histamine formation by *Klebsiella pneumoniae* and *Proteus morgani*, which are the main histamine-forming bacteria in tuna. In batches E to H the high histamine contents in the first-week liquids could indicate serious mishandling of fish prior to and/or during processing. The histamine levels could have been much higher without the addition of saturated brine in the tanks. A great increase in histamine levels could have occurred on board the fishing boats without the use of ice or salt, as confirmed by the increase in histamine values when anchovies were left at ambient temperatures for 12 h. Frank and others (1981) reported that histamine-forming bacteria could grow and produce histamine over a wide range of temperatures and their growth was more rapid at high-abuse temperatures. Improper mixing of fish and salt through layering in the collection vessels could be another contributing factor to the high histamine contents. Pockets of fish that are not in contact with salt could allow the growth of histamine-forming bacteria (Saisithi 1987).

The increase in histamine contents during the first 2 mo in batches A to D and during the first 6 to 10 mo in batches E to H could be due to the presence of histidine decarboxylase that can continue to convert histidine to histamine even after the fish is mixed with salt. It was reported that histidine decarboxylase already produced by bacteria that have ceased growing can still convert histidine to histamine (Kimata and Kawai 1953; Baranowski and others 1985). Halotolerant histamine-forming bacteria may have also produced the enzyme during fermentation, however, bacteria found in the first-week liquids of every batch were very low and the numbers decreased to almost undetectable levels after 1 mo. Further testing also confirmed that these bacteria could not produce histamine (data not shown). This agrees with the findings of Sato and others (1995) that halophilic bacteria at 7.9×10^2 /ml found in Japanese fish sauce (*shottsuru* and *moromi*) could not produce histamine. Similarly, halophilic histamine-forming bacteria was reported to produce high his-

amine levels in fermented sardine (at 12 to 13% salt) only when their numbers exceeded 10^4 /g while at 10^2 /g no histamine was detected (Yatsunami and Echigo 1993; Yatsunami and others 1994). The drop in histamine levels after 2 mo in batches A to D may indicate that the enzymes had been depleted while proteolysis continued to produce more liquid. Riaz and others (1986) concluded from their work that proteolysis continued until 160 d (5.3 mo) while Beddows and others (1976) found the volume of liquid produced during fermentation of Malaysian fish sauce, *budu*, reached a maximum after 120 to 140 d (4.0 to 4.7 mo). The drop in histamine could also be due to the depletion of histidine. However, Sanceda and others (1996) reported that histamine content in fish sauce did not increase even after histidine was added with the purpose of accelerating the fermentation process. If there were remaining enzymes, they could have converted more histamine from the added histidine.

The stable histamine levels in batches E to H after 6 to 10 mo may indicate that histidine decarboxylase enzymes had been depleted while there was no further increase in liquid. The much longer histamine formation in these batches could be due to higher levels of enzymes that were produced in the raw materials prior to salting as well as after salting (in the collection vessel's holds) as indicated by the much higher initial histamine values in the first-week liquids. Histamine contents at every stage were much higher than in batches A to D, with increases in histamine during fermentation about 10 times higher. All finished products from batches E to H exceeded the maximum 500 ppm histamine limit set by the USFDA while batches A to D were below 200 ppm. The differences between the results of these 2 production methods were mainly due to the differences in quality of fish raw material as well as differences in salting techniques. The good correlation ($P < 0.05$) between histamine values in raw material and first-week liquids, between raw materials and final products, and lastly between first-week liquids and final products indicate that histamine levels in the final products are influenced by the levels of histamine in the raw material. Sato and others (1995) hypothesized that the high histamine levels that were found in the final product were already produced prior to the salting process. However, the findings in this study show that in addition to the histamine levels in the raw material, histamine was also produced during the fermentation period, which was mostly likely from the action of histidine decarboxylase enzymes that were produced prior to the fermentation stage. It is thus reasonable to conclude that preserving the freshness of the raw material can control histamine levels in the final products. This can be achieved by proper icing of fish, maintaining them at low temperatures, and/or by immediate and proper mixing of fish with salt. Fishermen who do not bring ice to sea must carry salt and immediately mix fish with salt as soon as fish are caught. If storage space is limited they can reduce the amount of salt to at least 5.5% of fish as histamine formation is inhibited at this rate. Proper mixing of fish and salt should be done in order to avoid the growth of histamine-forming bacteria.

Initial productions of total N were relatively slow in batches A to D, which took about 1 mo to reach the same levels of total N in the first-week liquids in batches E to H. This could be because of the fresh quality of the raw material in batches A to D and the immediate and complete mixing of fish and salt. The high salt content at 25 to 30% could have also slowed down the rate of protein hydrolysis by both fish and bacterial enzymes. According to many works (Van Veen 1953; Voskresenski 1965; Saisithi 1987), fresh fish mixed with salt exceeding 15% yield lower total N contents as the growth of spoilage bacteria as well as fish tissue hy-

hydrolysis by bacterial enzymes were prevented. The rate of protein hydrolysis was found to decrease rapidly from 25 to 10.8% when salt concentrations increased from 0 to 10% (Poosaran 1986) and a 7 h delay in the addition of salt increased protein conversion by 35% (Beddows and others 1979).

In batches A to D, from low levels at the start, total N increased dramatically ($P < 0.05$) especially in the first 4 mo of fermentation followed by insignificant ($P > 0.05$) increases after 6 mo, which is in agreement with other works (Orejana and Liston 1981; Riaz and others 1986). It was also reported that the rate of increase in soluble N decreased after 4 mo due to the great reduction of trypsin-like enzyme caused by the accumulation of amino acids and small peptides during the fermentation (Orejana and Liston 1981). All final products in this study could be classified as Grade I *nampla* (total N > 20 g/L) under Thai standards (TISI 1983).

In batches E to H, except for batch E, total N contents in the first-week liquids were significantly higher ($P < 0.05$) than batches A to D in spite of the addition of saturated brine. The lower total N content in batch E could be due to a better quality raw material as indicated by the significantly ($P < 0.05$) lower histamine contents. The high total N in the first-week liquids in these batches could indicate that high amounts of amino acids and peptides were already present. These substances could have retarded the rate of protein hydrolysis during the rest of the fermentation period as shown by the slow increase in total N during the 12 mo. In spite of the slow production during the fermentation period, all batches except batch G could still qualify as Grade I sauce.

Salt contents and pH values from both experiments were relatively stable during the fermentation period and within normal levels (pH at 5 to 6, salt $> 23\%$) found in commercial products (TISI 1983).

Conclusion

THE QUALITY OF FISH RAW MATERIAL AS WELL AS SALTING techniques are the key factors in controlling histamine in fish sauce products. Histamine in the finished product was a combination of histamine in the raw material and histamine produced during fermentation. It is speculated that histidine decarboxylase enzymes that were formed in the raw material prior to the fermentation stage were responsible for histamine formation during fermentation.

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Authors Brillantes, Paknoi, and Totakien are with the Fish Inspection and Quality Control Div., Dept. of Fisheries, Kaset-klang, Chattuchak, Bangkok 10900, THAILAND. Direct inquiries to author Brillantes (E-mail: supapunb@fisheries.go.th).