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Detection and Confirmation of Histamine-producing Bacteria

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Consumption of fish containing a high level of free histamine is associated with scombroid food poisoning. Symptoms of this type of poisoning include a burning sensation in the throat, facial flushing, headache, nausea, and vomiting (EDMUNDS & EITENMILLER 1975). When fish are not properly refrigerated or frozen soon after being caught, histidine decarboxylating bacteria can rapidly multiply and convert the histidine to histamine as shown in Figure 1.

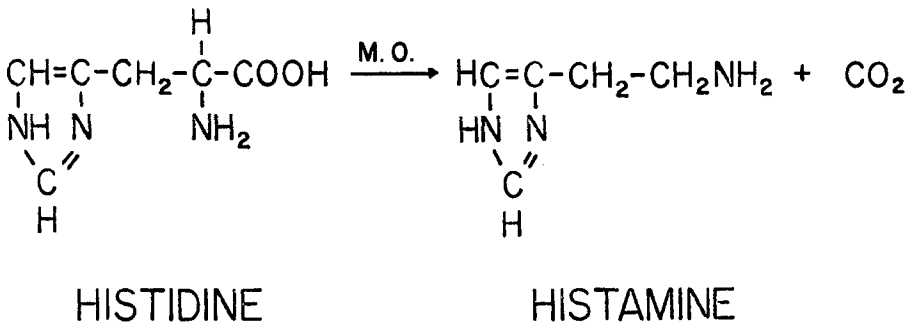

Figure 1. Decarboxylation reaction of histidine to histamine by microorganisms (M.O.).

It is thought that this reaction is the source of histamine in fish that causes the aforementioned symptoms associated with scombroid food poisoning.

High concentrations of free histidine were reported by LUKTON & OLCOTT (1958) in the tissue of tuna, mackerel, mahi mahi, bonito, butterfly kingfish, and saury. These authors also showed that skipjack tuna contained 1192 mg free histidine, yellowfin tuna, 740 mg, and mackerel 600 mg per 100 g fish. Organisms such as Enterobacter aerogenes, Klebsiella pneumoniae, and Proteus morganii were reported by CORLETT et al. (1978) to form more than 1500 mg free histamine in homogenates of tuna spiked with 2500 mg histidine per 100 g fish.

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Traditionally, fish suspected of causing scombroid poisoning have been chemically analyzed for histamine. Organoleptic analyses for evidence of decomposition are not adequate since the presence of histamine does not always correlate well with organoleptic results. Therefore, this study was conducted to detect and confirm the presence of histamine producing bacteria in fish that are responsible for the formation of histamine from histidine. Recovery rates of histamine producing bacteria (HPB) from fish were also determined.

MATERIALS AND METHODS

Samples and cultures: Fresh fillet of white snapper, red snapper (gills, fins, skin, and small amount of intestines), Chinese mackerel and mullet (gills and intestines), as well as chunks of tuna and frozen mahi mahi were used in this investigation. All fish samples except mahi mahi were obtained from local markets. The mahi mahi was a Japanese import sample collected by a FDA inspector. All of the fish samples were assayed for the total intrinsic HPB present, whereas only the tuna and mahi mahi were used for recovery analyses following spiking with the desired number of test organisms. Both E. aerogenes and K. pneumoniae, positive HPB, were utilized as test organisms. These cultures were activated, prior to use, by repeated inoculation in tryptic soy broth and incubation at 35 ± 2°C for 24 ± 2 h. The bacteria were inoculated on the surface of tryptic soy agar (TSA) plates which were then incubated at 35 ± 2°C for 24 ± 2 h. Cells were harvested from the plates using sterile saline, centrifuged (10,000 x g), washed twice with saline, and resuspended in same to the desired concentration. The latter was ascertained by serial dilution and pour plate technique utilizing TSA medium.

Differential and confirmation media: The differential agar medium (DAM), a modification of that reported by NIVEN et al. (1981), contained the following (g/L of distilled water): L-histidine 2HCl (ICN), 27; tryptone (Difco), 5; yeast extract (Difco), 5; sodium chloride, 5; calcium carbonate, 1; agar, 30; and 10 ml brom cresol purple solution (0.6 g per 100 ml of 95% ethanol). The pH of the medium was adjusted to 5.25 prior to sterilization (10 min at 121°C). The confirmation medium is an adaptation of the decarboxylase test agar (DTA) developed by MACCANI (1979, 1980) and had the following formulation (g/L of distilled water): L-histidine 2HCl (ICN), 27; ammonium chloride, 0.1; yeast extract (Difco), 1; D-glucose, 0.5; sodium chloride, 2; dextran T 500 (Pharmacia), 10; agar, 30; and 3.3 ml brom cresol purple solution (0.6 g per 100 ml of 95% ethanol). The pH was adjusted to 5.0 ± 0.1, and the medium was sterilized (15 min at 121°C), tempered, and approximately 40 ml were poured into petri plates (15 x 100 mm).

Isolation of intrinsic HPB: This was conducted by homogenizing a 50 g fish sample in 450 ml sterile phosphate buffer (AOAC 1980a) for 2 min using a Waring blender. Serial dilutions were made and 1 ml of each desired dilution was placed in a sterile petri dish to which DAM was added and swirled gently to mix. The medium was

allowed to solidify then overlayered with 12-15 ml DAM. The plates were incubated aerobically at 35 ± 2°C for 24 ± 2 h and examined for colonies of HPB. The latter are characterized by a purple zone surrounding the colonies, against the yellow background color of the DAM. The change in color from yellow to purple is due to the shift in pH caused by the alkaline histamine formed affecting the brom cresol purple indicator. Colonies of HPB were confirmed for the presence of the histidine decarboxylase enzyme by shallow stab inoculation of DTA plates followed by aerobic incubation at 35 ± 2°C for 24 ± 2 h. Histidine decarboxylase activity was detected by the development of a purple zone surrounding the bacterial growth as previously indicated. Each isolate was characterized morphologically and identified biochemically using API 20E strips (Analytab Products) for Enterobacteriaceae and other Gram negative bacteria.

The presence of histamine formed by the action of HPB in DAM plates was confirmed using the AOAC procedure (1980b). A known amount of the DAM agar was blended with 50 ml methanol. The homogenate was heated for 15 min in a 60°C water bath, cooled to 25°C, diluted to the original volume with methanol, and filtered. The filtrate containing the histamine was allowed to pass through an ion exchange column (Bio-Rad AG 1-X8, 50-100 mesh). The histamine, adsorbed onto the column, was eluted with water and the eluate collected in a flask containing 5 ml 1N HCl. An aliquot of this eluate was permitted to react with o-phthalicdicarboxaldehyde (OPT) solution to form a fluorescent derivative of histamine. The fluorescent intensity was determined photofluorometrically using a Perkin-Elmer fluorescence spectrophotometer model MPF-2A at excitation and emission wavelengths of 350 and 444 nm, respectively. The concentration of histamine in the sample eluate was ascertained by comparison to a histamine standard curve based on various concentrations of free histamine.

Recovery rates of HPB from fish: Fish samples (tuna and mahi mahi) that were found to contain no detectable intrinsic HPB were used in this phase of the investigation. Homogenates (99 ml) of the fish were spiked with 1 ml saline cell-suspensions containing high or low levels of the desired test organisms (*E. aerogenes* or *K. pneumoniae*). The fish homogenates were then assayed for the total population of the test organism recoverable using DAM. The positive histamine producing bacterial colonies from DAM were confirmed on DTA.

RESULTS AND DISCUSSION

Data for the total intrinsic histamine producing bacterial population of white and red snapper, Chinese mackerel, mullet, tuna, and mahi mahi are presented in Table 1. Both red snapper and mullet exhibited significant numbers of HPB, whereas Chinese mackerel, white snapper, tuna, and mahi mahi did not. The red snapper had 200 and mullet 20 HPB per g fish. Failure to isolate intrinsic HPB from Chinese mackerel, white snapper, tuna, and mahi mahi may be due to the presence of low numbers of these organisms that could not be detected by this procedure or the absence of HPB

in the fish samples tested. It is also possible that the isolation of HPB from red snapper and mullet is due to the nature of the samples themselves (gills, skin, fins, and intestines) that have been implicated to contain HPB (TAYLOR 1980). Four isolates (representative of 40 HPB) from the red snapper homogenate were identified as Proteus morganii, and 2 isolates (representative of 20 HPB) from the mullet were identified as Klebsiella oxytoca.

TABLE 1. Detection and identification of histamine producing bacteria (HPB) in fish. The fish were assayed for intrinsic HPB and the isolates identified using the API system.

Fish Sample	No. HPB/g Fish	Isolates
White Snapper	0	
Red Snapper	200	Proteus morganii
Chinese Mackerel	0	
Mullet	20	Klebsiella oxytoca
Tuna	0	
Mahi Mahi	0	

DAM plates containing known HPB (E. aerogenes and K. pneumoniae) and an uninoculated control plate were assayed photofluorometrically for free histamine. The results indicated that DAM plates containing HPB had high levels of free histamine whereas the control had no detectable free histamine. The rate of conversion of histidine to histamine in DAM plates containing the HPB was more than 45%.

Recovery rates of HPB from fish homogenates that were spiked with the test organisms are shown in Table 2. The percent recovery ranged from 86 to 95. Failure to achieve maximum recovery (100%) may be due to adherence of the organisms to the fish tissue. This adherence may also explain the failure to recover intrinsic HPB from Chinese mackerel, white snapper, tuna, and mahi mahi samples.

TABLE 2. Recovery of histamine producing bacteria from fish spiked with test organisms.

Fish	Test Organism	No. Cells Added/ml	Recovery (%)[a]
Tuna	E. aerogenes	6450	92
	" "	630	94
	K. pneumoniae	1005	95
	" "	112	91
Mahi Mahi	E. aerogenes	5055	88
	" "	380	90
	K. pneumoniae	1350	92
	" "	169	86

[a]Mean recovery based on 5 replicates at each cell concentration.

In conclusion, this study established that DAM differentiates between histamine producing and non-producing bacteria in fish, and that DTA can be used for confirming histamine production by the isolates. Confirmation of HPB in suspect fish samples and chemical verification of the presence of high levels of histamine in fish provide convincing evidence of scombroid food poisoning.

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