AN ABSTRACT OF THE THESIS OF

Sureerat Phuvasate for the degree of Master of Science in Food Science and Technology presented on December 5, 2007.

Title: Electrolyzed Oxidizing Water Treatment as a Post-harvest Process for Controlling Histamine Formation in Fish

Abstract approved:

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Scombroid poisoning, caused by histamine intoxication, is one of the most prevalent illnesses associated with seafood consumption in the United States. The illness is usually accompanied with a variety of symptoms, such as rash, nausea, diarrhea, flushing, sweating, and headache. Incidence of scombroid poisoning has been consistently reported in the U.S. through surveillance and is often underestimated due to mild and transient symptoms.

Histamine can be formed in fish through enzymatic decarboxylation of histidine. Many bacteria include *Morganella morganii*, *Proteus vulgaris*, *Klebsiella pneumoniae*, and *Enterobacter aerogenes* are known to be prolific histamine formers and have been frequently isolated from fish. Among them, *Morganella morganii* is the most prolific histamine former and plays the major role in histamine formation in fish that is improperly handled. The U.S. Food and Drug Administration's seafood

regulations limit histamine in fish at a level of 5 mg/100g (50 ppm) for assuring the safe consumption of fish.

This study was conducted to determine growth of histamine-producing bacteria (*Enterobacter aerogenes*, *Enterobacter cloacae*, *Proteus hauseri*, *Morganella morganii*, and *Klebsiella pneumoniae*) and histamine formation in yellowfin tuna (*Thunnus albacares*) stored at 5, 15 and 25°C as well as effects of treatments of electrolyzed oxidizing (EO) water and in ice form on reducing histamine-producing bacteria on food contact surfaces (ceramic tile and stainless steel) and fish skin (Atlantic salmon and yellowfin tuna).

Enterobacter aerogenes and *Morganella morganii* were the most prolific histamine formers capable of producing >1,000 ppm of histamine in broth culture after 12 h at 25°C. Both species grew rapidly at elevated temperatures (15-25°C), but the growth was inhibited at 5°C. Histamine was produced by the bacteria in medium broth and tuna meat held at 15 and 25°C when bacterial populations increased to $\geq 10^6$ CFU/ml (or CFU/g). However, storing yellowfin tuna inoculated with *M. morganii* or *E. aerogenes* at 5°C resulted in slight decreases of the bacteria over 14 days of storage and no histamine formation. Low-temperature (≤5°C) storage was critical to prevent histamine formation in fish.

Enterobacter aerogenes and *Morganella morganii* could survive well on food contact surfaces (glazed ceramic tile and stainless steel) and fish skin. However, a treatment of electrolyzed oxidizing (EO) water (50 ppm chlorine) for 5 min was capable of removing the bacteria completely from the surfaces (>1.7 to >5.4 log CFU/cm^2 reductions). Soaking salmon skin in EO water containing 100 ppm for 120

min could reduce *E. aerogenes* and *M. morganii* on salmon skin by 1.3 and 2.2 log CFU/cm^2, respectively. Holding fish skin in EO ice containing 100 ppm of chlorine for 24 h could reduce *E. aerogenes* by 1.6 log CFU/cm^2 on salmon skin and 2.4 log CFU/cm^2 on tuna skin and *M. morganii* by 2.0 log CFU/cm^2 on salmon skin and 3.5 log CFU/cm^2 on tuna skin.

EO water can be used as a sanitizer for decontaminating histamine-producing bacteria on food contact surfaces. Holding fish in EO ice (100 ppm chlorine) could be used as a post-harvest treatment to reduce histamine-producing bacteria contamination on fish skin and decrease probability of histamine formation in fish during storage.

Electrolyzed Oxidizing Water Treatment as a Post-harvest Process for Controlling Histamine Formation in Fish

by
Sureerat Phuvasate

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ELECTROLYZED OXIDIZING WATER TREATMENT AS A POST-HARVEST PROCESS FOR CONTROLLING HISTAMINE FORMATION IN FISH

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Fish is a vital source of food which contains high protein (15-20%) with significant amounts of all essential amino acids and provides at least 20 percent of animal protein consumed by 2.6 billion people around the world (FAO 2004). In certain regions where livestock is relatively scarce, fish protein could be an important source of essential nutrients. In certain countries such as Bangladesh, Cambodia, Indonesia, Sri Lanka and Japan, fish protein contributes more than 50 percent of total animal protein consumed in the diets (FAO 2004).

Fish can be harvested from marine sources as well as aquaculture with marine capture generally accounting for greater than 80 percent of the world's fish supply (Tidwell and Allan 2001). With increased worldwide consumption of seafood, the total worldwide fisheries production has been steadily increasing from 112 million tons in 1994 to 125 million tons in 1999 (FAO 2000) and to 142 million tons in 2005 (FAO 2006). The majority (approximately 75 percent) of the production is usually consumed by humans and the remaining 25 percent is utilized for preparation of non-food products, such as fishmeal and fish oil.

Fish is subjected to rapid deterioration upon harvest because it can be easily contaminated with bacteria in the marine environment. Spoilage bacteria are commonly present on the skin, in the gill and digestive tract of fish. Raw fish should be chilled in ice or frozen immediately upon harvest to prevent growth of bacteria or toxin production by bacteria. However, rapid freezing or chilling in ice of freshly caught fish on board fishing vessels may not always be feasible because of the large amount of ice required for chilling or limited capacity of the freezing chamber. It is

possible that a portion of the fish could be left on the deck after catch and exposed to ambient temperatures before being chilled or frozen. The delay in icing or freezing of fish after catch allows bacteria on fish to multiply and, in some case, produce toxic substances. Therefore, the quality of fish is dependent largely on the on-board handling after catch.

1.2 The United States Fisheries Industry

The United States has more than 13,000 miles of coastlines bordering the Pacific and Atlantic Oceans, the Gulf of Mexico, the Gulf of Alaska, the Bering Sea, and the Arctic Ocean. This occupies the world's largest exclusive economic zone (EEZ) with approximately 3.36 million square nautical miles of oceans for fishing and marine resources beyond the coastlines of 200 nautical miles (NMFS 2007). The U.S. fisheries industry, including domestic and foreign markets, contributed $35.1 billion to the Gross National Product in 2006, increasing dramatically from 2 billion reported in 2005 (NMFS 2007; NOAA 2007a).

The U.S. is the third largest market in the world for seafood consumption and American people consumed 4.9 billion pounds of fish and shellfish in 2006 with most of them (83%) being imported from foreign countries (NOAA 2007b). The U.S. per capita consumption of fish and shellfish increased in 2006 by 2% from the 2005 consumption of 16.2 pounds to 16.5 pounds (edible meat) (NOAA 2007b). Among them, 12.3 pounds were fresh and frozen fish and shellfish, 3.9 pounds were canned seafood, and 0.3 pound was cured. Tuna was the leading product accounting for 74% (2.9 of 3.9 pounds) of canned seafood consumed per capita (NOAA 2007b).

1.3 Nutritional and Health Benefits of Fish Consumption

It has been known for decades that fish is a source of top-quality protein with certain essential nutrients, particularly omega-3 polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Recent studies have suggested that eating fish or intake of fish oil on a regular basis can reduce the risk of coronary heart disease in both men and women (Daviglus and others 1997; Hu and others 2002). The American Heart Association's dietary guidelines have recommended eating some fatty fish (mackerel, sardines, albacore tuna and salmon) at least twice a week for benefits on the heart of healthy adults and those with coronary heart disease or at high risk.

In addition to EPA and DHA, vitamin D is rich in fatty fish (such as mackerel, salmon and sardine) and fish liver oil. Frequent consumption of fatty fish such as mackerel (11 μg of vitamin D/100g), salmon (10-32 μg of vitamin D/100g) and sardine (46 μg of vitamin D/100g) in Japan has been reported to provide 90.7% of daily vitamin D intake for elderly people (Nakamura and others 2002). Brustad and others (2004) reported that eating 3 traditional fish dish meals (*mølje*) containing cod liver and cod liver oil for a week provided 272 μg of vitamin D and could increase vitamin D production in skin of northern Norway people during winter.

Other than those, many small fish are good sources of vitamin A (mola) and iron (darkina and trey changwa plieng) (Roos and others 2007). Therefore, frequent consumption of these small fish could prevent vitamin A and mineral deficiencies. Moreover, marine fish is a great source of iodine though the iodine content varies among species. Chandrashekar and Deosthale (1993) studied iodine contents in Indian

marine fish such as anchovy, mackerel, lesser sardine, threadfins and Bombay duck as well as in freshwater fish (murrel, rohu, and catla) and found that marine species contained relatively higher amounts of iodine (6.8–97.1 μg/100 g) than freshwater fish (5–15.5 μg/100 g).

Despite the nutritional benefits to human health, the nutritional value of fish can diminish quickly if fish is not properly handled upon harvest. Fish lipid is subjected to oxidation and hydrolysis, which can lead to development of rancidity. Fish protein can be hydrolyzed by proteases, such as cathepsins, calpains and trypsin, to small peptides resulting in softening fish tissue (Huss 1995). In addition, enzymatic reaction converting trimethylamineoxide (TMAO) to trimethylamine (TMA) in fish muscle by bacteria produces fishy smell (Jay and others 2005).

1.4 Seafood Safety

1.4.1 Bacterial Pathogens

Fish can be a carrier of *Vibrio parahaemolyticus* that occurs naturally in the marine environments. Consumption of raw or undercooked fish contaminated with *V. parahaemolyticus* may lead to development of acute gastroenteritis characterized by diarrhea, vomiting, and abdominal cramps (Rippy 1994). In addition, fish can be contaminated with *Listeria monocytogenes* during handling and processing (Jørgensen and Huss 1998). This human pathogen is widely distributed in environment, including marine and fresh waters as well as seafood processing plants. Consumption of fish, particularly ready-to-eat (RTE) products such as smoked fish, contaminated with *L. monocytogenes* can cause serious health problems for consumers, especially pregnant

women, newborns, the elderly, and people with suppressed immune systems (FSIS 2000). The United States Department of Agriculture estimated that approximately 2,500 cases of listeriosis occur in the U.S. each year with 2,300 hospitalizations and 500 deaths (FSIS 2000).

In addition to *V. parahaemolyticus* and *L. monocytogenes*, fish can also serve as a vehicle for other foodborne pathogens. Many studies have reported that raw fish and fish products could be sources of pathogens such as *Escherichia coli* O157:H7, *Staphylococcus aureus* and *Salmonella* spp. (Ayulo and others 1994; Wallace and others 1999; Heinitz and others 2000). Fish consumption has been reported a leading cause of foodborne diseases in Japan and Southeast Asian countries where fish are commonly eaten raw or with little cooking (Sakazaki 1979).

1.4.2 Viruses

Seafood can also serve as a vehicle for transmission of viruses such as hepatitis A virus (HAV) and norovirus because of contamination of marine environments by discharge of sewage from plants and fishing boats near harvest area (CDC 2004; CID 2004). The infections caused by these two viruses generally lead to development of nausea, diarrhea, headache, fever and abdominal pain. However, the norovirus infection typically produces a sudden illness with a short onset time (12-48 h) and short duration (12-60 h) (CDC 2004), while infection with HAV has a longer incubation time (10 to 50 days) and can cause liver disease with a typical sign of jaundice (FDA 1992).

1.4.3 Methylmercury

In addition to microbial pathogens, fish can be contaminated with toxic chemicals such as methylmercury in the marine environment. Methylmercury is an organic form of mercury, generated through the methylation of mercury by aquatic bacteria. Exposure to methylmercury in humans usually comes from consumption of fish and shellfish, which can cause serious health problems affecting the normal functions of the brain and nervous system. The U.S. Food and Drug Administration has set an action level for methylmercury in commercial fish of 1.0 ppm (FDA 2001) and advised pregnant women, nursing mother and young children to avoid eating four fish species (king mackerel, swordfish, shark and tilefish) that may contain an average level of mercury closed to or exceeding 1.0 ppm (FDA 2004).

1.4.4 Marine Toxins

Fish and shellfish can contain marine toxins such as domoic acid, saxitoxin, and ciguatoxin produced by certain species of algae. These toxins can be accumulated in fish or shellfish if the algae are consumed. Human consumption of shellfish containing domoic acid will result in amnesic shellfish poisoning (ASP) with gastrointestinal (vomiting, diarrhea, abdominal pain) and neurological (confusion, memory loss, disorientation, seizure, coma) symptoms (FDA 1992). Consumption of shellfish containing saxitoxin will result in paralytic shellfish poisoning (PSP) characterized by tingling of tongue, numbness, vomiting and abdominal pain (CDC 1991). Eating fish containing ciguatoxin will result in illness (ciguatera fish poisoning)

in the gastrointestinal and neurological systems: numbness, tingling, nausea, vomiting, diarrhea and itching (CDC 2006a).

To prevent being poisoned by these marine toxins, consumers should avoid eating fish or shellfish harvested from the regions contaminated by the toxin-producing algae.

1.4.5 Histamine

Histamine is a biogenic amine formed through the decarboxylation of the amino acid, histidine, by the enzyme histidine decarboxylase. This enzyme occurs naturally in living animals including fish and can be produced by a number of microorganisms commonly associated with marine environments (Silla Santos 1996). Fish species, such as tunas, mackerel, mahi mahi, bonito, bluefish, and sardine, which contain high levels of histidine in muscle, are more likely to contain histamine (formed by bacterial enzymatic activity) if the fish is not properly stored before consumption (Yoshinaga and Frank 1982; Frank and others 1985; López-Sabater and others 1996b; Du and others 2002).

Consumption of fish containing histamine may result in illness known as scombroid poisoning with a variety of symptoms, including rash, facial flushing, sweating, vomiting, diarrhea, headache, burning sensation and metallic taste in the mouth (CDC 2000b). The onset of symptoms is rapid, ranging from immediate to 30 minutes after ingestion of fish. The duration of the illness is typically a few hours but may last for days. To prevent occurrence of scombroid poisoning associated with fish

consumption, the FDA established an advisory level that limits histamine at a level of <50 ppm in fish for consumption (FDA 2001).

While scombroid poisoning is usually a mild illness, the severity of symptoms greatly varies and depends on the amount of histamine consumed and individual sensitivity to histamine (Taylor 1986). Most patients can recover from the illness without medications; antihistamine, such as diphenhydramine or cimetidine, is often used to relieve the symptoms (Becker and others 2001). Since the formation of histamine in fish is mainly related to bacterial enzymatic activity, the FDA recommended rapid chilling of fish on board a vessel and keeping fish at low temperatures (≤4.4°C) throughout distribution to minimize formation of histamine in fish (FDA 2001).

1.4.6 Microbiological Limits of Fish and Fishery Products

To reduce hazards associated with consumption of fish and fishery products, the FDA established safety levels for fish and fishery products (Table 1.1). Fish and fisheries products exceeding the action level will be recalled. In addition to FDA's regulations, the International Commission on Microbiological Specifications for Foods (ICMSF 1986) also recommended microbiological limits of fish and fishery products for international trade (Table 1.2). The specifications recommend both aerobic plate counts and *Escherichia coli* be tested for all seafood products. The specifications also suggest that *Salmonella* spp. and *V. parahaemolyticus* be tested for raw fish and *Staphylococcus aureus* be tested for smoked fish. Moreover, the ICMSF suggested

Table 1.1 FDA and EPA safety levels for fish and fishery products (FDA 2001)

Products	Action Level
All fish	1. *Staphylococcus aureus* - positive for staphylococcal enterotoxin 2. *Staphylococcus aureus* level - equal to or greater than 10^4/g (MPN). 3. *Clostridium botulinum* - Presence of viable spores or vegetative cells or presence of toxin 4. Methyl mercury - 1.0 ppm 5. Paralytic shellfish poison - 0.8 ppm (80 μg/100g) saxitoxin equivalent
Ready-to-eat fishery products	1. Enterotoxigenic *Escherichia coli* (ETEC) - 1 x 10^3 ETEC/g 2. *Listeria monocytogenes* - presence of organism 3. *Vibrio cholerae* - presence of toxigenic 01 or non-01 4. *Vibrio parahaemolyticus* - levels equal to or greater than 1 x 10^4/g (Kanagawa positive or negative). 5. *Vibrio vulnificus* - presence of pathogenic organism
Tuna, mahi mahi, and related fish	Histamine - 50 ppm

Note: For a more thorough listing of FDA and EPA safety levels, see http://www.cfsan.fda.gov/~comm/haccp4x5.html.

Table 1.2 Sampling plans and microbiological limits for fresh/frozen and cold-smoked fish (ICMSF 1986).

Test	n	c	Limit / g or cm^2	
			m	M
Aerobic plate counts	5	3	$5x10^5$	10^7
Escherichia coli	5	3	11	500
Salmonella	5	0	0	-
Vibrio parahaemolyticus	5	2	10^2	10^3
Staphylococcus aureus	5	2	10^3	10^4

n = Number of representative sample units.
c = Maximum number of acceptable sample units with bacterial counts between m and M.
m = Maximum recommended bacterial counts for good quality products.
M = Maximum recommended bacterial counts for marginally acceptable quality products.

individuals and agencies to be aware of the potential hazard of toxins such as histamine in susceptible species and conduct toxin tests even though the specifications did not include recommendations for toxins.

1.5 Scombroid Poisoning

Scombroid poisoning, caused by histamine intoxication, is one of the most prevalent illnesses associated with seafood consumption in the United States (FDA 2001). The illness is usually accompanied with a variety of symptoms, such as rash, nausea, diarrhea, flushing, sweating, and headache. Incidence of scombroid poisoning has been consistently reported in the U.S. through surveillance. Between 1988 and 2002, a total of 263 outbreaks of scombroid poisoning involving 1,274 patients were reported to the Centers for Disease Control and Prevention (Table 1.3) (CDC 1996; 2000b; 2006b). However, the incidents are often underreported due to mild and transient symptoms.

Recently, two outbreaks reported in Louisiana and Tennessee in late 2006 were associated with eating tuna steaks imported from Indonesia and Vietnam, respectively (CDC 2007). In one incident in Louisiana, 6 of 23 persons were hospitalized after 2 hours of eating tuna steaks at a cafeteria. High levels of histamine (>500 ppm) were detected in tuna steaks received by the cafeteria as the same shipment from a local distributor with records of no temperature abuse. The investigation suspected that temperature abuse of fish was likely to occur sometimes after the fish was caught and before being delivered to the wholesale distributor in Boston. The other outbreak

Table 1.3. Numbers of reported scombroid poisoning outbreaks in the U.S., 1988-2002 (CDC 1996; 2000b; 2006b)

Year	Outbreaks	Cases	Deaths
1988	16	65	1
1989	17	80	0
1990	11	194	0
1991	17	40	0
1992	15	135	0
1993	5	21	0
1994	21	83	0
1995	16	91	0
1996	12	37	0
1997	15	65	0
1998	27	124	0
1999	21	67	0
2000	20	81	0
2001	29	132	0
2002	21	59	0

Note: For a more annual listing of foodborne outbreaks in the U.S., see http://www.cdc.gov/foodborneoutbreaks/outbreak_data.htm

at a restaurant in Tennessee caused 5 illnesses and one victim suffered from severe symptoms requiring medical treatment. While the investigation did not detect high level of histamine (>50 ppm) in any leftover tuna samples in the restaurant, inappropriate handling of the fish at some points between distribution and preparation of fish was suspected a major factor contributing to this outbreaks.

Outbreaks of scombroid poisoning are commonly associated with consumption of scombroid fish, including various species of tuna (albacore, big-eye, blackfin, bluefin, yellowfin and skipjack), mackerel, bonito and saury, because of high levels of histidine in the muscle (Taylor 1986). However, non-scombroid fish such as mahi mahi, bluefish, herring, anchovy and sardines have also been incriminated in the incidents (Rawles and others 1996). In the U.S., mahi mahi, tuna and bluefish have been frequently implicated in scombroid poisoning (CDC 1989). Between 1980 and 1994, 84% (77 of 92) of the scombrotoxin outbreaks reported in New York State were caused by eating tuna, bluefish and mahi-mahi (Wallace and others 1999). Between 1998 and 1999, 26 cases of histamine poisoning occurred in North Carolina and Pennsylvania were associated with consumption of tuna fish, tuna burger, and tuna salad (Becker and others 2001).

In the United Kingdom, tuna (fresh, frozen and canned) and mackerel are reported the major fish associated with scombroid poisoning (Scoging 1998). Fresh and hot-smoked kahawai have been implicated in scombroid poisoning in New Zealand (Bremer and others 1998). Several outbreaks occurred in Taiwan that were linked to the consumption of sailfish and canned mackerel (Hwang and others 1995; Tsai and others 2005).

Fish implicated in scombroid poisoning outbreaks usually containing histamine above 200 ppm, often above 500 ppm (FDA 2001). However, much higher levels of histamine (up to 5,000 ppm) have been reported in fish implicated in the outbreaks (Table 1.4). It is believed that a histamine level of 200 ppm in fish is sufficient to cause symptoms of the scombroid poisoning (CDC 1988).

1.6 Histamine Formation in Fish

1.6.1 Histamine-producing bacteria

Fresh fish usually contain negligible amounts of histamine. However, histamine can be formed in fish muscle postmortem by activity of bacterial histidine decarboxylase converting histidine to histamine through decarboxylation. Therefore, fish species, including tunas, mackerel, bonito, saury, mahi mahi, bluefish, herring, anchovy and sardines, with high levels of free histidine in muscle are more susceptible to histamine formation during storage (Taylor 1986; Antoine and others 1999; Antoine and others 2001)

Members of *Enterobacteriaceae* are the primarily bacteria commonly associated with fish. Many of them are capable of producing histidine decarboxylase and are the major bacteria involved in decomposition of scombroid fish (Frank and others 1981). These bacteria are referred to as histamine-producing bacteria because of their ability to produce histamine in fish. Among them, *Morganella morganii*, *Klebsiella pneumoniae* and *Hafnia alvei* have been frequently isolated from fish implicated in scombroid poisoning (Rawles and others 1996). In addition, other bacteria in including species of *Proteus* and *Enterobacter* (particular *E. aerogenes*)

Table 1.4 Examples of high histamine levels in fish implicated in scombroid outbreaks

Fish species	Histamine (ppm)	Location	Reference
Yellowfin	5,830-7,280	South Carolina, 1988	(CDC 1989)
tuna	>50	Pennsylvania, 1988	(CDC 2000a)
	500-600	Illinois, 1988	(CDC 1989)
	5,430	Japan, 1982	(Yamanaka and others 1982)
Tuna	2,740-3,240	North Carolina, 1999	(Becker and others 2001)
Amberjack	2,570-4,300	Alabama Tennessee	(CDC 1986)
Mahi-mahi	200	New Mexico, 1987	(CDC 1988)
	1,070-1,950	Japan, 1984	(Yamanaka and others 1987)
Marlin	3,080-4,560	Japan	(Yamanaka and others 1982)
Sailfish	1,680-1,800	Taiwan, 1994	(Hwang and others 1995)
Anchovy	3,060	Spain	(Murray and others 1982)
Herring	300-1,300	Netherlands	(ten Brink and others 1990)
Mackerel	100-3,000	Netherlands	(ten Brink and others 1990)
	100	Taiwan	(Chen and Malison 1987)
Kahawai	190-3,940	New Zealand	(Bremer and others 2003)
Canned			
Mackerel	1,539	Taiwan	(Tsai and others 2005)
Pilchard	2,900	Morocco	(Murray and others 1982)
Tuna	1,050	Peru	(Murray and others 1982)
Tuna	2,900	Malaysia	(Murray and others 1982)
Tuna	260	Thailand	(Murray and others 1982)
Tuna	6,400	Philippines	(Murray and others 1982)
Anchovies	680	Spain	(Murray and others 1982)
Sardine	720	Morocco	(Murray and others 1982)

have been isolated from temperature-abused fish such as mackerel and skipjack (Yoshinaga and Frank 1982; Middlebrooks and others 1988).

Histamine-producing bacteria (HPB) can be present in the marine environments and, therefore, be isolated from skin, gills and gut of fish (FDA 2001). Kim and others (2003) reported isolation of *M. morganii* from gills and skin of fresh mackerel and sardines. An earlier study showed that *Hafnia alvei* and *Enterobacter cloacae* were the major HPB isolated from albacore tuna (Kim and others 2001b). Taylor and Speckhard (1983) reported isolating *M. morganii* and *Citrobacter freundii* from gill of frozen skipjack tuna.

In addition to be part of natural microbial flora associated with fish, HPB can be present on processed fish as a result of cross-contamination during handling and processing. Studies have demonstrated that HPB such as *M. morganii* and *C. freundii* isolated from fish skin were also detected on direct contact surfaces such as conveyer belts, plastic totes and knifes when the fish was processed (Kim and others 2003; Allen and others 2005). Therefore, food contact surfaces should be sanitized frequently during fish processing to prevent introducing HPB from contaminated surfaces to fish.

1.6.2 Temperature effect on histamine formation in fish

Since the formation of histamine in fish is mainly related to the histidine decarboxylase produced by bacteria, a storage temperature allowing HPB to multiply would promote histamine formation in fish. It is important to realize that many of the HBP are psychrotrophs and able to grow slowly at refrigeration temperatures. Kim and

others (2001a) reported that *Actinobacillus ureae*, *Vibrio alginolyticus* and *Aeromonas hydrophila* isolated from mackerel stored at 4°C were able to produce histamine (111-256 ppm) in culture broth at 37°C. López-Sabater and others (1996b) studied growth of HPB and histamine formation in tuna meat stored at 8°C and found no changes in HPB populations in the inoculated tuna meat (10^5 CFU/g) after 84 h. However, low levels of histamine (23-43 ppm) were detected in the tuna meat after the storage. These studies demonstrated that psychrotrophic HPB in fish could survive at low temperature storage and produce histamine over time.

The formation of histamine by HPB in fish is greatly enhanced when fish is exposed to higher temperatures (20-25°C). Several studies have reported that HPB, particularly *M. morganii*, could produce greater than 3,000 ppm of histamine in culture broth at 25 or 37 °C after 18 h (López-Sabater and others 1996a; Kim and others 2001a; Kim and others 2001b). Klausen and Huss (1987) demonstrated that *M. morganii* was able to grow rapidly at 20 to 25°C but no growth was observed at 0 to 5°C. Kim and others (2002) reported that mackerel, albacore and mahi-mahi inoculated with *M. morganii* and held at 25°C had higher histamine levels than those held at 37°C, even though no differences in bacterial populations were observed. These results suggest that 25°C is the most favorable temperature for growth of *M. morganii* and production of histamine in fish.

While freezing is an effective means to inhibit growth of HPB and prevent histamine formation in fish, frozen fish may sometimes contain high levels of histamine if the fish had been exposed to elevated temperatures, even for a short period of time, before becoming frozen. Although freezing may inhibit growth of

HPB, the decarboxylase enzyme produced by the bacteria during the temperature abuse before fish became frozen remains stable in frozen stage and reactivated rapidly after thawing. Therefore, detection of large amount of histamine in frozen fish immediately after thawing indicates temperature abuse or improper chilling of the fish at some points between catching and freezing. Moreover, once histamine is produced in fish, cooking, freezing and smoking are ineffective in removing the toxin.

1.7 Control of Histamine Formation

1.7.1 United States Food and Drug Administration Regulations

In December 1997, FDA issued seafood regulations based on principles of Hazard Analysis and Critical Control Point (HACCP) system requiring processors to identify potential hazards, establish critical control points and critical limits, monitor procedures, establish corrective actions and keep records for process verification (FDA 2001). The regulations also established guidelines for controlling histamine production in fish with recommended time and temperature for handling fish after catch.

Rapid chilling of fish immediately after death is the most critical strategy for preventing histamine formation in fish, especially for fish that have been exposed to warmer waters or air as well as large fish that generate heat in the tissues of the fish (FDA 2001) Therefore, the FDA's Fish and Fisheries Products Hazards and Controls Guidance (2001) recommended the following:

- Fish should be placed in ice or in refrigerated seawater or brine at 40°F (4.4°C) or less within 12 hours of death, or placed in refrigerated seawater or brine at 50°F (10°C) or less within 9 hours of death.
- Fish exposed to air or water temperatures above 83°F (28.3°C), or large tuna (i.e., above 20 lbs.) that are eviscerated before on-board chilling, should be placed in ice (including packing the belly cavity of large tuna with ice) or in refrigerated seawater or brine at 40°F (4.4°C) or less within 6 hours of death.
- Large tuna (i.e., above 20 lbs.) that are not eviscerated before on-board chilling should be chilled to an internal temperature of 50°F (10°C) or less within 6 hours of death.

In addition, the guidelines also limit histamine in fish at a level of 5 mg/100g (50 ppm) for assuring the safe consumption of fish (Table 1.1) (FDA 2001). This guidance level of 50 ppm was set because histamine is generally not uniformly distributed in a decomposed fish. If 50 ppm is found in one section, there is the possibility that other sections may exceed 500 ppm.

The FDA's acceptable level of histamine in fish varies from international regulations. The European Community has established a maximum histamine content of 200 ppm and allowed two out of the nine samples to contain a level of between 100 and 200 ppm (EC 2005). Australia New Zealand Food Standards Code set by Australia New Zealand Food Authority states that the level of histamine in fish or fish products must not exceed 200 ppm (ANZFA 2001).

In addition to human health concern, presence of histamine in fresh fish is an indicator of bacterial spoilage. Fish containing histamine above the regulations should be discarded and not used as a human food. Thus, histamine formation is fish is a concern not only for the health of consumers but also for financial loss of the seafood producers.

1.7.2 Effects of Processing

Histamine is a very stable toxin. Once formed in foods, it can not be removed or destroyed by normal food processing procedures. Thus, the best strategy for preventing scombroid poisoning is to prevent formation of histamine in fish. A number of methods, including cooking, smoking, freezing and canning has been investigated to eliminate and control histamine in fish.

1.7.2.1 Thermal processing

Baranowski and others (1990) investigated effects of cooking methods on inactivating histamine in mahi mahi patties and reported that baking or steaming had no significant effects on reducing histamine levels in the patties. The histamine levels in baked and steamed mahi mahi patties (397 and 374 ppm, respectively) were almost unchanged after the processes when compared with uncooked samples (363 ppm). Bremer and other (1998) showed that a smoking process at 57°C for 12 s reduced 90% of *Hafnia alvei* inoculated to kahawai, but had no effect on reducing histamine.

Canning is a commercial process used to produce food products that can last for long time when stored at ambient temperature. However, the canning process is not

effective in inactivating histamine. Glória and others (1999) studied histamine levels in canned albacore tuna and reported that levels of histamine in the albacore tuna were not significantly reduced after the canning process. Fish that is not properly handled between harvest and canning may allow high levels of histamine accumulated in the fish before being canned. This is why the scombroid poisoning has been frequently linked to the consumption of canned tuna throughout the world.

1.7.2.2 Low-temperature storage

Kim and others (2001a) investigated effects of low-temperature storage on growth of HPB and histamine contents of fish and found that growth of prolific HPB (*M. morganii*) in fish was controlled by storing fish at 4°C. Du and other (2002), who investigated histamine formation in yellowfin tuna fillet stored at 0 and 4°C, observed a higher level of histamine (90 ppm) in the fillet stored at 4°C than those stored at 0°C contained (20 ppm) after 9 days of storage. In addition, several studies reported finding no histamine in albacore tuna stored at 0°C for 18 days (Kim and others 1999b) and very low levels (20 ppm) of histamine in mackerel stored in ice for 6 and 10 days (Jhaveri and others 1982; Bennour and others 1991). These results demonstrated that holding fish at 0-4°C is an effective means to control histamine formation in fish.

Despite the FDA's recommendations that fish should be cooled down to achieve a core temperature of 4.4°C or less and maintain this temperature throughout handling, processing and distribution (FDA 2001), studies had reported that 60% of yellowfin tuna did not reach the FDA's recommended cooling temperature of ≤10°C

within 6 hour of harvest because of relatively large size of tuna and insufficient amount of ice used to chill the fish. The delay in cooling of large fish upon catch gives bacteria on fish an opportunity to grow and produce histamine. Therefore, special effort should be given to cool down temperature of large fish upon harvest. Once the internal temperature of the fish is decreased to 4°C, production of histamine in fish by bacteria can be properly controlled. Rossi and others (2002) reported that whole skipjack tuna cooled in ice and held at 4°C had very low levels of histamine (≤6 ppm) after 18 days of storage.

In addition to low-temperature storage, frozen storage is also effective in controlling histamine formation in fish because growth of bacteria is inhibited during the storage. Ben-Gigirey and others (1998) reported no significant increases in levels of histamine were observed in albacore tuna during frozen storage at -25°C for 9 months. Therefore, fish that will not be consumed within days should be stored in a freezer.

1.7.3 Sanitation and Personal Hygiene

In addition to natural contamination of HPB, fish can sometimes be contaminated with HPB during processing through contact with processing surfaces and equipments that have been contaminated with the bacteria. Since HPB is commonly present on skin or in gills and intestines of fish, post-harvest contamination of fish with HPB may occur at the fishing vessel, processing plant, and distribution. In addition, contamination can also occur during food preparation as a result of poor personal hygiene, particularly through contact of fish without hand washing. Fish

served at restaurants has been involved in scombroid poisoning outbreaks due to mishandling of fish (CDC 2000b). Therefore, proper storage of fish at low temperature (≤4.4°C), frequent sanitation of food processing surfaces, and good personal hygiene all are important in preventing scombroid fish poisoning.

1.8 Development of a New Strategy for Reducing Histamine Formation in fish

The formation of histamine in fish is mainly related to the growth of HPB in fish. Although keeping fish at low-temperatures (≤4.4°C) is known effective in preventing HPB from producing histamine, temperature abuse of fish between harvest and consumption can not be totally avoided. Therefore, the best strategy to prevent histamine formation in fish is to eliminate HPB (or reduce the populations to low levels) on fish upon harvest so no bacterial histidine decarboxylase will be produced even if fish is exposed to certain degrees of temperature abuse before consumption. This might be achieved by an immediate post-harvest treatment of fish with a solution or ice containing antimicrobial activity.

1.8.1 Electrolyzed Oxidizing (EO) Water

Electrolyzed oxidizing water, produced through electrolysis of a dilute salt solution (0.05-0.2% NaCl) has recently been introduced as a sanitizer for the food industry. Through the saltwater electrolysis in a cell containing positively and negatively charged electrodes separated by a membrane, two kinds of water are produced with different properties of pH, chlorine concentration and oxidation-reduction potential (ORP) values. The water (electrolyzed reducing water) produced at

the cathode side is an alkaline solution with a pH of about 11.4 and high reduction potential (ORP value of -795 mV) while the water produced at the anode side (electrolyzed oxidizing (EO) water) is an acidic solution and contains a pH of about 2.5, high oxidation potential (ORP value of >1100 mV) and free chlorines mainly in the form as hypochlorous acid. Numerous studies have reported that the acidic electrolyzed oxidizing (EO) water exhibit strong bactericidal effects against many pathogens including *E. coli* O157:H7, *L. monocytogenes*, *Bacillus cereus* and *S. enteritidis* (Venkitanarayanan and others 1999a; Kim and others 2000b; Park and others 2004).

1.8.1.1 Antimicrobial activity of EO water

Venkitanarayanan and others (1999a) reported that populations of *E. coli* O157:H7, *Salmonella enteritidis*, and *L. monocytogenes* were reduced by approximately 7-log (CFU/ml) after exposing the bacterial cultures in EO water (43-86 ppm free chlorine) at 4 or 23°C for 5 min. Kim and others (2000b) also reported that a treatment of EO water (pH:2.6, ORP:1,160 mV, chlorine:56 ppm) for 10 s resulted in reduction of *E. coli* O157:H7 and *L. monocytogenes* by >8.6 log CFU/ml. The investigators also found that *Bacillus cereus* was more resistant than *E. coli* O157:H7 or *L. monocytogenes* to the EO water treatment and required a 30 s treatment for a complete inactivation (>6.6 log CFU/ml) of vegetative cells. In the same study, spores of *B. cereus* exhibited much more resistance to the EO water treatment than the vegetative cells. However, EO water (56 ppm chlorine) treatment for 120 s was capable of reducing *B. cereus* spores by approximately 3.5-log units. In addition, Liu

and others (2006) also reported that populations of a five-strain cocktail of *L. monocytogenes* decreased rapidly from 9.5 to <1.0 log CFU/ml in EO water containing 50 ppm chlorine within 10 s at room temperature. Park and others (2004) found that treatment of EO water containing 0.1 and 0.2 ppm of residual chlorine for 30 s with agitation reduced populations of *E. coli* O157:H7 F500 by 5.0 and 7.0 log CFU/ml and *L. monocytogenes* Scott A by 3.5 and 5.8 log CFU/ml, respectively. The strong bactericidal effects of the EO water against many foodborne pathogens suggest that EO water treatment could be used to deactivate HPB in fish.

1.8.1.2 Antimicrobial properties of EO water

The antimicrobial properties of EO water have been reported to relate to its low pH value, free chlorine contents, and high ORP value. Growth for most bacteria, except a few such as lactic acid bacteria is limited in a pH range between 4 and 9. Therefore, the low pH value (usually around 2.5) of EO water is inhibitory to growth of many bacteria. Park and others (2004) reported that bactericidal activity of EO water increased with decreasing pH. However, the bactericidal effect of low pH is limited to certain degree. Liu and others (2006) compared the bactericidal effects of EO water with acidic water with same pH of EO water and reported that a treatment of EO water for 5 min was capable of reducing *L. monocytogenes* on surface of stainless steel by >4.7 log CFU/chip, while the treatment with acidic water could only reduce *L. monocytogenes* by about 1.4 log CFU/chip. These results indicate that other factors such as free chlorine contents and high ORP value play important roles in EO water's antimicrobial properties.

Chlorine is the most widely used sanitizer and is most active in hypochlorous acid form for inactivating bacteria. During the saltwater electrolysis, hypochlorous acid (HOCl) is produced and the amounts of hypochlorous acid being produced can be increased by increasing salt concentrations in the water for electrolysis. It is known that hypochlorous acid inactivates growth of bacteria by inhibiting glucose oxidation through oxidizing sulfhydryl groups of certain enzymes in carbohydrate metabolism (WaterReview Technical Briefs 1997). Len and others (2000) reported that the antibacterial activity of EO water is strongly correlated ($r = 0.95$) with concentrations of hypochlorous acid (HOCl). However, concentrations of hypochlorous acid in EO water are affected by chloride ion (Cl^-) in the water. Chlorine gas can be formed through interaction of HOCl and Cl^-.

$$HOCl + Cl^- + H^+ \Leftrightarrow H_2O + Cl_2$$

Evaporation of free chlorine gas results in reduction of HOCl concentration in EO water, which leads to decrease in EO water's bactericidal effects. However, the loss of free gas in EO water can be minimized by using the water immediately after producing or by keeping EO water in a closed container until use. Another factor affecting bactericidal effects of EO water is the increase of pH value in a solution. As pH increases, HOCl will dissociate into a hydrogen ion (H^+) and a hypochlorite ion (OCl^-) that is 20 times less effective than HOCl (White 1999).

In addition to chlorine contents, high oxidation-reduction potential (ORP) has also been identified as a key factor affecting the antimicrobial property of EO water. Most aerobic bacteria require a positive ORP (+200 to +800 mV) to grow. The high ORP (> 1,100 mV) of EO water creates an environment not suitable for their growth.

Kim and others (2000a) investigated the role of ORP in EO water's for inactivating *E. coli* O157:H7 and found populations of *E. coli* 0157:H7 decreased sharply by 9.88 CFU/ml in EO water (56 ppm of residual chlorine, pH 2.7, ORP: 1122) but remained fairly constant (0.13 log CFU/ml reduction) in modified acetic acid solution (60 ppm of residual chlorine, pH 2.4, ORP: 256) with similar pH value and chlorine content. A similar study conducted by Liu and others (2006) reported that a 5-min treatment of modified EO water (pH:2.51, ORP:561 mV, chlorine:40 ppm) was less effective in inactivating *L. monocytogenes* on food contact surfaces (1.55-log reduction) when compared with 4.23-log reduction of the bacteria after treatment with EO water (pH:3.43, ORP:1033 mV, chlorine:40 ppm) containing same chlorine content but a higher ORP value.

1.8.2 Application of EO water in Food Products

1.8.2.1 Fresh fruits and vegetables

Washing fresh fruits and vegetables with clean water is the most commonly used method to remove dirt from the surface. However, the process is not effective in removing bacteria attached on surfaces. Many studies have reported that EO water treatments were effective in reducing bacterial contamination on fresh fruits and vegetables (Koseki and others 2001, 2004b).

Koseki and others (2004b) reported that washing strawberries with tap water did not reduce the microbial load on surfaces while a treatment of EO water (30 ppm of residual chlorine) for 10 min was capable of reducing aerobic mesophilic, coliform bacteria and fungi by 1.0-1.5 log CFU/strawberry. The study also reported that greater

reductions of bacteria and fungi on surfaces of cucumber were observed after EO water treatment. Park and others (2001) reported that washing fresh lettuce with EO water (45 ppm of residual chlorine, pH 2.6 and ORP: 1130 mV) for 3 min reduced counts of *E. coli* O157:H7 and *L. monocyogenes* by 2.41 and 2.65 log CFU/g, respectively, and the treatment did not cause significant changes in quality of lettuce during 2 weeks of storage at 4°C.

In addition to liquid form, ice made from EO water (EO ice) was also studied for its antimicrobial activity against *E. coli* O157:H7 and *L. monocyogenes* on lettuce. Holding lettuce in EO ice generating 70 and 150 ppm of chlorine gas (Cl_2) for 24 h resulted in significant reductions of *E. coli* O157:H7 (2 log CFU/g) and *L. monocyogenes* (1.5 log CFU/g) inoculated to the lettuce (Koseki and others 2004a).

1.8.2.2 Poultry

Poultry products are commonly contaminated with pathogens, such as *Campylobacter jejuni* and *Salmonella typhimurium*, and can cause infections if not properly cooked before consumption. In the poultry industry, carcasses are often sprayed with an organic acid or an alkaline compound followed by immersion in chlorinated chilled water to reduce pathogens on the carcasses. Fabrizio and others (2002) demonstrated that submerging poultry carcasses in 10% trisodium phosphate or 2% acetic acid solution (4°C, 45 min) ($P<0.05$) reduced *S. typhimurium* by 1.41 log (CFU/ml of rinsate) while EO water treatment (50 ppm of residual chlorine, pH 2.6, ORP: 1150 mV) resulted in 0.83-log (CFU/ml of rinsate) reduction of *S. typhimurium*. Park and others (2002a) reported that washing chicken carcasses with EO water (50

ppm of residual chlorine) at 4°C for 10 min was as effective as chlorinated water in reducing *C. jejuni* on chicken carcasses and could reduce populations of *C. jejuni* by 3 log CFU/g with no viable cells being detected in the washed EO water. These reports suggested that EO water could be used as a chlorine alternative for processing poultry carcasses and reducing pathogenic bacteria.

1.8.2.3 Surface sanitizer

Cutting boards and gloves can easily be contaminated with foodborne pathogens after handling raw materials and subsequently serve as sources of contamination during food preparation. Thus, frequent sanitation of gloves and processing equipments and surfaces is recommended to minimize cross-contamination. Venkitanarayanan and others (1999b) studied effects of EO water treatments: (1) 23°C for 10 and 20 min (80 –87 ppm chlorine), (2) 35°C for 10 and 20 min (87-90 ppm chlorine), (3) 45°C for 5 and 10 (87-93 ppm chlorine), and (4) 55°C for 5 min (45 ppm chlorine) on reducing *E. coli* O157:H7 as well as (1) 23°C for 20 min (72 ppm chlorine), (2) 35°C for 10 min, (66 ppm chlorine), and (3) 45°C for 10 min (52 ppm chlorine) on reducing *L. monocytogenes* on cutting boards. The results showed greater reductions (≥5-log CFU/100 cm^2) of *E. coli* O157:H7 and *L. monocytogenes* were observed on contaminated cutting boards after being soaked in EO water for all treatments while only a small reduction (1.0-1.5 log CFU/100 cm^2) of both pathogens were observed after soaking the boards in deionized water. Similarly, treatment of EO water (40 ppm of residual chlorine, pH 2.6, ORP: 1125 mV) for 5 min completely eliminated *L. monocytogenes* (4.46 to 6.54 log CFU/cm^2) on gloves

commonly used for food handling and significantly ($p<0.05$) reduced the counts by 1.60 to 3.87 log CFU/cm^2 on glove contained shrimp meat residues (Liu and Su 2006).

Liu and others (2006) studied effects of EO water treatments on reducing *L. monocytogenes* contamination on surfaces of three types of materials (stainless steel, ceramic tile and floor tile) commonly used in seafood processing environments. Results showed that soaking contaminated materials cut into chips (5×5 cm^2) and contained crabmeat residue in EO water (50 ppm of residual chlorine, pH 2.5, ORP: 1150 mV) for 5 min significant reduced (1.52 to 2.33 log CFU/chip) *L. monocytogenes* populations inoculated to the surfaces, while smaller reductions (0.75 to 1.33 log CFU/chip) were observed after tap water treatment. Similarly, immersing materials inoculated with *Enterobacter aerogenes* and *Staphylococcus aureus* on surfaces in EO water for 5 min without agitation resulted in 2.2 to 2.4 and 1.7 to 1.9 log CFU/cm^2 reduction of *E. aerogenes* and *S. aureus*, respectively (Park and others 2002b). However, a same treatment with water could only reduce populations of both bacteria by 0.1 to 0.3 log CFU/cm^2 (Park and others 2002b). These indicated that EO water could be used to sanitize cutting boards and processing gloves as well as materials commonly used in food processing environments.

1.8.2.4 Seafood

Many treatments including aqueous chlorine, trisodium phosphate, chlorine dioxide and acidified sodium chloride have been evaluated for their abilities to reduce bacterial contamination on fish postharvest, preserve quality, extend shelf life, and enhance safety (Lin and others 1996; Mu and others 1997; Kim and others 1999a; Su

and Morrissey 2003). Aqueous chlorine dioxide treatment (200 ppm) of fish cubes for 5 min has been reported capable of reducing inoculated *L. monocytogenes* by 53.4-64.4% (Lin and others 1996). Dipping trout fillets in 20% (w/v) trisodium phosphate solution (10 min) significantly lowered *L. monocytogenes* and psychrotrophic counts (Mu and others 1997). Dipping raw salmon fillets in acidified sodium chlorite solution (50 ppm chlorine) for 1 min reduced *L. monocytogenes* populations by 0.52 log CFU/g (Su and Morrissey 2003).

Application of EO water for preservation of fish has recently been investigated (Mahmoud and others 2004; Huang and others 2006b; Ozer and Demirci 2006). Dipping whole carp and fillet in EO water (40.8 ppm of residual chlorine) for 15 min reduced total and aerobic plate count on the skin and the fillet by 2.8 and 2.0 log CFU/cm^2, respectively (Mahmoud and others 2004). Washing inoculated salmon fillets with EO water (50 ppm of residual chlorine) at 35°C for 64 min resulted in 1.07- and 1.12-log reduction of *E. coli* 0157:H7 and *L monocytogenes*, respectively (Ozer and Demirci 2006). The combination of EO water (50 or 100 ppm of residual chlorine) and carbon monoxide gas treatment exhibited was reported effective in inhibiting aerobic bacteria on yellowfin tuna stored at refrigeration temperatures (Huang and others 2006b). The treated samples showed lower aerobic plate count (0.80-log CFU/g for 50 ppm of residual chlorine and 1.27-log CFU/g for 100 ppm of residual chlorine) than those detected in samples receiving no treatments after 8 days of storage.

In addition to EO water, EO ice was also studied for preserving freshness of fish. Holding Pacific saury in EO ice containing 34 ppm of active chlorine retarded

growth of aerobic bacteria and psychrotrophs and, according to sensory evaluation, gave 5 more days for shelf life when compared with saury held in regular ice Pacific saury (Kim and others 2006). These results suggest that EO ice can be used for temporary storage of fish and reduce bacterial contamination.

1.8.3 Advantage of EO water

While chlorine is the most widely used sanitizer due to its broad-spectrum bactericidal activities and relatively low cost (Eifert and Sanglay 2002), a major disadvantage of using chlorine-based compounds as sanitizers is that workers must prepare a diluted working solution by handling concentrated chemicals, which is a safety concern. This disadvantage can be overcome by use of EO water, which can be easily generated on-site without the need to handle concentrated chemicals. In addition, studies have demonstrated that EO water is more effective than chlorine water in inactivating bacteria and could be used as chlorine alternative for sanitation.

1.9 Objectives

The objectives of this study are to determine (1) effects of storage temperature on growth of HPB and histamine formation in tuna meat, (2) the effectiveness of EO water against HPB on food-contact surfaces and fish skin under various conditions (times and chlorine concentrations), and (3) bactericidal activity of EO water ice against HPB on fish skin.

CHAPTER 2

GROWTH OF HISTAMINE-PRODUCING BACTERIA AND HISTAMINE FORMATION IN YELLOWFIN TUNA STORED AT VARIOUS TEMPERATURES

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(Submitted to Food Microbiology)

2.1 ABSTRACT

Growth of histamine-producing bacteria (*Enterobacter aerogenes*, *Enterobacter cloacae*, *Proteus hauseri*, *Morganella morganii*, and *Klebsiella pneumoniae*) and histamine formation in yellowfin tuna (*Thunnus albacares*) were investigated at 5, 15 and 25°C. All the bacteria were capable of producing histamine in broth culture at 25°C after populations increased to $>10^6$ CFU/mL. Among them, *M. morganii* and *E. aerogenes* appeared to grow faster than others and identified as the most prolific histamine formers (>1,000 ppm after 12 h in broth culture). When the tuna was inoculated with *M. morganii* or *E. aerogenes* and held at 15 and 25°C, formation of histamine were observed when the bacterial population increased to 10^6 CFU/g. Storing yellowfin tuna inoculated with *M. morganii* or *E. aerogenes* at 5°C resulted in slight decreases of the bacteria over 14 days of storage and no histamine formation. Low-temperature (≤5°C) storage was critical to prevent histamine formation in fish even the fish was contaminated with histamine-producing bacteria at a level of 10^4 CFU/g and exposed to mild-temperature (15°C) for 6 h after harvest.

2.2 INTRODUCTION

Scombroid poisoning, caused by histamine intoxication, is one of the most prevalent illnesses associated with seafood consumption in the United States (FDA 2001). The illness is usually accompanied with a variety of symptoms, including rash, nausea, diarrhea, flushing, sweating, and headache. Incidence of scombroid poisoning has been consistently reported in the U.S. through surveillance and is often underestimated due to mild and transient symptoms (CDC 2000b).

Histamine can be formed through enzymatic decarboxylation of histidine. Many bacteria include *Morganella morganii*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Hafnia alvei*, and *Enterobacter aerogenes* are known to be prolific histamine formers. These histamine-producing bacteria (HPB) have been frequently isolated from a variety of fish such as tunas (skipjack and yellowfin), mackerel, mahi-mahi, bonito, bluefish, and sardine (Yoshinaga and Frank 1982; Frank and others 1985; López-Sabater and others 1996a; Du and others 2002). Among them, *Morganella morganii* is known to be the most prolific histamine former and plays a major role in histamine formation in fish during storage (Kim and others 2001a).

Scombroid fish, particularly tunas, mackerel and bonito, as well as some non-scombroid fish, such as mahi-mahi, bluefish, sardine, anchovies and herring, are frequently implicated in outbreaks of scombroid poisoning because of high contents of free histidine in muscles (Taylor 1986). Although these fish generally contain negligible amounts of histamine when caught, mishandling of fish at some points between capture and consumption allows the histamine-producing bacteria (HPB) to proliferate and produce histamine. It has been reported that histamine can be produced

rapidly by *M. morganii* in fish during storage if temperature abuse occurs (Kim and others 2002).

The United States Food and Drug Administration (FDA 2001) recommended rapid chilling of fish after catch on board a vessel and keeping fish at lower than 4.4°C throughout distribution to minimize formation of histamine in fish. However, it is possible that harvest fish be left on the deck of a vessel for a while (some time up to 4 h) before being chilled (Craven and others 2001). The delay in icing of fish after catch allows the proliferation of HPB and production of the histidine decarboxylase in fish. Once the enzyme is formed, it can continue to produce histamine in the fish at or near refrigeration temperatures even if the HPB are inactivated. Jeyasekaran and others (2004) reported that delay in icing of tropical fish, Barracuda (*Sphyraena barracuda*), for 6 h at 32°C resulted in an increase of HPB in the fish by 1-log CFU/g.

Several studies have reported the formation of histamine in a variety of fish stored at different temperatures (López-Sabater and others 1996b; Silva and others 1998; Du and others 2002; Kim and others 2002). However, the correlation between growth of HPB and histamine formation in fish at various temperatures has not been concluded. This study investigated the growth of HPB and production of histamine in yellowfin tuna stored at various temperatures (5, 15 and 25°C). A mild temperature abuse (15°C for 6 h) of the fish followed by storage at 5°C was also examined for risk of histamine formation during refrigerated storage.

2.3 MATERIALS AND METHODS

2.3.1 Bacterial cultures

Enterobacter aerogenes (ATCC 13048), *Enterobacter cloacae* (ATCC 23355), *Proteus hauseri* (ATCC 13315), *Morganella morganii* (ATCC 25830) and *Klebsiella pneumoniae* (ATCC 13883) obtained from the American Type Culture Collection (ATCC, Manassas, VA) were used in this study. Each bacterium was individually grown in 10 mL of tryptic soy broth (TSB; Difco, BD, Spark, MD) at 37°C for 18-24 h. Enriched culture was centrifuged (3,000×g, Sorvall RC-5B, Kendro Laboratory products, Newtown, CT) at 5°C for 15 min. Pellet cells were re-suspended in phosphate-buffered saline (PBS) to obtain a bacterial culture suspension of approximately 10^{8-9} CFU/mL.

2.3.2 Growth of bacteria and histamine formation in medium broth

To determine the capability of producing histamine in a histidine-containing environment, each bacterial strain was grown in 10 mL of TSB containing 1% L-histidine (Sigma-aldrich, St. Louis, MO) at two initial inoculating levels of 10^3 and 10^5 CFU/mL. The inoculated media were incubated at 25°C for 12 h. Changes in bacterial populations and formation of histamine in the broth cultures were analyzed at 0, 3, 6, 9 and 12 h of incubation. Two bacterial strains that were the most prolific in producing histamine were selected for histamine formation studies in fish.

2.3.3 Growth of histamine-producing bacteria and histamine formation in fish

Frozen yellowfin tuna fillets were obtained from a local seafood company and thawed overnight in a refrigerator (5°C). The skin of fillet was removed with a sterile knife and the flesh was chopped into mince. The tuna mince was inoculated with *Enterobacter aerogenes* or *Morganella morganii* each at two levels (10^3 and 10^5 CFU/g). Inoculated samples were put into sterile plastic bags (25 g/bag) stored at 5, 15 and 25°C. Growth of the bacteria and histamine formation were determined every 3 h for samples stored at 25°C, every 12 h for samples stored at 15°C, and on days 0, 1, 3, 5, 7, 10 and 14 for samples stored at 5°C, or until the level of histamine in a sample increased to >100 ppm.

2.3.4 Effects of temperature abuse on histamine formation in fish

To determine the effect of a mild temperature abuse of fish on formation of histamine in fish during processing, minced yellowfin tuna was inoculated with a mixed culture of *E. aerogenes* and *M. morganii* at a level of 10^{4-5} CFU/g and held at 15°C for 6 h before being stored at 5°C. Growth of bacteria and formation of histamine in the mince were analyzed immediately after the temperature abuse and during storage at 5°C on days 1, 3, 5 and 7.

2.3.5 Determination of bacterial populations in broth culture

Populations of bacteria in the broth cultures were determined by serial dilutions of a broth culture (1.0 mL) with 9.0 mL of Phosphate Buffered Saline (PBS) to prepare a 1:10 sample suspension and pour-plate method using tryptic soy agar

(TSA; Difco, BD, Spark, MD). Colonies formed on the TSA plates were counted after incubation at 37°C for 48 h.

2.3.6 Determination of histamine-producing bacteria in fish

Total bacterial populations and HPB of yellowfin tuna were determined before HPB inoculation and during storage. Each sample (11 g) was added to a bottle of 99 mL of PBS and shaken for 30 sec to prepare a 1:10 sample suspension. The sample suspension was serially diluted with PBS buffer. HPB in a sample was determined with the pour-plate method using a differential plating medium designed for quantitative detection of HPB (Niven and others 1981). The plates were incubated at 37°C for 48 h and growth of HPB on the medium was identified by the formation of colonies with purple halo. Total bacterial populations were determined with the pour-plate method using TSA plates and incubation at 37°C for 48 h. All experiments were conducted in triplicate.

2.3.7 Determination of histamine with Neogen Veratox histamine test kit

Histamine in a sample was determined with Veratox histamine test kit (Neogen Corp., Lansing, MI). For determination of histamine in broth cultures, each broth culture after incubation was centrifuged (3000×g) at 5°C for 15 min and a portion (1.0 mL) of the supernatant was mixed with 9 mL of distilled water to prepare a sample extract. For determination of histamine in tuna mince, 10 g of each sample were mixed with 90 mL of distilled water in a plastic container and shaken vigorously three times each for 15 to 20 sec and pulsed by a 5-min interval to release histamine from fish

tissue. The sample mixture was allowed to settle for 30 sec and filtered through a Neogen syringe packed with a glass wool to obtain a sample extract. Each sample extract (100 μL) was then diluted with 10 mL of extract diluent buffer (provided by Veratox kit) to obtain a final sample solution for test. Histamine concentration (ppm) in a sample was determined using 100 μL of the final sample solution according to manufacturer's procedures (Neogen, Lansing, MI).

2.4 RESULTS AND DISCUSSION

2.4.1 Growth of bacteria and histamine formation in broth culture

Growth of five bacterial species and histamine formation in TSB supplemented with 1% histidine at 25°C are shown in Tables 2.1 and 2.2. All five bacteria grew well in the broth and detectable levels of histamine were observed when bacterial populations increased to near 10^6 CFU/mL. Among the five bacterial species studied, *E. aerogenes* and *M. morganii* appeared to grow faster than other three at 25°C.

When the medium broth was inoculated with each strain at approximately 10^3 CFU/mL and incubated at 25°C, populations of *E. aerogenes* and *M. morganii* increased by 4.0- and 3.5-log units, respectively, after 12 h while increases less than 3.0-log increases were observed for the other three species (Table 2.1). Histamine was detected in the 9-h *M. morganii* culture (5.7 log CFU/mL) and in the 12-h *E. aerogenes* culture (7.6 log CFU/mL) at low levels of 5 and 15 ppm, respectively, but not detectable in the other three cultures even after 12 h at 25°C.

Similar results were obtained when the medium broth was inoculated with each strain at a higher level of approximately 10^5 CFU/mL and incubated at 25°C. Both *E.*

aerogenes and *M. morganii* grew faster than others and histamine was detected in the 6-h cultures when populations of *E. aerogenes* and *M. morganii* cultures increased to 7.0 and 6.4 log CFU/mL, respectively (Table 2.2). At the end of 12 h at 25°C, levels of histamine in cultures of *E. aerogenes* (8.9 log CFU/mL) and *M. morganii* (8.5 log CFU/mL) increased to 1,890 and 1,277 ppm, respectively. However, no histamine was detected in the *E. cloacae* culture (6.9 log CFU/mL) and only low levels (<26 ppm) of histamine were detected in cultures of *P. hauseri* and *K. pneumoniae* after 12 h at 25°C when the populations increased to > 7.6 log CFU/mL.

It has been reported that the activity of histidine carboxylase produced by bacteria might vary depending on bacteria isolated from different species. Kim and others (2001a) reported that both *M. morganii* and *Proteus vulgaris* isolated from Pacific mackerel were capable of producing >3,000 ppm of histamine in the mackerel after storage at 15°C for four days or at 25°C two days, while *E. aerogenes* isolated from the same fish was a weak histamine former (produced 23.4 ppm of histamine in culture broth after 24 h at 37°C). Lopez-Sabater and others (1996b) also reported that *M. morganii* and *Klebsiella oxytoca* isolated from tunafish (*Thunnus thynnus*) were capable of producing ≥3,000 ppm of histamine in culture broth after 18 h at 37°C.

In this study, we demonstrated that *E. aerogenes* (ATCC 13048) and *M. morganii* (ATCC 25830) are fast growers at 25°C and could contribute rapid formation of histamine once the populations increased to 10^6 CFU/g. Therefore, both strains were selected for studying histamine formation in yellowfin tuna.

Table 2.1 Growth of histamine-producing bacteria (10^3 CFU/mL) and histamine formation (ppm) in culture broth at 25°C.

Time (h)	Bacterial Populations (Log CFU/mL) [Histamine concentration (ppm)]				
	Enterobacter aerogenes	*Enterobacter cloacae*	*Proteus hauseri*	*Morganella morganii*	*Klebsiella pneumoniae*
0	3.56±0.06[a] [N.D.[b]]	3.21±0.03 [N.D.]	3.06±0.07 [N.D.]	3.21±0.06 [N.D.]	3.20±0.05 [N.D.]
3	4.07±0.14 [N.D.]	3.28±0.02 [N.D.]	3.27±0.03 [N.D.]	3.64±0.18 [N.D.]	3.28±0.04 [N.D.]
6	5.01±0.06 [N.D.]	4.17±0.03 [N.D.]	4.15±0.06 [N.D.]	4.46±0.02 [N.D.]	3.77±0.03 [N.D.]
9	6.51±0.04 [N.D.]	5.51±0.14 [N.D.]	5.14±0.04 [N.D.]	5.70±0.02 [5]	4.66±0.02 [N.D.]
12	7.56±0.16[15]	6.15±0.05 [N.D.]	6.00±0.04 [N.D.]	6.71±0.08 [25]	5.51±0.12 [N.D.]

[a] Bacterial populations (Log CFU/mL) reported as means of triplicate determinations ± standard deviation.
[b] Not detectable with a detection limit of <2.5 ppm.

Table 2.2 Growth of histamine-producing bacteria (10^5 CFU/mL) and histamine formation (ppm) in culture broth at 25°C.

Time (h)	Bacterial Populations (Log CFU/mL) [Histamine concentration (ppm)]				
	Enterobacter aerogenes	*Enterobacter cloacae*	*Proteus hauseri*	*Morganella morganii*	*Klebsiella pneumoniae*
0	5.43±0.04[a] [N.D.[b]]	4.70±0.11 [N.D.]	5.04±0.05 [N.D.]	5.23±0.01 [N.D.]	4.89±0.00 [N.D.]
3	5.92±0.02 [N.D.]	4.80±0.05 [N.D.]	5.26±0.05 [N.D.]	5.51±0.03 [N.D.]	5.09±0.03 [N.D.]
6	6.99±0.04 [5]	5.16±0.03 [N.D.]	6.06±0.06 [N.D.]	6.44±0.06 [15]	5.75±0.02 [N.D.]
9	8.26±0.05 [202]	6.44±0.06 [N.D.]	7.12±0.02 [N.D.]	7.48±0.12 [285]	6.49±0.07 [N.D.]
12	8.85±0.04 [1890]	6.86±0.10 [N.D.]	8.02±0.03 [26]	8.46±0.02 [1277]	7.60±0.08 [5]

[a] Bacterial populations (Log CFU/mL) reported as means of triplicate determinations ± standard deviation.
[b] Not detectable with a detection limit of <2.5 ppm.

2.4.2 Growth of histamine-producing bacteria and histamine formation in yellowfin tuna

When yellowfin tuna mince was inoculated with *E. aerogenes* or *M. morganii* and stored at various temperatures, detectable levels of histamine were reported in the mince when growth of *E. aerogenes* and *M. morganii* in the mince increased to populations of greater than 10^6 CFU/g (Tables 2.3 and 2.4). Since the formation of histamine in fish is related to the growth of HPB, detectable levels of histamine could be formed quickly in fish contaminated with high levels of HPB at an elevated temperature. This study demonstrated that detectable levels of histamine (15-24 ppm) could be formed in yellowfin tuna inoculated with 10^5 CFU/g of *E. aerogenes* or *M. morganii* and held at 25°C for 9 h (Table 2.3). Although the levels of histamine in the tuna mince were lower than the FDA's advisory level (50 ppm), they quickly increased to 200-419 ppm after 12 h of holding at 25°C. However, only low levels of histamine (3-8 ppm) were detected in the yellowfin tuna inoculated with *E. aerogenes* or *M. morganii* at a lower level of 10^3 CFU/g and held at 25°C for 12 h. These results suggested that delay in icing of fish after catch should be limited to 6 h, particularly in summer time, to prevent multiplication of HPB to a level that is high enough to produce histamine. This study demonstrated that high levels of histamines (>200 ppm) could be formed in fish if it is contaminated with high levels (10^5 CFU/g) of *E. aerogenes* or *M. morganii* and left at 25 °C for more than 9 h (Table 2.3).

The correlation between the populations of HPB and histamine formation in yellowfin tuna was also observed when inoculated tuna mince was held at 15°C. Detectable levels of histamine were found in the mince after the populations of *E.*

aerogenes and *M. morganii* increased to 10^6 CFU/g or higher (Table 2.4). The multiplications of *E. aerogenes* and *M. morganii* in the tuna mince at 15°C were both slower than at 25°C. Low levels of histamine (3-14 ppm) became detectable in the mince inoculated with high level (10^5 CFU/g) of *E. aerogenes* or *M. morganii* after 24 h at 15°C and in those inoculated with 10^3 CFU/g after 36 h (10-18 ppm). However, the levels of histamine in the mince increased rapidly to >500 ppm after additional 12 h. Du and others (2002) reported a high level of histamine (1,000 ppm) was detected in yellowfin tuna after 3 days of storage at 22°C. A similar study reported histamine at a level of 3,000 ppm in skipjack stored at 22°C for 2 days (Silva and others 1998). These results indicate that temperature abuse of fish, even at 15°C, during storage or distribution for one or two days may result in high levels of histamine formation in the fish.

2.4.3 Effects of refrigeration temperature on growth of HPB and histamine formation in yellowfin tuna

Growth of *E. aerogenes* and *M. morganii* in yellowfin tuna was inhibited at 5°C. Populations of both bacteria decreased gradually during the refrigerated storage and no histamine was detected in any of the samples during the 14 days of storage (Table 2.5). It is known that both *E. aerogenes* and *M. morganii* are mesophilic bacteria with *M. morganii* being frequently isolated from fish stored at 25 than 15°C (Kim and others 2001a). Therefore, keeping fish at low temperature (≤5°C) could inhibit growth of both organisms and prevent histamine formation during storage unless fish was contaminated with very high levels (10^6 CFU/g) of HPB.

Table 2.3 Growth of *Enterobacter aerogenes* and *Morganella morganii* and histamine formation in yellowfin tuna inoculated with the bacteria at a level of 10^3 or 10^5 CFU/g and stored at 25°C.

Bacteria	Time (h)	Bacterial Populations (Log CFU/g) [Histamine concentration (ppm)]	
Enterobacter aerogenes	0	3.31±0.11[a] [N.D.[b]]	5.04±0.08[N.D.]
	3	3.49±0.04 [N.D.]	5.31±0.09 [N.D.]
	6	4.31±0.04 [N.D.]	6.29±0.04 [N.D.]
	9	5.58±0.12 [N.D.]	7.40±0.12 [24]
	12	6.96±0.14 [3]	8.58±0.02 [419]
Morganella morganii	0	3.30±0.03 [N.D.]	5.03±0.07[N.D.]
	3	3.44±0.04 [N.D.]	5.30±0.06 [N.D.]
	6	4.03±0.03 [N.D.]	5.84±0.08 [N.D.]
	9	5.04±0.05 [N.D.]	6.79±0.17 [15]
	12	6.20±0.12 [8]	7.79±0.04 [201]

[a] Bacterial populations (Log CFU/g) reported as means of triplicate determinations ± standard deviation.
[b] Not detectable with a detection limit of <2.5 ppm

Table 2.4 Growth of *Enterobacter aerogenes* and *Morganella morganii* and histamine formation in yellowfin tuna inoculated with the bacteria at a level of 10^3 or 10^5 CFU/g and stored at 15°C.

Bacteria	Time (h)	Bacterial Populations (Log CFU/g) [Histamine concentration (ppm)]	
Enterobacter aerogenes	0	3.02±0.04[a] [N.D.[b]]	5.09±0.05 [N.D.]
	12	4.11±0.05 [N.D.]	5.91±0.05 [N.D.]
	24	5.55±0.06 [N.D.]	7.46±0.09 [3]
	36	7.25±0.05 [10]	8.63±0.04 [513]
	48	8.69±0.14 [787]	
Morganella morganii	0	3.19±0.01 [N.D.]	5.27±0.04[N.D.]
	12	3.68±0.06 [N.D.]	5.71±0.25 [N.D.]
	24	4.71±0.03 [N.D.]	6.83±0.14 [14]
	36	6.12±0.15 [18]	8.05±0.07 [718]
	48	7.61±0.11 [927]	

[a] Bacterial populations (Log CFU/g) reported as means of triplicate determinations ± standard deviation.
[b] Not detectable with a detection limit of <2.5 ppm.

Table 2.5 Survival of *Enterobacter aerogenes* and *Morganella morganii* in yellowfin tuna stored at 5°C.

Bacteria	Time (Day)	Bacterial Populations (Log CFU/g)	
Enterobacter aerogenes	0	3.03±0.03 [a]	5.00±0.08
	1	3.05±0.04	5.04±0.06
	3	2.92±0.03	5.01±0.09
	5	2.88±0.01	4.92±0.06
	7	2.61±0.07	4.74±0.11
	10	2.20±0.10	4.65 ±0.07
	14	2.26±0.06	4.69±0.23
Morganella morganii	0	2.98±0.01	4.92±0.06
	1	2.88±0.06	4.91±0.04
	3	2.79±0.13	4.76±0.06
	5	2.77±0.16	4.62±0.11
	7	2.54±0.16	4.53±0.11
	10	2.00±0.04	4.26±0.12
	14	1.75±0.13	3.95±0.13

[a] Bacterial populations (Log CFU/g) reported as means of triplicate determinations ± standard deviation.

While low-temperature (≤5°C) storage appears to be a means to control histamine formation in fish, certain HPB may still be able to grow slowly in fish during refrigerated storage and produce histamine over time. Du and others (2002) reported that the populations of HPB (*Enterobacter agglomerans*, *Enterobacter intermedium*, *Pseudomonas fluorescens*, *Proteus valgaris* and *Serratia liquefaciens*) in yellowfin tuna could all increase to 10^6 CFU/g after 9 days of storage at 4°C and produce histamine (60 ppm) in the fish. Kim and others (2002) studied histamine formation in mackerel, mahi-mahi and albacore tuna inoculated with *M. morganii* (10^6 CFU/g) and stored at 4ºC. The investigators reported a gradual increase of histamine in the fish from <10 ppm after 6 days of storage to 78, 54, and 46 ppm in mackerel, mahi-mahi, and albacore tuna, respectively, after 14 days of storage. Another study also reported that HPB (*Actinobacillus ureae*, *Pseudomonas putida, Aeromonas hydrophila, Vibrio alginolyticus* and *Photobacterium damsela*) isolated from mackerel were capable of producing 100 and 500 ppm of histamine in mackerel after 8 and 14 days of storage, respectively, at 4°C (Kim and others 2001a).

To further investigate the effectiveness of low-temperature (≤5°C) storage on preventing histamine formation in fish that has been subjected to mild temperature-abuse during processing, yellowfin tuna were inoculated with a mixture of *E. aerogenes* and *M. morganii* at a level of approximately 10^4 CFU/g and held at 15°C for 6 h before being stored at 5°C. Results showed that no histamine was detected in the fish after being held at 15°C for 6 h (Table 2.6). Growth of *E. aerogenes* and *M. morganii* in the fish appeared to be retarded at 15°C and their populations remained

Table 2.6 Changes of bacterial populations and histamine formation in yellowfin tuna held at 15°C for 6 h and stored at 5°C.

Test	Time					
	Initial populations	After 6 h at 15°C	Day 1	Day 3	Day 5	Day 7
Total counts	4.26±0.06[a]	4.27±0.05	4.18±0.06	3.97±0.10	3.92±0.06	4.83±0.02
HPB	4.15±0.12	4.17±0.06	4.11±0.01	3.79±0.02	3.84±0.05	3.87±0.14
Histamine	N.D.[b]	N.D.	N.D.	N.D.	N.D.	N.D.

[a] Bacterial populations (Log CFU/g) reported as means of triplicate determinations ± standard deviation.
[b] Not detectable with a detection limit of <2.5 ppm.

almost unchanged after the mild temperature-abuse and were much lower that the levels required to allow the bacteria to produce detectable histamine in fish. Growth of the bacteria in the fish was further inhibited when the fish was kept at refrigerated storage and, therefore, production of histamine was prohibited. These results further confirm that low-temperature storage (≤5°C) is an effective means for preventing histamine formation in yellowfin tuna even after a short exposure to an elevated temperature.

In summary, *E. aerogenes* and *M. morganii* were prolific histamine formers and can produce high levels of histamine in yellowfin tuna at elevated temperatures (15 and 25°C). Histamine should be expected to be formed in fish once the growth of *E. aerogenes* and *M. morganii* in the fish increased to a population of 10^6 CFU/g. Therefore, avoiding delay of icing of fish after catch and keeping fish stored at ≤5°C during storage and distribution could effectively prevent histamine formation.

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CHAPTER 3

EFFECACIES OF ELECTROLYZED WATER TREATMENTS IN REDUCING HISTAMINE-PRODUCING BACTERIA ON FOOD CONTACT SURFACES AND FISH SKIN

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3.1 ABSTRACT

Scombroid poisoning associated with fish consumption is caused by histamine produced through bacterial enzymatic decarboxylation of histidine. This study investigated efficacy of electrolyzed oxidizing (EO) water and in ice form (EO ice) in reducing histamine-producing bacteria (*Enterobacter aerogenes*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Morganella morganii*, and *Proteus hauseri*) on food contact surface (glazed ceramic tile and stainless steel) and fish skin (Atlantic salmon and yellowfin tuna). Treatments of ceramic tile and stainless steel sheet with EO water (50 ppm chlorine) for 5 min completely inactivated histamine-producing bacteria (>1.7 to >5.4 log CFU/cm^2) inoculated to surface. Soaking fish skin inoculated with histamine-producing bacteria in EO water (50 ppm chlorine) for 120 min resulted in small reductions (0.57-0.94 log CFU/cm^2) of the bacteria. Treatments of inoculated skin in EO water containing 100 ppm of chlorine for 120 min increased the reductions of bacterial populations to 1.32-2.17 log CFU/cm^2. Holding fish skin inoculated with *Enterobacter aerogenes* or *Morganella morganii* in regular ice for 24 h resulted in reductions of *Enterobacter aerogenes* (0.56-0.71 log CFU/cm^2) and *Morganella morganii* (1.17-1.38 log CFU/cm^2) on the skin. Storing inoculated fish skin in EO ice (50 ppm chlorine) for 24 h increased the reductions of *E. aerogenes* to 1.27-2.06 log CFU/cm^2, but did not increase reductions of *M. morganii* when compared with regular ice treatment. However, keeping fish skin in EO ice containing 100 ppm of chlorine for 24 h resulted in much greater reductions of *E. aerogenes* (1.62 log CFU/cm^2 on salmon skin and 2.43 log CFU/cm^2 on tuna skin) and *M morganii* (2.02 log CFU/cm^2 on salmon skin and 3.50 log CFU/cm^2 on tuna skin). Holding fish in EO ice (100 ppm

chlorine) could be used as a post-harvest treatment to reduce histamine-producing bacteria contamination on fish skin and decrease probability of histamine formation in fish during storage.

3.2 INTRODUCTION

Scombroid poisoning is a common illness resulted from consumption of fish containing histamine, a toxic chemical produced by enzymatic decarboxylation of histidine. Several bacterial species, such as *Morganella morganii*, *Proteus vulgaris*, *Klebsiella pneumoniae*, and *Enterobacter aerogenes*, are known capable of producing histidine decarboxylase and can convert free histidine in fish muscle to histamine in improperly handled fish (Rawles and others 1996). These bacteria are known as major histamine producers associated with fish and have been isolated from skin and gill of fresh fish (Kim and others 2001a; Kim and others 2001b). Scombroid fish including tuna, mackerel, and bonito that contain high levels of free histidine in muscles have been frequently implicated in scombroid poisoning. However, some nonscombroid fish, such as mahi-mahi, bluefish, herring, sardine, and anchovy, that were not properly stored have also been linked to scombroid outbreaks (Taylor 1986; Rawles and others 1996).

Histamine is produced in fish mainly by the activity of bacterial histidine decarboxylase when fish is exposed to temperatures of 7.2°C (45°F) or higher (FDA 2001). Therefore, keeping fish at <4.4°C (<40°F) at all times is the most effective means to prevent scombroid poisoning. Study has reported that histamine could be formed in tuna when stored at 8°C (López-Sabater and others 1996b). Once histamine

is produced in fish, it will not be destroyed by cooking, freezing or smoking process (Bremer and others 1998). Since the formation of histamine in fish is mainly related to the growth of bacteria that produce histidine decarboxylase, a fish could contain histamine before it is spoiled by growth of other bacteria. Therefore, sensory examination (smell) commonly used to evaluate freshness of fish can not be used to predict presence of histamine in fish. In addition, distribution of histamine in fish is usually not consistent within the muscle, and the differences across sections can reach as high as ten-fold (Baranowski and others 1990).

To reduce incidence of scombroid poisoning associated with fish consumption, the United States Food and Drug Administration suggests that fish should be chilled immediately upon death and established an advisory level that limits histamine at a level of <50 ppm in fish for consumption (FDA 2001). However, exposure of fish to elevated temperatures (>8°C) during retail storage can not be totally avoided. Exposure of fish to temperatures above 8°C could allow the histamine-producing bacteria to grow and produce histidine decarboxylase in fish. Therefore, development of post-harvest treatments for reducing histamine-producing bacteria in fish would be an important intervention strategy to prevent histamine formation in fish and control scombroid poisoning.

Electrolyzed oxidizing (EO), produced through electrolysis of a dilute salt solution (0.05-0.2% NaCl), was recently introduced as a new sanitizer with strong antibacterial activities against many foodborne pathogens including *Escherichia coli* 0157:H7, *Salmonella enteritidis, Listeria monocytogenes* and *Bacillus cereus* (Venkitanarayanan and others 1999a; Kim and others 2000b). Application of EO

water as a disinfectant for reducing microbial contaminations has been reported for fresh fruits and vegetables (Izumi 1999; Koseki and others 2001, 2004b), poultry carcasses (Fabrizio and others 2002; Park and others 2002a), cutting boards (Venkitanarayanan and others 1999b; Chiu and others 2006), and food or non-food contact surfaces (Park and others 2002b; Liu and others 2006; Liu and Su 2006).

Mahmoud and others (2004) reported that soaking whole carp in EO water (40.8 ppm chlorine) at room temperature (25°C) for 15 min was capable of reducing total bacterial populations on the skin by 2.8 log CFU/cm^2. Huang and others (2006a) studied EO water for reducing microbial population on tilapia and reported that EO water (120 ppm chlorine) treatments reduced *Vibrio parahaemolyticus* on tilapia by 1.5 and 2.6 log CFU/cm^2 after 5 and 10 min, respectively. In addition, Kim and others (2006) studied effects of regular tap water ice and ice prepared from EO water (47 ppm chlorine) on preserving freshness on Pacific saury (mackerel pike) and reported that EO ice storage significantly retarded growth of aerobic and psychrotrophic bacteria on the fish. Sensory and microbiological analyses showed that the saury stored in EO ice had a shelf life that was 4 to 5 days longer than fish stored in regular ice. These results indicate that EO water might be used as a disinfecting agent for reducing histamine-producing bacteria (HPB) in fish and in seafood processing environments. Our preliminary study confirmed that EO water (pH: 2.74, chlorine: 30 ppm, ORP: 1211 mV) was effective in inactivating HPB (*Enterobacter aerogenes, Enterobacter cloacae, Klebsiella pneumoniae, Morganella morganii* and *Proteus hauseri*). Populations of these HPB were all reduced by greater than 5.0 log CFU/ml after 30 s in the EO water solution (unpublished data). This study was conducted to

determine the effectiveness of EO water in liquid or ice form on inactivating HPB on food-contact surfaces and fish skin.

3.3 MATERIALS AND METHODS

3.3.1 Bacterial cultures

Five histamine-producing bacteria (HPB), including *Enterobacter aerogenes* (ATCC 13048), *Enterobacter cloacae* (ATCC 23355), *Klebsiella pneumoniae* (ATCC 13883), *Morganella morganii* (ATCC 25830), and *Proteus hauseri* (ATCC 13315), obtained from the American Type Culture Collection (ATCC, Manassas, VA) were used in this study. Each strain was individually grown in 10 ml of tryptic soy broth (TSB; Difco, BD, Spark, MD) at 37°C for 18-24 h. Each culture was centrifuged in a sterile centrifuge tube at 3000×g (Sorvall RC-5B, Kendro Laboratory products, Newtown, CT) at 5°C for 15 min. Pellet cells were re-suspended in phosphate-buffered saline (PBS) to obtain a bacterial culture suspension of approximately $10^{8\text{-}9}$ CFU/ml.

3.3.2 Material preparation

Two types of materials, glazed ceramic tiles (FC30, Wanke Cascade, Portland, OR) and stainless steel sheet (SS304, K-Manufacturing, Astoria, OR), commonly used in food processing environments as either food or non-food contact surfaces were used in this study. Both materials were cut into small chips (5×5 cm^2), washed with cleaning detergent, and autoclaved. Each chip was inoculated with one strain of HPB by spreading 50 µl of the culture suspension over the chip with a sterile colony

spreader. Inoculated chips (10^{5-6} CFU/cm^2) were held inside a biological safety cabinet (NuAire, Plymouth, MN) for 45 min at room temperature to allow HPB to attach to surfaces.

3.3.3 Fish skin preparation

Skins of Atlantic salmon (*Salmo salar*) and yellowfin tuna (*Thunnus albacares*) were used in this study. Fresh Atlantic salmon fillet was purchased from a local supermarket and frozen yellowfin tuna fillet was obtained from a local seafood company. The skin on salmon or tuna fillet was removed using a sterile knife and rinsed briefly under tap water. The skin was then cut into small pieces (3x3 cm^2) and allowed to dry in the biological safety cabinet at room temperature for 10 min. Each fish skin was inoculated with one strain of HPB by spreading 100 µl of a HPB culture suspension over the skin with a sterile colony spreader to produce a contamination level of 10^{6-7} CFU/cm^2. Inoculated skins were allowed to dry inside the biological safety cabinet at room temperature for 15 min.

3.3.4 Electrolyzed oxidizing (EO) water and ice preparation

EO water containing 50 or 100 ppm chlorine was prepared by electrolysis of 1% sodium chloride (NaCl) solution using an electrolyzed water generator (model V-500, Electric Aquagenics Unlimited, Inc., Lindon, UT) with a setting of 2.2 or 3.2 Amperes (A). EO water was produced on the day of experiments, kept in a sealed container, and used within 2 h of production. Chlorine contents, pH and oxidation-reduction potential (ORP) of EO water were determined with a commercial chlorine

detection kit (HACH Company, Loveland, CO), a pH meter (model 420A, Orion Research, Inc., Boston, MA) and an ORP meter (CheckmateII Systems with Redox Sensor, Corning, Inc., Corning, NY), respectively.

EO ice containing 50 or 100 ppm of chlorine was prepared by freezing EO water containing 120 or 250 ppm of chlorine in plastic ice trays immediately after production. The ice trays were sealed with adhesive plastic films and held inside a -18°C freezer overnight. The EO ice was crushed using a rubber hammer and used within 30 min. The chlorine content in EO ice was measured with ice melt after holding ice in a sealed container in a water bath (48°C) for 20 min.

3.3.5 EO water treatment of ceramic tile and stainless steel

Each ceramic tile or stainless steel chip inoculated with HPB was soaked in a plastic beaker containing 200 ml of EO water (50 ppm chlorine) for 5 min at room temperature. The EO water-treated chips were transferred to individual beakers containing 200 ml of sterile Butterfield's phosphate buffer and held for 1 min to neutralize the acidity before conducting microbiological analysis. Inoculated chips soaked in distilled water followed by in sterile Butterfield's phosphate buffer were used as controls. Populations of HPB on each chip were analyzed before and after treatments. All treatments were conducted in triplicate.

3.3.6 EO water treatment of fish skin

Efficacy of EO water treatment on reducing HPB on fish skin was conducted with Atlantic salmon. Each fish skin inoculated with one strain of HPB (10^{6-7}

CFU/cm^2) was soaked in 200 ml EO water in a sealed plastic jar (500 ml) and held at room temperature for 120 min with a change of EO water after 60 min. Reductions of HPB on fish skin treated with EO water were compared with those treated with distilled water. Fish skin not inoculated with HPB was analyzed for presence of HPB before inoculation. To further investigate effects of chlorine contents in EO water on reducing HPB on fish skin, HPB that survived well on fish skin were inoculated to salmon skin and treated with EO water containing 50 or 100 ppm at room temperature for 120 min. Populations of HPB on the skin were analyzed after 60 and 120 min of treatments. All treatments were conducted in triplicate.

3.3.7 EO ice treatment of fish skin

Efficacy of EO ice in reducing HPB on fish skin was determined by placing salmon and tuna skin inoculated with strains that survived well on fish skin. HPB-inoculated fish skin (10^{6-7} CFU/cm^2) was placed scale side up on a bed (5.5 cm from the bottom) of EO ice (50 or 100 ppm chlorine) in a 500-ml plastic jar and covered with a layer (4 cm) of EO ice. All jars were sealed and stored in polystyrene coolers (L 43 × W 30 × H 30 cm) covered with regular ice to prevent fast melting of EO ice. Populations of HPB on fish skin were determined after 6, 12 and 24 h of treatments. Inoculated skins stored in regular ice treatment were used as controls. All tests were conducted in triplicate.

3.3.8 Microbiological analysis

Populations of HPB on surface of ceramic tile and stainless steel sheet were determined by swabbing entire surface of a chip (5×5 cm^2) with 3-4 sterile swabs until it became totally dry. All swabs were placed in a sterile centrifuge tube containing 25 ml of PBS. The tube was vigorously vortexed for 1 min to allow bacterial cells to detach from swabs. For determination of HPB on fish skin, each piece of fish skin was placed in a bottle containing 90 ml of sterile PBS. The bottle was shaken for 30 s to prepare 1:10 sample suspension. Additional serial dilutions of samples were prepared with sterile Butterfield's buffer. Presence of HPB in each sample suspension was determined by the pour-plate method using a differential plating medium designed for quantitative determination of histamine-producing bacteria based on histidine decarboxylation activity (Niven and others 1981). The medium plate was incubated at 35°C for 48 h. Colonies formed on the plate with purple halo were considered as HPB. Results were reported as means of triplicate determinations.

3.3.9 Statistical analysis

Results of microbiological tests were transformed into log values and analyzed with two-sample *t* test and ANOVA using S-PLUS (Insightful Corp., Settle, WA). Significant differences between treatments were established at a level of p=0.05.

3.4 RESULTS AND DISCUSSION

3.4.1 Survival of HPB on food-contact surface

All the HPB bacteria, except *Proteus hauseri*, were able to survive on stainless steel (SS) to some degrees (Table 3.1). Among them, *E. aerogenes* and *M. morganii* appeared to be more resistant to dry condition than others. Populations of *E. aerogenes* and *M. morganii* decreased by about 1 log CFU/cm^2 on SS after 45 min at room temperature while greater reductions of 1.9 and 4.0 log CFU/cm^2 were observed for *E. cloacae* and *K. pneumoniae*, respectively. No vegetative cells of *P. hauseri* were recovered from the SS after 45 min at room temperature.

When the HPB were inoculated to ceramic tile (CT), only *E. aerogenes*, *E. cloacae*, and *K. pneumoniae* were recovered after 45 min at room temperature (Table 3.1). No vegetative cells of *M. morganii* or *P. hauseri* were recovered from the CT and the reductions of *E. aerogenes* (2.1 log CFU/cm^2), *E. cloacae* (3.1 log CFU/cm^2), and *K. pneumoniae* (4.8 log CFU/cm^2) on CT were all greater than those observed for SS. These results indicate that certain HPB, such as *E. aerogenes*, *E. cloacae*, and *M. morganii*, could survive well on food contact surfaces even after 45 min at room temperature. This is similar to a previous report showing that populations of *E. aerogenes* (6.10 log CFU/cm^2) on glazed ceramic tile and stainless steel remained almost unchanged after 30 min of drying at 30°C (Park and others 2002b). Therefore, food contact surfaces should be sanitized immediately after handling of raw seafood to avoid transfer of HPB to subsequently handled products.

3.4.2 Survival of HPB on fish skin

In addition to their abilities to survive on CT and SS, *E. aerogenes* and *M. morganii* also survived well when inoculated to salmon skin. Populations of these two HPB on inoculated fish skin remained almost unchanged after 15 min at room temperature (Table 3.2). However, *E. cloacae*, *K. pneumoniae* and *P. hauseri* could not survive well on the fish skin. Populations of *E. cloacae* and *K. pneumoniae* on the skin decreased rapidly by 3.5 and 4.9 log CFU/cm^2, respectively, after drying inoculated fish skin at room temperature for 15 min. *Proteus hauseri* was once again confirmed very sensitive to dry condition. No vegetative cells of *P. hauseri* were recovered from the skin after 15 min. These results suggested that both *E. aerogenes* and *M. morganii* would be the major species of concern for causing scombroid poisoning because of their abilities to survive on food contact surfaces and fish skin. Many studies have reported isolating *E. aerogenes* and *M. morganii* from a variety of fish with *M. morganii* capable of producing large amounts of histamine (>1,000 ppm) in fish when exposed to elevated temperatures (Middlebrooks and others 1988; López-Sabater and others 1996b; Kim and others 2001a; Kim and others 2001b, 2002).

3.4.3 Efficacy of EO water treatment in inactivating HPB on food-contact surface

Soaking HPB-inoculated CT and SS in DI water for 5 min at room temperature resulted in some reductions of *E. aerogenes*, *E. cloacae*, and *K. pneumoniae* on CT (0.14–0.93 log CFU/cm^2) as well as *E. aerogenes*, *E. cloacae*, *K. pneumoniae*, and *M. morganii* on SS (0.87-1.37 CFU/cm^2) (Table 3.1). These results indicate that rinsing food contact surfaces with water would not be an effective means for eliminating HPB

from contaminated surfaces. The reductions of HBP on food contact surfaces after soaking in distilled water observed in this study were probably due to a washing off effect of HPB cells that were loosely attached to surfaces. However, soaking HPB-inoculated CT and SS in EO water (pH 2.7, chlorine: 50 ppm, ORP: 1,160 mV) for 5 min completely eliminated HPB on both materials (Table 3.1). The reductions of HPB ranged from >0.92 log CFU/cm^2 for *K. pneumoniae* on CT to >5.41 log CFU/cm^2 for *E. aerogenes* on SS depending on the populations survived on the chips after inoculation. These results demonstrated that EO water could be used as a surface sanitizer to eliminate HPB contamination.

3.4.4 Effects of EO water treatment on reducing HPB on fish skin

Soaking fish skin inoculated with HPB in distilled water for 120 min at room temperature resulted in very little reductions (0.10-0.37 log CFU/cm^2) of the bacteria (Table 3.2). Treatments of fish skin with EO water (50 ppm chlorine) for 120 min resulted in slightly greater reductions (0.57-0.94 log CFU/cm^2) of the bacteria when compared with distilled water treatments. However, the reductions were limited to <1.0 log CFU/cm^2. These results indicate that treatment of fish with EO water containing 50 ppm of chlorine would not be effective on reducing HPB on the skin.

The antibacterial activity of EO water is known to relate to its low pH, available chlorine (Cl_2, HOCl, OCl^-), and high ORP (Kim and others 2000a; Oomori and others 2000). Therefore, the antibacterial activity of EO water is expected to decrease when organic compounds are present because of interaction between organic substances and available chlorine. Liu and others (2006) reported that a 5-min EO

Table 3.1 Efficacy of EO water (50 ppm chlorine) treatment in reducing histamine-producing bacteria on ceramic tile and stainless steel.

Treatments	*Enterobacter aerogenes*	*Enterobacter cloacae*	*Klebsiella pneumoniae*	*Morganella morganii*	*Proteus hauseri*
Glazed ceramic tile					
Initial inoculation	6.41[a]	5.12	5.70	4.58	4.72
After drying	4.29 ± 0.11[b]A	2.03 ± 0.12 A	0.92 ± 0.48	ND	ND
Distilled water	3.36 ± 0.06 B (0.93)[c]	1.89 ± 0.09 A (0.14)	ND	ND	ND
EO water	ND[d] (>4.29)	ND (>2.03)	ND	ND	ND
Stainless steel					
Initial inoculation	6.41	5.12	5.70	4.58	4.72
After drying	5.41 ± 0.03 A	3.19 ± 0.05 A	1.74 ± 0.15 A	3.47 ± 0.04 A	ND
Distilled water	4.54 ± 0.03 B (0.87)	2.23 ± 0.04 A (0.96)	0.37 ± 0.10 B (1.37)	2.39 ± 0.05 B (1.08)	ND
EO water	ND (>4.29)	ND (>3.19)	ND (>1.74)	ND (>3.47)	ND

[a] Bacterial populations (log CFU/cm^2).
[b] Data are means of three determinations ± standard deviation. Means with the same letter in the same column are not significantly different ($P > 0{\cdot}05$).
[c] Reductions of bacterial populations (log CFU/cm^2) after treatments.
[d] Not detectable by plate count method with a detection limit of <1 CFU/cm^2.

Table3.2 Effects of EO water (50 ppm chlorine) treatments on reducing histamine-producing bacteria on fish skin.

Bacteria	Bacterial populations (log CFU/cm^2) after treatments			
	Initial inoculation	After drying	Distilled water	EO water
Enterobacter aerogenes	6.75	6.52 ± 0.02[a] A	6.31 ± 0.08 B (0.21)[b]	5.58 ± 0.07 C (0.94)
Enterobacter cloacae	5.16	1.70 ± 0.09 A	1.60 ± 0.11 A (0.10)	ND (>0.70)
Klebsiella pneumoniae	6.29	1.37 ± 0.30	ND[c]	ND
Morganella morganii	5.88	5.93 ± 0.18 A	5.74 ± 0.11 AB (0.19)	5.36 ± 0.28 B (0.57)
Proteus hauseri	5.58	ND	ND	ND

[a] Data are means of three determinations ± standard deviation. Means with the same letter in the same row are not significantly different ($P > 0{\cdot}05$).
[b] Reductions of bacterial populations (log CFU/cm^2) after treatments.
[c] Not detectable by plate count method with a detection limit of <10 cfu/cm^2.

water (50 ppm chlorine) treatment was much more effective in reducing *Listeria monocytogenes* contamination on clean food-contact surfaces (3.73-4.24 log CFU/cm^2) than on crabmeat-containing surfaces (2.3 log CFU/cm^2). Since fish skin contains large numbers of organic materials such as glycoproteins from mucus (Ebran and others 2000), EO water treatment will be less effective on inactivating bacteria on the skin unless the chlorine concentration in the water is increased. Su and Morrissey (2003) reported that dipping whole salmon in acidified sodium chlorine solution containing 50 ppm of chlorine for 1 min could only reduce total bacterial populations on skin by 0.43 log CFU/cm^2.

To evaluate the effect of EO water with higher chlorine content on inactivating HPB on fish skin, salmon skin was inoculated with *E. aerogenes* or *M. morganii* that survived well on food-contact surface and fish skin and treated with EO water containing 100 ppm of chlorine (pH, 2.5, ORP: 1173 mV). Results showed that treatments of EO water containing 100 ppm of chlorine for up to 120 min did not result in increased reductions of *E. aerogenes* on fish skin when compared with EO water treatments containing 50 ppm of chlorine (Table 3.3). However, the treatment did increase the reduction of *M. morganii* on fish skin by 0.93 log CFU/cm^2 after 120 min when compared with the treatment containing 50 ppm of chlorine. These results indicate that a treatment of EO water (50-100 ppm chlorine) for 120 min could decrease HPB contamination on fish skin by 1-2 log units and reduce the probability of histamine production by HPB in fish during storage.

Table 3.3 Effect of EO water treatments on *Enterobacter aerogenes* and *Morganella morganii* on fish skin.

Treatment	Time (min)	Bacterial populations (log CFU/cm^2)	
		Enterobacter aerogenes	*Morganella morganii*
Initial populations		7.00 ± 0.03[a] A	6.86 ± 0.03 A
EO water (50 ppm chlorine)	60	6.07 ± 0.08 B (0.93)[b]	5.88 ± 0.09 B (0.98)
	120	5.72 ± 0.06 C (1.28)	5.62 ± 0.12 B (1.24)
EO water (100 ppm chlorine)	60	5.83 ± 0.12 BC (1.17)	5.68 ± 0.21 B (1.18)
	120	5.68 ± 0.16 C (1.32)	4.69 ± 0.17 C (2.17)

[a] Data are means of three determinations ± standard deviation. Means with the same letter in the same column are not significantly different ($P > 0{\cdot}05$).
[b] Reductions of bacterial populations (log CFU/cm^2) after treatments.

3.4.5 Effect of EO ice treatment on reducing HPB on fish skin

Holding salmon skin inoculated with *E. aerogenes* and *M. morganii* in regular ice for 24 h resulted in small reductions of *E. aerogenes* (0.56 log CFU/cm^2) and *M. morganii* (1.17 log CFU/cm^2) (Table 3.4). Storing inoculated salmon skin in EO ice containing 50 ppm of chlorine for 24 h resulted in a similar reduction of *M. morganii* (1.15 log CFU/cm^2) but increased the reduction of *E. aerogenes* to 1.27 log CFU/cm^2 on the skin. These results showed that keeping fish in regular ice inhibited growth of HPB and chlorine in ice provided additional power for inactivating bacteria on fish skin. This is evidenced by greater reductions of *E. aerogenes* (1.62 log CFU/cm^2) and *M. morganii* (2.02 log CFU/cm^2) on the skin after 24 h of treatment with EO ice containing 100 ppm of chlorine.

To validate the effectiveness of EO ice on reducing HBP on fish skin, the study was repeated with yellowfin tuna skin because yellowfin tuna is more frequently involved in scombroid poisoning than salmon. Holding tuna skin inoculated with *E. aerogenes* and *M. morganii* in regular ice for 24 h resulted in similar reductions of *E. aerogenes* (0.71 log CFU/cm^2) and *M. morganii* (1.38 CFU/cm^2) when compared with the salmon skin study (Table 3.5). Treatments of EO ice containing 50 ppm of chlorine for 24 h increased the reductions of *E. aerogenes* and *M. morganii* to 2.06 and 1.63 log CFU/cm^2, respectively. The reductions of *E. aerogenes* and *M. morganii* on tuna skin increased to 2.43 and 3.50 log CFU/cm^2 after keeping the skin in EO ice containing 100 ppm of chlorine after 24 h. These results demonstrated that storing fish in EO ice containing 100 ppm of chlorine could be used as a post-harvest treatment for reducing HPB on yellowfin tuna.

Table 3.4 Reductions of *Enterobacter aerogenes* and *Morganella morganii* on salmon skin stored in ice.

Bacteria	Time (h)	Bacterial populations (log CFU/cm^2) on fish skin		
		Regular ice	EO ice (50 ppm chlorine)	EO ice (100 ppm chlorine)
Enterobacter aerogenes	0	7.06 ± 0.03[a] A	7.18 ± 0.02 A	7.25 ± 0.09 A
	6	6.79 ± 0.02 B (0.37)[b]	6.59 ± 0.15 B (0.59)	6.34 ± 0.35 B (0.91)
	12	6.76 ± 0.18 B (0.40)	5.82 ± 0.36 C (1.36)	6.30 ± 0.35 B (0.95)
	24	6.60 ± 0.07 B (0.56)	5.91 ± 0.25 C (1.27)	5.63 ± 0.12 C (1.62)
Morganella morganii	0	7.00 ± 0.11 A	7.15 ± 0.02 A	7.15 ± 0.02 A
	6	6.54 ± 0.09 B (0.46)	6.24 ± 0.28 B (0.91)	5.75 ± 0.33 B (1.40)
	12	6.34 ± 0.28 B (0.66)	5.88 ± 0.30 B (1.27)	5.84 ± 0.33 B (1.31)
	24	5.83 ± 0.17 B (1.17)	6.00 ± 0.28 B (1.15)	5.13 ± 0.12 C (2.02)

[a] Data are means of three determinations ± standard deviation. Means with the same letter in the same column are not significantly different ($P > 0{\cdot}05$).
[b] Reductions of bacterial populations (log CFU/cm^2) after treatments.

Table 3.5 Reductions of *Enterobacter aerogenes* and *Morganella morganii* on yellowfin tuna skin stored in ice.

Bacteria	Time (h)	Bacterial populations (log CFU/cm^2) on fish skin		
		Regular ice	EO ice (50 ppm chlorine)	EO ice (100 ppm chlorine)
Enterobacter aerogenes	0	6.08 ± 0.18[a] A	6.08 ± 0.18 A	6.20 ± 0.06 A
	6	5.40 ± 0.08 B (0.68)[b]	4.54 ± 0.38 B (1.54)	4.17 ± 0.18 B (2.03)
	12	5.46 ± 0.18 B (0.62)	3.86 ± 0.11 B (2.22)	3.64 ± 0.13 C (2.56)
	24	5.37 ± 0.25 B (0.71)	4.02 ± 0.14 B (2.06)	3.77 ± 0.16 C (2.43)
Morganella morganii	0	6.32 ± 0.02 A	6.32 ± 0.02 A	6.16 ± 0.03 A
	6	5.37 ± 0.14 B (0.95)	5.00 ± 0.09 B (1.32)	3.99 ± 0.42 B (2.17)
	12	5.18 ± 0.19 B (1.14)	4.72 ± 0.19 B (1.60)	3.93 ± 0.47 B (2.23)
	24	4.94 ± 0.09 B (1.38)	4.69 ± 0.40 B (1.63)	2.66 ± 0.27 C (3.50)

[a] Data are means of three determinations ± standard deviation. Means with the same letter in the same column are not significantly different ($P > 0{\cdot}05$).
[b] Reductions of bacterial populations (log CFU/cm^2) after treatments.

Holding fish in ice immediately after catch is a common practice to prevent rapid growth of bacteria on fish. Although keeping fish in ice retards growth bacteria on fish, the practice generally does not inactivate bacteria associated with fish skin. Once fish is removed from ice and exposed to temperature-abused environments before consumption, the bacteria including HPB will be able to multiply and cause spoilage or produce histamine if the fish is contaminated with high levels of HPB. Kim and others (2002) studied histamine formation in mackerel, mahi-mahi and albacore tuna inoculated with *M. morganii* (10^6 CFU/g) and stored at 4ºC. The investigators reported a gradual increase of histamine in the fish from <10 ppm after 6 days of storage to 78 (mackerel), 54 (mahi-mahi), and 46 ppm (albacore tuna) after 14 days of storage.

While fish flesh is generally free of bacteria before fish dies, contamination with HPB can occur through contact with skin or processing surface during the filleting. Therefore, reducing HPB on fish skin after catch will reduce the possibility of cross-contamination when fish fillet is prepared. This study demonstrated EO ice (100 ppm of chlorine) could be used to store fish and reduce HPB on skin. Even a short 6-h of holding fish in the EO ice was capable of reducing HPB on the fish by 2-log units.

In conclusion, EO water containing 50 ppm of chlorine could be used as a sanitizer to reduce HPB contamination on food-contact surfaces and fish skin. Soaking fish in EO water (50-100 ppm chlorine) for 120 min could reduce HPB contamination on skin by 1-2 log CFU/cm^2. Storing fish in EO ice containing 100 ppm of chlorine could reduce HPB on yellowfin tuna by 2 log CFU/cm^2 in 6 h.

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CHAPTER 4

CONCLUSION AND FUTURE STUDY

Scombroid poisoning is a common foodborne illness associated with consumption of finfish containing a high level of histamine, a toxic chemical that can be formed through decarboxylation of histidine in fish muscle by histidine decarboxylase. Many bacteria, including *Enterobacter aerogenes*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Morganella morganii* and *Proteus hauseri*, are known to be capable of producing this enzyme. Among them, *M. morganii* and *E. aerogenes* are frequently isolated from Scombroid fish, particularly tunas, mackerel and bonito, and play a major role in histamine formation in fish during storage.

The production of histamine in fish is related to the growth of histamine-producing bacteria (HPB). This study demonstrated that histamine could be produced by *E. aerogenes* or *M. morganii* in fish held at 15 and 25°C when the bacterial populations increased to 10^6 CFU/g. However, growth of HBP in fish can be controlled by storing fish at refrigeration temperatures. This study showed that growth of *E. aerogenes* and *M. morganii* in fish were inhibited at 5°C. No histamine was detected in yellowfin tuna inoculated with *E. aerogenes* or *M. morganii* and stored at 5°C for 14 days. Therefore, keeping fish stored at low temperatures (<5°C) is the best strategy for preventing histamine formation by bacteria in fish.

Histamine-producing bacteria are commonly found on fish skin or in gill and intestines of fish. They can be spread to fish flesh and food contact surfaces during

processing and survive on the surfaces even after 45 min at room temperature. Therefore, reducing histamine-producing bacteria on fish skin immediately after harvest and eliminating contamination of food contact surfaces is the best strategy to prevent histamine formation in fish during storage. This study demonstrates that rinsing food contact surfaces, such as glazed ceramic tile and stainless steel, with electrolyzed oxidizing (EO) water containing 50 ppm is an effective means to eliminate histamine-producing bacteria and prevent cross-contamination of fish during handling and processing.

Soaking fish in EO water after catch can also be used to reduce HPB contamination on the skin. This study showed that populations of *E. aerogenes* and *M. morganii* on fish skin could be reduced by 1.32-2.17 log CFU/cm^2 after being treated with EO water containing 100 ppm of chlorine for 120 min. The reductions of *E. aerogenes* and *M. morganii* on fish skin could be enhanced by storing fish in EO ice (ice made from EO water). Holding fish skin in EO ice containing 100 ppm of chlorine for 24 h could reduce *E. aerogenes* by 1.62 and 2.43 log CFU/cm^2 on skin of salmon and yellowfin tuna, respectively. The EO ice treatment also resulted in 2.02 and 3.50 log CFU/cm^2 reductions of *M morganii* on salmon and tuna skin, respectively. These results demonstrated that EO water either in liquid or ice form could be used as a post-harvest treatment to reduce HPB contamination on fish skin and decrease probability of histamine formation in fish during storage.

In summary, fish should be held in refrigerated seawater (≤ 5°C) or ice within 6 h of catch and maintained at the low temperature until processing to prevent multiplication of HPB. Food contact surfaces should be frequently sanitized to

eliminate HPB contamination to prevent cross-contamination of fish fillet during fish processing. Keeping fish in EO water or EO ice after harvest can be used to control histamine formation in fish and reduce incidence of scombroid poisoning associated with seafood consumption.

Fresh fish may contain a low amount of histamine-producing bacteria (HPB) and are generally free of histamine. Temperature abuse during storage allows multiplication of HPB and production of histamine in fish. This study demonstrates that holding fresh fish in electrolyzed oxidizing (EO) water or EO ice can reduce HPB contamination on fish skin, which provides an extra barrier to prevent formation of histamine in fish. Additional study may be conducted to verify the efficacy of EO water or ice treatment in reducing HPB on whole fish.

While the EO water or EO ice treatment could reduce HPB on fish skin, the treatment was less effective in reducing HPB on contaminated fish fillet because of reaction of chlorine with organic substances. Future study may be conducted to determine effectiveness of other post-harvest processing procedures, such as high pressure process, on reducing HPB on fish fillet.

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