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Short communication

Analysis of the growth of histamine-producing bacteria and histamine accumulation in fish during storage at low temperatures

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ABSTRACT

We have previously isolated psychrophilic histamine (Hm)-producing bacteria from fresh fish at low temperatures. Among these were strains that produce high levels of Hm even under low temperatures. In the present study, we inoculated 2 strains of psychrophilic Hm-producing bacteria into swordfish (10^4 cfu/g), and examined their behavior during storage at low temperatures (4 °C, 10 °C, and 15 °C). The average counts of normal viable bacteria and Hm-producing bacteria, and cumulative Hm levels in the fish were measured over time and analyzed. We detected Hm levels of 300 mg/kg or more in 5 days, and 800 mg/kg or more in 7 days, during storage at 4 °C. At 10 °C, we detected Hm levels of more than 150 mg/kg in 3 days, while at 15 °C, we detected Hm production of ≥200 mg/kg after 36 h. These data indicate that Hm accumulates in fresh fish during storage at low temperatures when psychrophilic Hm-producing bacteria are present. Further, we observe that inadequate storage temperatures cause the accumulation of Hm within a short time.

1. Introduction

Histamine (Hm) food poisoning (HFP) is characterized by allergic symptoms such as urticaria, cutaneous flushing, headache, and nausea (Taylor & Eitenmiller, 1986). Hm, the agent of this type of food poisoning, is produced by decarboxylation of free histidine present in fish meat due to the action of microbes (Hm-producing bacteria) that possess histidine decarboxylase (HDC) (Lerke & Bell, 1976).

HFP develops mainly in fresh fish that have high amounts of free histidine, such as bonito, tuna, sardine, saury, and mackerel, and in processed fish products (Taylor & Eitenmiller, 1986). Once Hm accumulates in food, it is difficult to prevent poisoning as Hm is very stable against heating and freezing. It is, therefore, important to prevent Hm accumulation. At present, Hm food poisoning does not occur often in Japan, due to thorough hypothermic storage and hygienic control (Toda, Yamamoto, Unayama, & Moriyama, 2009). In addition, dozens of such cases occur on a yearly basis in the US (Centers for Disease Control and Prevention, 2006–2008), and it is considered an important issue throughout the world.

Bacterial strains, including enteric bacteria such as *Morganella morganii* (Kawabata, Ishizaka, Miura, & Sasaki, 1956), *Enterobacter aerogenes*, and *Raoultella planticola* (Enjalbert, Richard, Attisso, & Crémieux, 1979), and marine bacteria such as *Photobacterium damselae* subsp. *damselae* (Kimura, Hokimoto, Takahashi, & Fujii, 2000), and *Photobacterium phosphoreum*(Kanki, Yoda, Ishibashi, & Tsukamoto, 2004) are reported as the main causes of HFP. Since many of these Hm-producing bacteria are mesophilic, strict temperature control is considered important in the prevention of HFP. However, even though the importance of temperature control is widely recognized, there are still sporadic occurrences of Hm poisoning. Inadequate temperatures during preservation and processing are considered the main cause of this; however, the presence of psychrophilic Hm-producing bacteria at low temperatures also causes the accumulation of Hm at these low temperatures (Kanki et al., 2004).

Of the various psychrophilic Hm-producing bacterial strains that have been isolated from fresh fish so far in our laboratory, some strains, namely *Photobacterium phosphoreum* and *Photobacterium iliopiscarium*, are capable of producing high amounts of Hm at low temperatures.

It is known that *P. phosphoreum* is the predominant Hm-producing bacterium at low temperatures, and its behavior has been previously characterized (Ishimoto, Kasama, & Morii, 1994; Kanki et al., 2004). Even though only a few reports exist of food poisoning by Hm-producing bacteria at low temperatures, there are reports of the isolation of *P. phosphoreum* from the ingredient causing HFP (Kanki et al., 2004).

P. iliopiscarium, a species related to *P. phosphoreum*, is a psychrophilic Hm-producing bacterium at low temperatures and

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its proliferative capacity at 4 °C has been reported (Rivas, García-Fraile, Mateos, Martínz-Moline, & Velázquez, 2006). It is considered as an important psychrophilic Hm-producing bacterium, similar to *P. phosphoreum*.

Reports related to Hm production in fish meat have increased in recent times (Ben-Gigirey, Vieites Baptista De Sousa, Villa, & Barros-Velazquez, 1998; Emborg, Laursen, & Dalgaad, 2005; Guizani, Al-Busaidy, Al-Belushi, Mothershaw, & Rahman, 2005; Jørgensen, Huss, & Dalgaard, 2000; López-Sabater, Rodoríguez-Jerez, Hernádez-Herrero, Roig-Sagués, & Mora-Ventura, 1996; Takahashi, Kimura, Yoshikawa, & Fujii, 2003; Tsai, Chang, Kung, Wei, & Hwang, 2005; Yoshinaga & Frank, 1982), but there is no report focused on behavior of strong Hm-producing bacteria in relation to the amount of Hm accumulated so far. In this study, we selected 2 *Photobacterium* strains that displayed strong Hm accumulation and inoculated them into swordfish in order to investigate Hm accumulation and behavior.

2. Materials and methods

2.1. Sample and bacterial strains

Frozen swordfish samples (*Xiphias gladius*) were obtained from the market, defrosted immediately before inoculation, and used in the study.

The 7D155 (Mackerel isolate, *P. phosphoreum*) and 7I1519 (Tuna isolate, *P. iliopiscarium*), the strongest Hm-producing bacteria among 9 strains tested according to the method described below, were used for inoculation.

2.2. Study of bacterial growth characteristics in fish meat

2.2.1. Screening of strong Hm-producing bacteria

A total of 9 *P. phosphoreum* and *P. iliopiscarium* isolated from fresh fish (Table 1) were used for Hm-producing ability. Briefly, 1 ml of inoculation cultures (4 °C, in histidine broth [10 g Bacto peptone (BD Biosciences, Franklin Lakes, NJ), 3 g Bacto Yeast Extract (BD Biosciences), 5 g glucose, and 5 g L-histidine in 1000 ml of 50% artificial sea water (ASW; consisting of 23.5 g NaCl, 0.66 g KCl, 3.9 g Na_2SO_4, 10.6 g $MgCl_2/6H_2O$, and 1.5 g $CaCl_2/2H_2O$ in 1000 ml of ddH_2O); pH5.5]) was filtered through a 0.2 μm-pore-size syringe filter (Advantec, Tokyo, Japan) and analyzed Hm accumulation by enzyme-linked color developing kit (Kikkoman, Chiba, Japan) (Sato, Horiuchi, & Nishimura, 2005). Measurement was made after 0, 72, 96, and 120 h of storage.

2.2.2. Inoculation of bacteria into fish

The frozen fish meat sample was defrosted, and 25 g of the sample was cut into small pieces and inoculated with the bacteria to a final concentration of 10^4 cfu/g. The inoculated fish meat was stored at different temperatures (4 °C, 10 °C, and 15 °C). At different times after storage, normal viable bacterial counts, Hm-producing bacterial counts, and the amount of Hm produced were measured. Similar measurements were also made in a non-inoculated control sample. Measurements were made after 0, 3, 5, and 7 days of storage at 4 °C, after 0, 1, 2, and 3 days of storage at 10 °C, and after 0, 12, 24, 36, and 48 h of storage at 15 °C. The experiment was repeated three times.

2.2.3. Microbial colony count

Twenty-five grams of swordfish were resuspended in 225 ml of histidine broth. The viable bacterial count was determined using a TSA plate containing 1.5% NaCl. After 72 h at 15 °C, the number of bacteria was counted. The number of Hm-producing bacteria was measured using the most probable number (MPN) method (Cochran, 1950). Additionally, the histidine broth culture was inoculated into 3 tubes at different dilutions, and cultured for 48 h at 15 °C. Hm production was confirmed by paper chromatography (Miyaki, 1954). We compared the most probable number with the amount of Hm to estimate the number of Hm-producing bacteria.

Table 1
Growth and histamine-producing activity at 4 °C of *P. phosphoreum* and *P. iliopiscarium* isolates used for the screening strong Hm-producers.

Isolate[a]		Bacterial count (log cfu/ml)				Histamine (mg/kg)			
		0 h	72 h	96 h	120 h	0 h	72 h	96 h	120 h
P. phosphoreum	4A1525	2.8	4.0	5.0	5.3	ND[b]	ND	103	373
	5G1524	2.7	5.4	5.3	6.7	ND	ND	ND	64
	6C1528	2.7	4.3	5.1	5.1	ND	ND	ND	ND
	6H1523	3.1	5.4	5.7	6.4	ND	ND	ND	29
	7D155*	2.8	5.0	5.4	6.1	ND	ND	ND	459
	9A154	2.7	4.1	5.5	5.7	ND	ND	146	417
	9D1518	3.5	5.6	6.6	6.9	ND	ND	ND	56
P. iliopiscarium	6C1521	2.9	3.7	4.0	4.3	ND	ND	ND	ND
	7I1519*	3.1	6.4	7.1	7.5	ND	ND	196	815

[a] Isolates with an asterisk were used for the further study.
[b] ND; not detected.

2.3. Measurement of Hm content

Twenty-four milliliters of an aqueous solution of 0.1 M EDTA/sodium (pH, 8) were added to 1 g of fish meat and heated in boiling water for 20 min. The samples were cooled and centrifuged at 10,000 × *g* for 5 min. The supernatant was collected for Hm measurement, which was carried out using an enzyme-linked color developing kit (Kikkoman, Chiba, Japan) according to the manufacturer's instructions (Sato et al., 2005).

3. Results

Figs. 1–3 show the results of inoculation of low temperature Hm-producing bacteria into swordfish.

When fish inoculated with the 7D155 strain was stored at 4 °C, the counts of Hm-producing bacteria and normal viable bacteria after 3 days exceeded 10^6 MPN/g and 10^6 cfu/g, respectively, with the number of Hm-producing bacteria reaching 10^8 MPN/g after 5 days. The amount of Hm in fish meat was about 400 mg/kg after 5 days, reaching up to 870 mg/kg after 7 days. Similar behavior was observed in samples inoculated with 7I1519 strain: the amount of Hm in these fish was up to 350 mg/kg after 5 days, and 1750 mg/kg after 7 days. In the control group, the counts of normal viable bacteria and Hm-producing bacteria that were already present in the fish meat were 10^6 cfu/g and 10^5 MPN/g, respectively, after 7 days. However, Hm production was not observed.

When fish inoculated with the 7D155 strain were stored at 10 °C, the counts of normal viable bacteria and Hm-producing bacteria after 2 days were 10^6 cfu/g and 10^6 MPN/g, respectively. The accumulated Hm level was ≥100 mg/kg after 2 days, and 220 mg/kg after 3 days. In samples inoculated with the 7I1519 strain, counts of Hm-producing bacteria were up to 10^6 MPN/g after 2 days, and Hm accumulation of 140 mg/kg was detected. In the control group, an increase in normal viable bacteria and Hm-producing bacteria was observed, but Hm accumulation was not observed.

When fish inoculated with either the 7D155 or 7I1519 strains were stored at 15 °C, counts of Hm-producing bacteria reached up to 10^6 MPN/g after 12 h and 10^8 MPN/g after 36 h in both cases. The Hm accumulation was >600 mg/kg after 48 h. In the control group, normal viable bacteria counts after 48 h were 10^8 cfu/g and the count of Hm-producing bacteria was up to 10^6 MPN/g, but Hm