Original Article

A Pilot Study of Histamine in Silkworm Pupae

Sitthiphon Bunman, PhD[1]; Sanghyun Kim, MSc [2,3] ; Pakorn Thaiyanant, MSc[4] ; Chatrawin Krithep, MSc[5]; Pannee Tanudechpong, PhD[5]

Sitthiphon Bunman, PhD

Abstract

OBJECTIVES: We evaluated the effects of storage temperature and time on the concentration of histamine and microbial growth in the silkworm pupae.

MATERIALS AND METHODS: Silkworm pupae were stored at -20, 10 and 25 °C respectively. We measured histamine levels and counted bacterial colonies on the microbiological culture plates in 0, 24, 48 and 72 hours after euthanizing the silkworm pupae.

RESULTS: The level of histamine in the silkworm pupae was increased when storing at 10 oC and 25 oC with the lapse of time. The histamine level has soared from 24 h to 48 h at 25 °C, which is nearly 5 times higher. Each bacterial colony was increased at 10 °C and 25 °C until 48 h and then rapidly decreased in 72 h. The highest level of bacterial colony appeared in the 48 h at 10 °C and 25 °C.

CONCLUSION: This study showed that the level of histamine and microbial growth in silkworm pupae were changed by the storage temperature and time. However, the histamine level in the silkworm pupae was no cause for concern on human health.

Keywords: food safety, histamine, silkworm pupae, DAO

[1] Department of Community and Family Medicine, Faculty of Medicine, Thammasat University, Thailand.
[2] Forest Research Institute, Université du Québec en Abitibi-Témiscamingue, Campus of Amos, 341 Rue Principal Nord, Amos, Quebec J9T 2L8, Canada.
[3] Center for Forest Research, Université du Québec à Montréal, P.O. Box 8888, Centre-ville Station, Montréal, Québec H3C 3P8, Canada.
[4] Faculty of Medical Technology, Nakhonratchasima College, Nakhonratchasima, Thailand.
[5] Department of Pharmacology, Faculty of Medicine, Chulalongkorn University, Thailand.

** Address Correspondence to author:*
Sitthiphon Bunman, Department of Community and Family Medicine, Faculty of Medicine, Thammasat University, Pathumthani, Thailand.
email: Acetaminophen55@gmail.com

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Scombroid poisoning is recognized as food poisoning that results from the ingestion of scombroid food with high levels of histamine.[1] Histamine was suggested as a causative agent of scombrotoxism.[2] Scombroid food and beverages such as fish, meat, wine, fermented vegetables, cheese and insects naturally contain high levels of amino acid histidine that can be decarboxylated to form toxic histamine by histidine decarboxylase-producing bacteria when processing or storage conditions are improper, causing bacterial growth.[3,4] The decarboxylation process is induced by enzymes produced by enteric gram-negative bacteria such as *Morganella morganii, Escherichia coli, Klebsiella* species and *Pseudomonas aeruginosa* that are found in foods and intestines.[5] Histamine concentrations vary extensively, not only between different food varieties, but also within the varieties themselves. Scombroid poisoning usually occurs within an hour after the ingestion of fish or other foods which contain high levels of histamine. The most common symptoms of scombroid poisoning are flushing, urticaria, headache, dizziness, sweating, and swelling of the face and tongue. Gastrointestinal symptoms of scombroid poisoning can include diarrhea, abdominal cramps, nausea and vomiting.[4] Bronchospasm, respiratory distress and vasodilatory shock can also occur. The severity of symptoms depends on the amount of histamine uptake and the individual's sensitivity to histamine.[6,7]

Scombroid poisoning can also occur from insect ingestion.[8,9] Silkworm pupae are widely consumed in Asia countries.[10] In Thailand, oil-fried silkworm pupae are common snacks often sold by traditional Thai food stall vendors. They are the main by-products of the silk industry after extracting threads. They are a low cost healthy food source of high nutritional value including protein, lipids and essential amino

acid.[11,12] High levels of histamine in food is known to cause food poisoning but it does not alter the organoleptic quality, leading to histamine-contaminated foods being viewed as normal. Therefore, scombroid poisoning is frequently misdiagnosed. Some Thai populations frequently ingest silkworm pupae, but there are few studies about the existence of histamine and bacteria in silkworm pupae. This study aimed to evaluate histamine level and the effect of storage time and temperature on the histamine levels and bacterial growth in silkworm pupae.

Material and methods

Reagents

The following substances were used: Tetramethylbenzidine (TMB) plus hydrogen peroxide (H_2O_2), phosphate buffered saline, histamine horseradish peroxidase conjugate, histamine standards, monoclonal antibody against histamine (H_1), and Neogen's red stop solution (product NO. 301474) (Neogen Corporation, USA and Canada).

Silkworm pupae preparations

The silkworm pupae were purchased from a silkworm farm (Figure 1). They were housed at the Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University under standard conditions. Silkworm pupae were kept under laboratory conditions for 2 days prior to the start of experiment. They were randomly divided into 3 groups of 10 silkworm pupae. The pupae were euthanised with carbon dioxide and stored at a temperature of -20°C, 10°C and 25°C. The histamine level and bacterial count of each group were measured immediately after being euthanized, at 24, 48 and 72 h after being stored at each storage temperature.

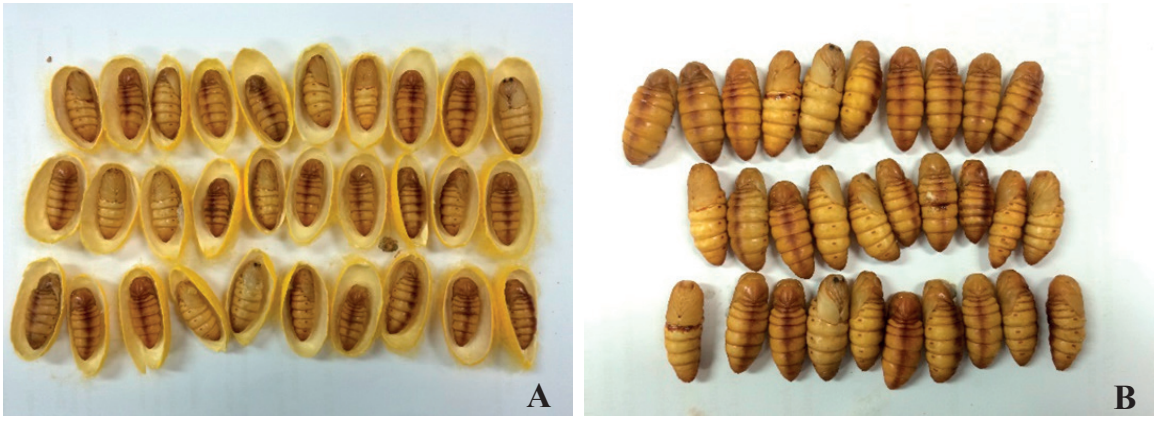

Figure 1: A silkworm pupae in cocoon, B silkworm pupae.

Silkworm pupae were crushed in a sterile plastic tube until homogenous. One gram of the homogenous mixture was added to a clean disposable extraction tube containing 9 mL of sterile extraction buffer (phosphate buffered saline). The extraction tube was tightly capped and vigorously shaken for 15 to 20 seconds to suspend the silkworm pupae tissue in the phosphate buffered saline. The tube was left for approximately five minutes, and was shaken again for 15 to 20 seconds to re-suspend the silkworm pupae tissue. This was repeated twice. The homogenates were centrifuged (3,000g, 15 minutes, 4°C) and filtered through Whatman No. 2 filter paper (Whatman, Maidstone, England). The filtrate was used to measure histamine level.

Determination of histamine level

Quantification of histamine was carried out using Neogen's Histamine ELISA test kit (Life Science Format) product number 409010. Using the stock standard histamine solution, diluted solutions are prepared in the final concentration at 0, 2.5, 5, 10, 20, 50 ng/ml. Added 50 µl of each standard solution, or the prepared sample, to separate duplicate wells. 50 µl of the enzyme conjugate was added to each well. The plate was covered with plate film and mixed thoroughly by shaking gently and incubated for 45 minutes at room temperature. After the enzyme conjugate incubation, the contents of the plate were dumped out. Each well was then washed with precisely 300 µl of the washing buffer and this was repeated 3 times. After that, 150 µl of the substrate was added to each well. It was mixed thoroughly and incubated for 30 min at room temperature and the absorbance was measured at 650 nm against blank.

Isolation of bacteria

Bacteria were isolated from the whole body of the silkworm pupae as follows.

Whole body of the silkworm pupae: To obtain samples from this batch of silkworm pupae, five grams of the silkworm pupae were added to 50 ml of sterile water and homogenized thoroughly with a glass rod under sterile conditions.

The mixture was vigorously shaken for about seven minutes. Each suspension was serially diluted to 10^2 or 10^4 with the same sterile water, and then 0.2 ml aliquots were inoculated to nutrient agar using the spread plate method and incubated at 37°C for 24h. This set up was observed for colony growths and 12 observed was recorded to determine the standard plate count from 2 replicates. Distinct colonies were subsequently isolated for further analysis.

Microbiological analysis: The distinct colonies on the plates were counted using a colony counter. The mean bacteria count per ml was obtained by dividing the average value of colony-forming units on enumerated plates by the volume of sample dispensed into nutrient agar and multiplied by the dilution factor. To identify the isolated organisms, distinct isolates were then screened based on the size, colony morphology, color, gram's staining reactions and biochemical characteristics of the isolates determined by subjecting the isolates to tests such as methyl red, Vogues-Praskauer, citrate, urease, indole, motility, catalase, oxidase and sugar fermentation tests. Differential media such as Mannitol salt and MacConkey agars were used to confirm the identification of suspected organisms. Identified organisms were further characterized by in *vitro* sensitivity testing (antibiogram) against 5 mcg novobiocin antibiotic disks. This was carried out using the Kirby-Bauer method and results compared with Clinical Laboratory Standards Institute (CLSI) values (CLSI, 2007).

Results

The level of histamine

The level of histamine stored at the temperature of -20°C in each group was measured immediately after the pupae were euthanized, at 24, 48, and 72 h, respectively which was unchanged when compared to control group. The level of histamine when stored at a temperature of 10°C and 25°C increased when compared to the control group (Table 1).

The level of bacteria colony

The level of bacteria colony of each group at the temperature of -20°C, 10°C and 25°C were counted immediately after the pupae were euthanized, at 24, 48 and 72 h, respectively. The level of bacteria colony of each group rose to the highest level after the pupae were euthanized at 48 h and it dropped at 72 h. The identification of bacteria growth is *Staph coagulase-negative* (Table 2).

Table 1: The levels of histamine of each group was measured immediately after being euthanized, at 24, 48, and 72 h, and stored at a temperature of -20°C, 10°C and 25°C (n = 10 for all groups).

Temperature	Control (ng/g)	24 h (ng/g)	48 h (ng/g)	72 h (ng/g)
-20°C	12.45	13.00	12.00	12.70
10°C	12.45	13.07	12.48	22.30
25°C	12.45	11.73	50.12	69.70

Table 2: The level of bacteria colony of each group was counted immediately after the pupae were euthanized, at 24, 48, and 72 h, and stored at the temperature of -20°C, 10°C and 25°C (n = 5 for all groups).

Temperature	Control (CFU/ml)	24 h (CFU/ml)	48 h (CFU/ml)	72 h (CFU/ml)
-20°C	250	110	100	95
10°C	250	230	310	70
25°C	250	520	570	300

** The identification of bacteria growth is Staph coagulase negative.

Discussion

Histamine is a primary heterocyclic amine formed by decarboxylation of the amino acid histidine. Histamine is an inflammatory mediator which is released from mast cells (granulocytes) but is typically stored in metachromatic granules of basophils.[13] Histamine is associated with the initial phase of immediate hypersensitivity response, and acts on blood vessels and smooth muscle causing vasodilation, bronchoconstriction and increased vascular permeability (erythema).[14] Furthermore, histamine is found from eating fish high in histamine due to inappropriate processing or storage, especially scombroid fish such as mackerel, tuna, sardine, mahi mahi, herring, anchovy, amberjack, bluefish, and marlin. For this reason, histamine is called "Scombrotoxin".[15] However histamine is also found in cheese, silkworms, and wine.[16-18]

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Silkworms *(Bombyx mori)* convert plant proteins into silk and use it as a source of food, with a rich nutritional profile, especially in terms of proteins, fat, and chitin contents.[12] Silkworm pupae has been used as a medicine and as an animal feed in many Asian countries for a long time.[19] Silkworm pupae has a high reproduction rate, short life period and short space requirements which provides a practicable alternative opportunity for food safety such as scombroid poisoning. This poisoning accounts for about 40% of all poisonings resulting from ingestion of fish[20] but the poisoning data from silkworm pupae were limited.

In our study, we determined the histamine levels of silkworm pupae in storage under different temperatures and times. Histamine levels seem to increase at temperatures of 25°C and during storage times greater than 24 h, whereas a temperature of -20°C did not affect histamine levels. Given that the histamine levels detected in silkworm pupae in this study were significantly lower than the toxic level set by Food and Drug Administration (50 ppm)[1], these levels could not induce food poisoning or intolerance. However, the susceptibility to histamine varies according to the diamine oxidase (DAO) activity of each individual. DAO is an enzyme responsible for the metabolism of histamine at the intestinal level, and impaired DAO activity is one of the main causes of histamine intolerance. Sensitive people with insufficient DAO activity could suffer from undesirable reactions after ingestion of foods containing low levels of histamine.[4] Good hygiene practices must be applied during the farming, processing and marketing of silkworm pupae intended for human consumption in order to control the microbiological growth and histamine level.

Conclusion

This study showed that the histamine level and microbiological growth in silkworm pupae can change according to storage time and temperature. However, the silkworm pupae investigated in this study has a relatively low concentration of histamine, which is thought not pose a health risk to people.

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