

## **Studies on amines of hurulla (*Amblygaster sirm*) Do they cause allergic reactions of hurulla?**

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### **Abstract**

Past studies stated that consumption of hurulla (*Amblygaster sirm*) gave an adverse reaction to tuberculosis patients treated with isoniazid drugs and that the cause may be tyramine. In order to test this hypothesis fresh hurulla samples were tested for histamine and tyramine with and without storage at room temperature using TLC and enzymatic methods. Only traces of tyramine (Rf 0.88) were found in only 5% of the samples of hurulla even in the presence of exogenous tyrosine. However, histamine (Rf 0.63) was detected and it was low, 0.34 mg/g of fish. Many other free amino acids were detected on keeping the fish at room temperature.

The bacteria producing these amines were isolated from the fish and cultured in a liquid medium. TLC was carried out from these bacterial supernatants to detect histamine. Histamine spots were then quantified by (I) ninhydrin reaction using a spectrophotometer at 570nm or densitometer. (II) Using an ELISA technique. (III) Histaminase assay. All forty-four bacterial colonies isolated were tested for histamine production from histidine. Eleven colonies showed > 0.8mg/ml. However studies with this culture in the log phase of the growth showed that the bacteria produced maximum histamine at 24 hours. However histamine content declined thereafter and was not detected in a 48 h culture. Adjusting pH to 1 to solubilize histamine complex increased histamine levels. Addition of EDTA (0.005mg.ml<sup>-1</sup>) increased histamine content with the age of supernatant indicating that chelation could prevent the breakdown of histamine decarboxylase. There was no evidence of breakdown of histamine or tyramine.

Studies show that tyramine is not the cause for adverse reactions with isoniazid drugs. Although the histamine content is low, it may contribute to allergic reactions.

**Keywords:** *Amblygaster sirm*, Allergenicity, Tyramine, Histamine

## 1. Introduction

*Amblygaster sirm* (hurulla) is a small round fish commonly found in waters around Sri Lanka and its maximum size is 20 cm. It is a very commonly used variety of fish to low income groups due to its low price. It is caught in large numbers from September to January of the coast of Sri Lanka and is also found off India, Singapore, Indonesia and China (Uragoda, 1978)

According to traditional medicine it has been classified as a heaty fish, but does not have as extreme a reputation as *balaya* (*Skipjack*). However, Uragoda (1978) found that allergic reaction similar to tyramine was produced by hurulla when consumed by people treated with isoniazid drugs. This was supported by Thadhani (2000), who tentatively identified one ninhydrin positive spot on TLC as tyramine. Past studies have clearly show that bacteria are responsible for histamine formation in fish (Gunaratne and Samarajeewa, 1994). Therefore, the objectives of this study were (I) To determine the quantity of tyramine released by bacterial action on tyrosine. (II) To determine any other reason for allergic reaction. (III) To isolate bacteria producing these biogenic amines and attempt to study the rate of formation by endogenous enzymes.

## 2. Methods and Materials

### Reagents

Histamine, Histidine, Histidine decarboxylase and Diamine oxidase, were purchased from the Sigma St. Louis, MO, USA. Peroxidase from the Aldrich Chemical Co., Leucocrystal violet from the Fluka chemicals, Yeast extract and Tryptone soy broth from the Hiemedia Laboratory, Mumbai, India, Peptone and Tryptone from Oxoid Ltd, England, Beef extract from Qualigens chemicals, India and Bacteriological agar from the Marine chemicals, India were purchased.

### Media

Niven's modified medium (Niven *et al.*, 1981) was used to isolate histamine producing bacteria and it comprised 0.5% glucose, 0.5% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% CaCO<sub>3</sub>, 2% agar and 1% histidine. HC1: The composition of the general medium used to isolate bacteria from hurulla fish was 0.4% peptone, 0.2% beef extract, 1.0% sucrose and 1.5% agar (when necessary).

### **Samples and Sample treatment**

Fresh hurulla were purchased from the Negombo fishery area and transported in ice containers to the University Research Laboratory. Samples were used immediately for analyzing amines or stored in  $-20^{\circ}\text{C}$  for further use. Head and viscera were removed. The flesh of the fish was used for experiments. Fish suspensions were prepared by homogenizing flesh of hurulla fish (5g) with sterilized distilled water (5 ml).

In the case of bacteriological studies all the glassware including petri palates, culture tubes were sterilized at  $160^{\circ}\text{C}$  in an oven for 3 hours before and after use. Culture media and distilled water were sterilized  $121^{\circ}\text{C}$  for 15 minutes in an autoclave.

### **Assay for amines**

Aliquots from the test and control (2-10 $\mu\text{l}$ ) were applied on laboratory prepared silica G60 (Merck, Germany) TLC plates (20cm x 20 cm) containing 0.1mm thickness with the standard histamine, histidine, tyrosine and tyramine solutions. Plates were chromatographed using  $\text{CHCl}_3$ :  $\text{CH}_3\text{OH}$ :  $\text{NH}_4\text{OH}$  (sp. gr.0.88) 2:2:1 (v/v) air-dried overnight to evaporate ammonia. Plates were heated to  $80^{\circ}\text{C}$  for 1 hour before being visualized with 1% ninhydrin in acetone. Rf values for histidine, histamine, tyrosine, tyramine were found to be 0.59, 0.63, 0.61, 0.88 respectively.

### **Quantification**

#### **Densitometer**

A Compaq deskpro Computer model 20005/23 3 MMX programmed with image master 1 D Elite software version 2.0 was used to obtain densitograms (Thadhani, 2000). Quantification of amines and amino acids were done using standard curves  $r^2$  values for histidine, histamine, tyrosine, tyramine were 0.9725, 0.9729, 0.9634, 0.9699 respectively.

#### **UV-Visible Spectrophotometer (Thadhani, 2002)**

The corresponding amines and amino acids spots were scraped from the TLC plates and dissolved in 70% acetone and absorbance were measured at 573 nm using spectrophotometer (Shimadzu, UV1601). All experiments were done in duplicate.

#### **ELISA Reader**

Histamine contents in one day old bacterial supernatants were quantified using Stat faxR 303 ELISA Reader with 650 nm filter using histamine specific microwells (Neogen Co. Ltd.). All readings were taken in duplicate.

### **Enzymic method**

This method of Lopez *et al.* (1993) based on the sequential action of diamine oxidase and peroxidase on the histamine and hydrogen peroxide respectively. Ammonia, imidazolacetaldehyde and hydrogen peroxide were formed as a result of the action of histaminase on histamine. The second enzyme peroxidase catalyzes oxidation of reduced form of the chromogen, 4-amino, antipyrine/phenol in to oxidized form. After incubation the reaction mixture 37°C for 2 hours readings were made using UV spectrophotometer at 510 nm. All experiments were done in duplicate.

### **Histamine analysis in tissue samples**

Histamine in fresh and aged hurulla fish samples (5g) were extracted into 0.4N perchloric acid (25ml) and 0.5 ml of the extract used to quantify histamine using the enzymic method after adjusting the pH to 6.8 with KOH

### **One step isolation of histamine producing bacteria using Niven's medium:**

The gill, skin, and intestine of fresh hurulla fish were swabbed with cotton and inoculated in to trypticase soy broth (2ml) and incubated 37°C for 24 hours. One loopful of this was streaked on to the Niven's agar plates and incubated 37°C for 24 hours. Positive presumptive histamine producing bacterial colonies were picked based on the morphological characteristic, purple colonies on the yellow background were considered as positive for histamine production while negative colonies were appeared as yellow or white colour.

### **Isolation of bacteria from hurulla fish using general medium**

Hurulla suspension was prepared and a loop from this was streaked under sterile conditions onto agar plates and the plates were incubated 37°C for 24 hours. Bacterial colonies were appeared as yellow and white colonies.

### **Maintenance of cultures**

Isolated bacterial colonies were inoculated into agar slant and sub-culturing was done every two weeks.

### **Quantification of histamine in bacterial supernatants**

One loop from the isolated bacterial colonies were inoculated into fresh sterile liquid medium (2ml aliquots) and incubated 37°C. Histamine was quantified using ELISA reader after 24 hours and using TLC-UV spectrophotometer.

### **Incubation of bacterial supernatants with histidine**

One loop from the highest histamine-producing bacteria was inoculated in to sterile liquid medium (100 ml) and incubated 37°C. Aliquots (1 ml) of the bacterial supernatants of 24 hours, 48 hours, and 72 hours old bacterial cultures were incubated with 1 mg of histidine. Aliquots (1 ml) of the bacterial supernatants without histidine were used as a control. These were incubated 37°C and TLC was carried out after 24 hours, 48 hours, and 72 hours. The plates were visualized with ninhydrin and 2, 4- DNP. Same experiment was repeated introducing tyramine (1mg) and histamine (1mg) in to bacterial supernatants.

### **Protease activity of the bacterial supernatants**

Test samples were prepared by mixing of histidine (5 mg.ml<sup>-1</sup>) with 2ml of each day's bacterial supernatants. Supernatant (2 ml) with distilled water (1 ml) was used as control. These were incubated 37°C and TLC was carried out after 24 hours of incubation before and after adjusting the pH to 1 (to free histamine from insoluble complexes).

### **Determining the effect of EDTA on protease activity .**

Histidine (5 mg. ml<sup>-1</sup>), supernatant (2 ml) and EDTA (0.015 mg) were transferred into test samples and histidine (5mg. m<sup>-1</sup>) and supernatant (2 ml) were taken as control. These were incubated 37°C and after 24 hours, TLC carried just and after adjusting the pH to 1.

## **3. Results**

### **Tyramine and histamine in Fresh hurulla**

During the course of the study more than twenty samples of fresh hurulla were tested for tyramine and histamine by the methods outlined. Fresh hurulla showed no tyramine and very low histamine (< 1 mg<sup>-1</sup>). On aging tyramine appeared only in trace quantities and that too in on about 5 % of the fish. Histamine increased on aging at room temperature. (Table 1).

### **Histamine released by bacteria**

Cultured bacteria in the log phase were used to quantify histamine released by one day supernatants of forty four bacterial colonies. Supernatants were used, as the histidine decarboxylase of bacteria is an exoenzyme (Rawles *et al.*, 1996). Results are shown in Table 2. A similar experiment was conducted using the ELISA technique in the microwell reader. Bacteria from the skin

gill and intestine were isolated from the fresh fish. Results are shown in Table 3. Results showed that the 24 h supernatants from bacterial colonies from the skin showed highest histamine content.

#### **Effect of incubation time on histamine content**

This is shown in figure 1. This clearly shows that histamine levels remains constant after 24 hours even in the presence of exogenous histidine decarboxylase. It also shows that adjustment of pH to 1 (Thadhani, 2000) improves quantification.

#### **Effect of supernatant from liquid culture of different age at different pH values on histamine release from exogenous histidine**

Results (figure 1) show that adjusting of pH to 1 improves quantification, but still two problems occur, increasing histidine and decreasing histamine with time. As it was possible that histidine decarboxylase was being destroyed by proteolytic action (Table 4). EDTA ( $0.005 \text{ mg ml}^{-1}$ ) was used to possibly chelate  $\text{Mn}^{2+}$  and  $\text{Mg}^{2+}$  Results (Table 5) showed that there was an increase in histamine levels with time. However histidine content also increased.

#### **Studies on possible amine destruction**

As histamine level declined in many instances it became important to test for results of any enzyme action that destroys histamine and tyramine. Results showed that no new spot was formed as a result of possible histamine lyase reaction. Further spraying with 2, 4-dinitrophenyl hydrazine of TLC's gave no evidence of histaminase or other deaminating action. Incubation of supernatants with both histamine and tyramine showed no loss of these additives showing that no enzyme is destroying the amines.

### **4. Discussion**

Uragoda (1978) suggested tyramine as the problem for allergic reaction with hurulla due to the similarity of responses seen with allergies after consuming certain cheeses containing tyramine. It is very clear that allergic reaction of hurulla is not due to tyramine. Only traces of tyramine were found in hurulla and that too in a very small number of instances. Therefore the spot suspected by Thadhani, (2000) was not tyramine (This author had no tyramine standard). However one other amine was found, which forms even in the presence of goraka (*Garcinia cambogia*) extract at very low pH.

Histamine content found was comparable to that of non-heaty fish. Several bacteria, isolated from various parts of fish, tested for histamine production from histidine from the ELISA technique, showed that bacteria from skin produces the highest amount of histamine indicating that bacteria grow during damage to skin, which is possible during catch by the nets used.

Histidine decarboxylase is an exoenzyme (Rawles and Flick, 1996). Therefore, bacterial supernatants used to detect histamine formation. Surprisingly it was found that the maximum histamine formation from the one day old bacterial supernatant. Thereafter histamine content declined. This suggests some histamine utilizing reaction. However, testing showed no new amine spot and nor DNP derivative. Therefore, this appears to be not due to a histaminase destroying activity. Further, incubation of histamine and tyramine with supernatant also showed no evidence of destruction of these amines.

Following method of Thadhani (2000) pH was reduced to 1. This was done to solubilize histamine complex shown in early studies (Thadhani, 2000). When this experiment was repeated a hyperbolic curve was obtained showing that little or no histamine was formed after 24 h. It was suspected that the bacteria release proteases that destroy histidine decarboxylase. Therefore histamine level does not increase even in the presence of histidine. Use of EDTA which chelate  $Mn^{2+}/Mg^{2+}$  (cofactor of bacterial proteases) showed an increase in histamine levels with time. Study of amino acid profile in hurulla showed that it is possible that a gizzerozine type reaction occurred. (Lopes, *et al.*, 1994) However lysine content was normal, but comparatively high levels of tryptophan, isoleucine, leucine, norleucine and methionine was found. The question then was to find out what is the reason for allergic reaction. From theory, the effect of isoniized drug is that it reacts with Vitamin B<sub>6</sub> which is necessary for the action of monoamine oxidases and diamine oxidases, thus giving rise to allergic reactions. Therefore it is possible to speculate that conditions of vitamin B<sub>6</sub> deficiency will lead to low MAO and DAO activity. Vitamin B<sub>6</sub> is commonly found in yeast extract, rice polishings, germinal portion of seeds, grains and egg yolk. It is possible certain Sri Lankan diets do not have enough vitamin B<sub>6</sub> to detoxify the little histamine formed and this causes a mild "heaty" (allergic reaction).

## 5. References

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Table 1: Effect of aging hurulla at room temperature on histamine content.

Fish sample	Amount of histamine mg/g of fish
Fresh hurulla	0.08
24h aged fish	0.94
48h aged fish	1.34

Method of assay Enzymatic histamine analysis method.

Volume of the reaction mixture 2.6 ml (Sample (0.5ml), Phosphate buffer p116.8

(1.0 ml), 4-amino, antipyrine/phenol (0.1 ml), diamine oxidase (0. 5ml), peroxi dase(0. 5ml).

Incubation time 2 h.

Incubation temperature 37°C.

Table 2: Release of histamine by bacterial isolate supernatants

Histamine (mg/ml)	Percentage bacterial colonies (%)
0.02- 0.19	2
0.2- 0.39	23
0.4 - 0.59	20
0.6 - 0.79	20
0.8 -1.5	9

One loopful of culture was inoculated into sterile liquid medium and incubated 37°C for 24 h. and determined the histamine content by TLC- UV Spectrophotometric method. Volume of the supernatant applied on TLC =10 µl. Histamine spots scraped and dissolved in 70% acetone (1 ml) and measured absorbance at 573 nm

Table 3: Release of histamine by bacterial isolate supernatants

Histamine ppm	Number of bacteria colonies		
	Skin	Gill	Intestine
0 -50	2	8	2
50—100	1	5	3
100—200	4	2	1
200-300	7	1	0
300-400	1	0	0

Gill, Skin and intestine were swabbed with cotton, inoculated into tryptone soy broth, and incubated 37°C for 24 h. One loop from this cultures streaked on to agar plates under sterile condition and plates were incubated 37°C for 24 h. Isolated bacterial colonies transferred into sterile liquid medium and incubated 37°C for 24h and histamine was determined by ELISA method using a microwell reader.

Table 4: Effect of age of supernatant of bacterial culture on histidine and histamine levels at pH 5.6 and 1

Age of bacterial culture	Amount of amine at pH 5.6 ( $\mu\text{moles ml}^{-1}$ )		Amount of amine at pH 1 ( $\mu\text{moles ml}^{-1}$ )	
	Control	Test	Control	Test
<b>Histidine</b>				
24 h old supernatant	3.5	11.9	4.5	11.7
48 h old supernatant	5.5	13.5	3.9	13.4
72 h old supernatant	3.4	14.2	4.2	14.5
96 h old supernatant	ND	13.2	2.2	16.7
<b>Histamine</b>				
24 h old supernatant	3.4	5.4	3.6	9.2
48 h old supernatant	ND	4.9	2.9	10.8
72 h old supernatant	ND	ND	ND	3.4
96 h old supernatant	ND	ND	ND	2.7

The supernatants of 24 h bacterial cultures were separated and determinations were made by TLC- Spectrophotometric method after adjusting the pH to 1 (unadjusted pH=5.6). The same treatment was performed using 2, 3 and 4 days old bacterial supernatants (2ml). Histidine was added at levels of  $5 \text{ mg ml}^{-1}$  in a total volume of 3 ml. Control-no histamine, test -histidine added. Adjustment of pH from the normal pH of 5.6 to 1 was carried out just before TLC.

ND — not detected.

Table 5: Effect of EDTA on histamine formation

Age of bacterial culture (Days)	Histamine content ( $\mu\text{moles ml}^{-1}$ )	
	Control.	Test
1	3.6	6.3
2	11.0	12.6
3	13.5	15.3
4	12.8	15.5

Method of assay TLC - UV Spectrophotometry. Total Volume 3ml. Control Histidine ( $5 \text{ mg m}^{-1}$ ), supernatant (2 ml). Test Histidine ( $5 \text{ mg m}^{-1}$ ), supernatant (2 ml), EDTA (0.015 mg). pH adjusted to 1.

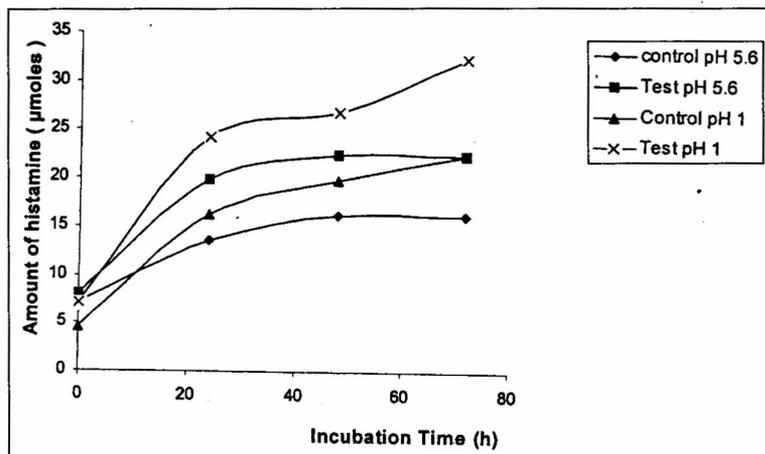


Figure 1 Effect of incubation time on histamine content.

The supernatant of a 24 h bacterial culture was separated and determinations were made by TLC-Spectrophotometry method after adjusting pH to 1 (Unadjusted pH = 5.6). Histidine decarboxylase enzyme ( $1.32 \text{ U ml}^{-1}$ ) and histidine ( $5 \text{ mg}$ ) were added to control and test. Control - No supernatant added, Test - Supernatant added (2ml), Total volume 3ml.