Non-volatile Amine Production in Mackerel Muscle during Growth of Different Bacterial Species

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The production of various non-volatile amines by *Morganella morganii*, *Klebsiella planticola*, *Enterobacter aerogenes* and *Hafnia alvei* in the dark and white muscle extracts of mackerel and in intact muscle was investigated during storage at 20°C. The amines were extracted with 2% perchloric acid and quantified by an HPLC method. The major amines produced were putrescine, cadaverine and histamine. The amine levels in the white muscle extracts were higher than those in the dark muscle extracts. All the bacterial species tested produced histamine in large quantities except *H. alvei*, which mainly produced cadaverine in the muscle extracts together with a little histamine. *M. morganii* did not produce any cadaverine in either of the extracts during storage. In the intact muscles, inoculated with *M. morganii* and/or *E. aerogenes*, there was an increase in amine levels followed by a gradual decrease. The results show that the strong histamine producers are also capable of producing the other amines in considerable amounts, and the rate and the type of the amine produced depend on the bacterial species, some of which show amine-degrading ability, especially during prolonged storage of the fish.

Key words: non-volatile amines; histamine poisoning; *Morganella morganii*; *Klebsiella planticola*; *Enterobacter aerogenes*; *Hafnia alvei*

Introduction

It is well documented that histamine poisoning can result from the ingestion of foods such as fish and cheese that contain high levels of histamine¹⁾. Various types of bacteria have been isolated from fish in connection with histamine formation^{2)~4)}. Among them, *Morganella morganii* and *Klebsiella planticola* are reported to be strong histamine producers³⁾. In addition, *Enterobacter aerogenes*, an enteric bacterial species has been found to be another prolific histamine producer³⁾. Lack of toxic symptoms after oral administration of pure histamine has led to the hypothesis that other diamines or polyamines present in the spoiled fish potentiate the histamine poisoning⁵⁾. The most common diamines in spoiled fish, putrescine and cadaverine, which are the decarboxylated products of ornithine and lysine, respectively, are known to be involved in such potentiation^{6), 7)}. A number of bacterial species possess lysine and ornithine

decarboxylase activities⁹⁾. Suzuki *et al.*⁸⁾ reported the ornithine decarboxylase activity of *Shewanella putrefaciens*, which produces only diamines in fish muscle. Okuzumi *et al.*⁴⁾ reported the decarboxylase activities of *Pseudomonas* and *Photobacterium* isolated from horse mackerel. However, the potential of producing such amines together with histamine during bacterial proliferation in fish muscle has not been reported yet.

The present study was undertaken to compare the capacity of various bacteria for polyamine production in fish muscle and to determine the difference in the patterns of bacterial non-volatile amine production between the dark muscle and the white muscle.

Materials and methods

1. Bacterial strains

Four microorganisms implicated in histamine poisoning incidents were used in this study. *Enterobacter aerogenes* (ATCC 43175) and *Klebsiella*

la planticola (ATCC 43176) were obtained from the American Type Culture Collection. *Morganella morganii* (IFO 3848) and *Hafnia alvei* (IFO 3731) were obtained from the Institute for Fermentation, Osaka, Japan. All the cultures were maintained on nutrient agar slants.

2. Preparation of inoculum

The strains were inoculated into 5 ml of trypticase soy broth and incubated at 37°C for 24 hours. A loopful of this culture was inoculated into a test tube containing 5 ml of trypticase soy broth and incubated at 37°C overnight. This culture was used as the inoculum for polyamine production experiments.

3. Preparation of fish muscle extracts

Very fresh mackerel were obtained from the central fish market in Kyoto and the dark and white muscles were separated immediately. These muscles were each homogenized with two volumes of water, steamed at 90°C for one hour, filtered, aliquoted (10 ml per test tube) and sterilized. Glucose was sterilized separately and added to each tube to give a final concentration of 1%¹⁰⁾.

4. Amine production in fish muscle extracts

Overnight bacterial cultures (0.2 ml) were inoculated into the fish muscle extracts and incubated at 20°C without shaking. Immediately after the inoculation, initial samples were taken for amine analysis. Further samples were then taken at 6 hour intervals for 30 hours.

5. Amine production in intact fish muscles

Pieces of dark and white muscles (5–6 cm in length) were aseptically inoculated with either *E. aerogenes* or *M. morganii*, placed in sterilized Petri dishes and stored at 20°C for 4 days. Uninoculated muscle pieces were also stored at the same temperature. Samples were taken for amine analysis at 24 hour intervals.

6. Bacterial growth

The test cultures (3 ml) were removed and growth was monitored spectrophotometrically at 600 nm as a function of time.

7. Aerobic plate counts

Aerobic plate counts were done using trypticase soy agar as the medium. Plates were incubated at 37°C for 24 hours. Duplicate plate counts were used in all instances.

8. Amine extraction

Perchloric acid (PCA) was added to the remainders of the extracts to give a final PCA concentration of 2% and then mixed well. Filtered solutions were made up to 25 ml and stored at –70°C until analyzed. The intact muscles were homogenized with 2% PCA and extracts were prepared as described previously¹¹⁾. All the experiments were repeated at least 2 times and similar results were obtained in each case.

9. Amine analysis

Mono- and polyamines were analyzed by high-performance liquid chromatography (HPLC) using post column derivatization of the amines with *o*-phthalaldehyde as described by Gamoh and Fujita¹²⁾, with slight modifications. A Waters liquid chromatograph with a system controller (model 600 E), a U6K injector and a Hitachi fluorescence detector were used. A reverse-phase microparticulate-type column (150 × 3.9 mm i.d., 5 µm particle size, Waters µ Bondasphere C₁₈) was used to separate the amines. Fluorescence excitation was done at 345 nm and emission was measured at 455 nm. The mobile phase consisted of two solvents; solvent A was sodium perchlorate (pH 4.0) with 0.01 M sodium 1-hexanesulfonate and solvent B was a mixture of 75% methanol and 25% solvent A (pH 3.0). The gradient elution system was controlled as described by Suzuki *et al.*¹³⁾. The flow rate was 1.0 ml/min. The PCA extracts, filtered through 0.22 µm membrane filters, were injected either directly or after appropriate dilutions into the chromatograph.

Results

1. Non-volatile amine production in fish muscle extracts

Figures 1, 2, 3 and 4 show the changes in amine concentration and the bacterial growth in the muscle extracts. During the experimental period, the bacteria grew rapidly in the fish

Fig. 1. Non-volatile amine production by *M. morgani* in dark and white mackerel muscle extracts at 20°C

○, ●, histamine; △, ▲, putrescine;
--○--, --●--, bacterial growth

muscle extracts; the growth rate was different for each species. In all instances, the growth was higher in the dark muscle extracts (DME) than in the white muscle extracts (WME), with *H. alvei* and *M. morgani* in particular showing a comparatively higher growth. Histamine, putrescine and cadaverine were found to be the major amines produced by the bacteria during storage at 20°C. The capacity to produce these amines in the muscle extracts varied according to the bacterial strains and the amine levels, except for putrescine, were always higher in the WME than in the DME. Of the three amines, histamine was produced in both WME and DME in large amounts while putrescine levels were very low. The three bacterial species other than *M. morgani* contained cadaverine and putrescine at the time of inoculation; *H. alvei* had a rather high amount of cadaverine. Only putrescine was detected in *M. morgani* culture.

As shown in Fig. 1, only histamine and putrescine were detected in both types of muscle extracts inoculated with *M. morgani*. The histamine level was around 7,500 nmol/ml after 30 hours in WME and 600 nmol/ml in DME. The putrescine levels were almost the same in both

Fig. 2. Non-volatile amine production by *E. aerogenes* in dark and white muscle extracts at 20°C

□, ■, cadaverine; ○, ●, histamine; △, ▲, putrescine; --○--, --●--, bacterial growth

types of muscles initially but they increased slightly in DME later. After 18 hours, histamine reached its maximum level in DME while it increased steadily in WME.

E. aerogenes produced cadaverine in addition to histamine and putrescine in the muscle extracts (Fig. 2). Its histamine-forming capacity was remarkably higher than that of *M. morgani*. After 30 hours, it had produced 11,000 nmol/ml of histamine and 900 nmol/ml of cadaverine. In DME, the histamine level was rather low compared to that of *M. morgani*. Another significant difference was the delay in histamine production; it started after about 6 hours. In the case of *M. morgani*, about 200 nmol/ml histamine was produced within 6 hours in WME. Accumulation of a large amount of cadaverine in the extracts was seen from a very early stage of the growth of *E. aerogenes*.

K. planticola was also able to produce all three amines (Fig. 3). Approximately 4,500 nmol/ml of histamine was found by the end of the incubation period in WME. In DME, histamine production was rather high compared to the

Fig. 3. Non-volatile amine production by *K. planticola* in dark and white muscle extracts at 20°C
 □, ■, cadaverine; ○, ●, histamine; △, ▲, putrescine; --○--, --●--, bacterial growth

other bacteria. Cadaverine levels were also higher than that of *E. aerogenes*. Putrescine levels were slightly higher in the dark muscle but remained at almost the same level at the end of the storage time.

Histamine-forming capacity of *H. alvei* was rather low and it was not until after 24 hours that substantial amounts of histamine was produced (Fig. 4). In DME, we could not detect any significant amount of histamine; *H. alvei* had a greater capacity to produce a large amount of cadaverine in the muscle extracts, and about 1000 nmol/ml of cadaverine was produced after one day in WME. At that time, the histamine level was only 70 nmol/ml. The patterns of the amine production in both extracts were very similar.

The patterns of cadaverine production were almost the same in three species, excluding *M. morganii*, which did not produce cadaverine. Histamine production patterns, however, varied from species to species. Spermidine and spermine present in the muscle extracts disap-

Fig. 4. Non-volatile amine production by *H. alvei* in dark and white muscle extracts at 20°C
 □, ■, cadaverine; ○, ●, histamine; △, ▲, putrescine; --○--, --●--, bacterial growth

peared gradually with increase in the storage period.

2. Non-volatile amine production in intact muscles

When the intact muscles were inoculated with *E. aerogenes*, or *M. morganii*, the amine levels increased at first and then started to decrease from the second day in the white muscle and from the first day in the dark muscle (Fig. 5). No amines were detected in the uninoculated samples during the storage time. The growth of *E. aerogenes* was slightly higher in the dark muscle than in the white muscle, whereas the growth of *M. morganii* was considerably higher, especially towards the end of storage. The sudden decrease in the amine levels (primarily histamine) was more pronounced in the dark muscle than in the white muscle. Putrescine and cadaverine levels fell almost to zero after four days.

Discussion

1. Non-volatile amine production in the muscle extracts

Of the four bacterial species, which are known

Fig. 5. Non-volatile amine levels in intact mackerel muscles spiked with bacteria during storage at 20°C

A: mackerel muscles spiked with *E. aerogenes*

B: mackerel muscles spiked with *M. morganii*

□, ■, cadaverine; ○, ●, histamine; △, ▲, putrescine; --○--, --●--, bacterial growth

to be involved in histamine poisoning, *M. morganii*, *K. planticola* and *E. aerogenes* were capable of producing large amounts of histamine in the mackerel muscle extracts. Among them *E. aerogenes* seemed to possess the highest potential for producing histamine. Only *H. alvei* was a slow histamine producer. The same finding has been reported previously in tuna fish infusion broth³. The present results further show that the ability of these bacteria to produce other diamines, putrescine and cadaverine was also high. The difference in the rate of amine production between the muscles can be attributed to the difference in substrate levels in the two types of muscles. The precursor amino acids of the amines, *i. e.*, lysine, histidine and ornithine, are present in much higher amounts in the white muscle^{11, 14}. Therefore higher decarboxylase activity could be expected in WME. On the other hand, dark muscle is known to be rich in vitamins and other factors (*e. g.*, minerals) necessary for the growth of bacteria^{15, 16}. However, the exact reason for the higher growth of bacteria in the dark muscle is not clear.

The cultures of *H. alvei*, *K. planticola* and *E. aerogenes* contained cadaverine and putrescine at the time of inoculation, suggesting that the enzymes were constitutionally produced in the bacterial cells. Polyamines are usually found in

microorganisms and they have been reported to be essential for bacterial growth in small quantities¹⁷. Bacterial histidine decarboxylases are known to be adaptive enzymes¹⁸. In the presence of a high level of histidine in fish muscle extracts, the decarboxylase of the bacteria was induced, resulting in the increase of histamine production. Histamine accumulation reached the maximum in the stationary phase. The time required for the induction varied according to the bacterial species. It seemed that the enzyme synthesis was induced in the early phase of the growth of *M. morganii*. Okuzumi *et al.*⁴ has reported that *M. morganii* is responsible for the accumulation of large amounts of histamine in common mackerel stored at high temperatures.

The results further indicated that the pattern of cadaverine production was always parallel to the bacterial growth. In the early phase of growth, there was a remarkable production of cadaverine while histamine levels remained low. During the stationary phase the cadaverine level became stable and histamine formation increased gradually. The ratio of histamine to cadaverine varied among the bacterial species as well as in the two kinds of muscles. Moreover, the ratio varied with time even in a single species. *M. morganii* culture did not accumulate

any cadaverine, suggesting its inability to produce the enzyme even if the substrate is available in abundance.

The pattern of the amine formation in DME was very similar to that in WME (Figs. 1~4). The only difference was the lower concentrations of the amines in DME, except for putrescine, which was formed at almost the same level as found in WME, or even slightly higher. Since ornithine occurs in very small amounts in fish muscles¹¹⁾, putrescine could be expected to be found in very small amounts. Further studies on the amine formation under different conditions are in progress.

2. Non-volatile amine production in intact muscle

The decrease in amine level in the intact muscles inoculated with the bacteria indicated the existence of histaminase activity. Since many amine oxidases are adaptive enzymes¹⁹⁾, their activity can be induced by large amounts of the respective amines. The overall histamine-producing capacity is dependent on both decarboxylase and diaminase activities. *Enterobacter*²⁰⁾ suggested that some bacterial strains possess histaminase and thus limit histamine concentration. The bacterial species tested in the present study seemed to possess the diaminase activity and the dark muscle had an ability to accelerate the amine degradation. Sudden decrease of the amine levels in the dark muscle suggested that a factor(s) contained in the dark muscle activated the diaminase activity. The amine-degrading activity was not observed in the extracts. A probable reason for this phenomenon is the static incubation condition of the extracts, which limits oxygen supply for the action of amine oxidase.

In histamine poisoning incidents, the fish sample implicated is usually tested for histamine. The toxic level is considered to be 50 mg/100 g of fish⁷⁾. The present results confirmed that the histamine level would be determined by the bacterial species as well as other factors such as temperature and substrate level. Some bacteria produced little histamine with large amounts of other diamines (Fig. 4). Sometimes, in an early phase of growth, the cadaverine level was much higher than the histamine level. If

there is a potentiating action by other amines, even a little histamine may be important in histamine poisoning incidents. It has been reported that histamine is more toxic when ingested with spoiled fish and considerable data are available on the potentiation²¹⁾. The potentiator would decrease the dose of histamine needed to induce the adverse reactions. Therefore, the determination of histamine level alone may not be enough to confirm that a sample is responsible for histamine poisoning. Simultaneous polyamine analysis would be required in such a case.

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