Simple Measurement of Gizzerosine in Fish Meals by High-performance Liquid Chromatography

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A simple method has been developed for analysis of gizzerosine in fish meals. A sample, hydrolyzed with 6 N HCl, was transferred into an Amberlite IR CG-50 column which was equilibrated with sodium borate solution (0.045 M, pH 8.25). After elimination of a large portion of amino acids from the column, gizzerosine was eluted with 0.5 N NH₄OH and then measured by HPLC. HPLC separation was done with an analytical column of Hitachi 2619-F and gradient elution with three solvents (two borate buffers—pH 9.2 and 10.7, [Na⁺] = 0.1 M, containing 3% (v/v) EtOH—and 0.8% NaOH). Gizzerosine was measured by the post-column reaction of OPA and fluorometric detection. Recovery of gizzerosine added to fish meal was over 90% and the detection limit was 1 ppm.

Gizzerosine (2-amino-9-(4-imidazolyl)-7-azanonanoic acid, Fig. 1) was identified as a causative substance for gizzard erosion in broiler chicks. It is assumed to be formed by reaction of the ε-amino group of lysine with free histidine during the heating of brown fish meal, especially when overheated. In feed production for chicken-raising, fish meal occupies a considerable role and nowadays it has become important to know the contents of gizzerosine in fish meal accurately before mixing for production of the feed. A rapid and simple procedure for gizzerosine measurement in the fish meal has been needed for the fish meal production and mixed feedstuff production.

Ito et al. reported a fluorometric measurement of gizzerosine with a pre-column reaction of α-phthalaldehyde (OPA) using high-performance liquid chromatography (HPLC) which includes 3 steps of chromatographic purification, and Wada et al. also reported the same HPLC method, using an automatic amino acid analyzer for the purification procedure. The former procedure is complicated and difficult to put into practice. In the latter procedure, large amounts of sample (3 ml) should be injected into a preparative amino acid analyzer at once, which is of a special type not available in the usual laboratory.

This paper reports a simple method of measuring the content of gizzerosine in fish meal. It involves a purification step with Amberlite IR CG-50 column chromatography and OPA post-column detection using HPLC.

MATERIALS AND METHODS

Materials. Synthetic DL-gizzerosine was purchased from Ajinomoto Co., and 10 mg of it was dissolved in 100 ml of 0.05 M HCl as a standard stock solution of gizzerosine. A standard working solution was prepared by diluting it with 0.02 M HCl. OPA was the biochemical use grade of Wako Pure Chemicals. Other chemicals were of analytical reagent grade. Water was redistilled.

As an eluent for Amberlite IR CG-50 column chromatography, 0.045 M sodium borate solution adjusted to pH 8.25 with 1 N HCl was prepared. For separation of
gizzerosine from fish meal hydrolyzate, two kinds of Amberlite IR CG-50 (Type 1) columns—named Amberlite column (A) and Amberlite column (B)—were prepared. Column (A) (1.5 x 20 cm) was packed with resin which was equilibrated with sodium borate solution (the same as the eluent), while column (B) (1.0 x 7 cm) was the \( \text{NH}_4^+ \) form.

In the HPLC, three solvents—Solvents A, B, and C—were used as the mobile phases. Solvent A was 0.05M sodium borate at pH 9.2 containing 30 ml of EtOH per 1 l of solution. Solvent B was prepared from solvent A by adjusting the pH to 10.7 with 0.1 M NaOH containing 3% EtOH (v/v). As solvent C, 0.8% NaOH was used.

For the post-column reactor, OPA reagent was prepared as follows: 19.1 g of Na₂B₄O₇ 10H₂O and 3 g of NaOH were dissolved in water, and mixing with 80 µl of 2-mercaptoprotoethanol, 4 ml of 25% Brij 35, and 161 mg of OPA in 10 ml of EtOH; it was brought to 1 l with water.

**Apparatus.** The HPLC system is a LC-6A (Shimadzu Corp.) with a gradient controller, post-column reaction equipment, a fluoromonitor (Shimadzu RF530), and a Rheodyne valve injector. For post-column reaction, a Teflon tubing coil (0.25 mm x 5 m) which is installed in the column oven and a Shimadzu LC-3A pump were used.

Hitachi Ion Exchange Resin 2619F (4 x 150 mm) was used as a stationary phase and Hitachi Ion Exchange Resin 2650 (4 x 120 mm) was used as an ammonia filter column which was put in front of an injector to stabilize the mobile phase conditions.

Chromatograms, peak areas, and retention times were obtained using a data processor (Shimadzu Chromatopac C-R3A).

**Sample preparation.** Five hundred milligrams of fish meal was hydrolyzed with 10 ml of 6 N HCl in a Pyrex tube (20 x 150 mm) sealed with a Teflon screw cap at 110°C for 24 hr in an oven. After the hydrolysis, the sample was filtered through sintered glass fiber filter paper (Toyo GB 140, size 47 mm, Toyo Roshi Co.) and evaporated under reduced pressure to remove HCl. The residue was dissolved with 30 ml of water, neutralized with 2 N NaOH to pH 7, and filtered through Toyo No. 5B filter paper. The precipitated residue on the filter paper was washed with 50 ml of water, and the combined sample solution was transferred into an Amberlite Column (A). The solution was drained into the top of the column at a flow rate of 1.5 ml/min. The column was washed with 350 ml of sodium borate solution to remove a large portion of the amino acids of the hydrolyzate. After washing the column with 100 ml of water (1.5 ml/min), the outlet of Amberlite column (A) was linked to an Amberlite column (B); then gizzerosine was recovered quantitatively by 100 ml of 0.5 N \( \text{NH}_4\text{OH} \) (1.5 ml/min). The eluate was evaporated to dryness under reduced pressure and dissolved in 2.5 ml of 0.02 N HCl. The solution was passed through a 0.45 µm membrane filter (Millipore Ltd.) before HPLC analysis.

**Measurement by HPLC.** A sample (20 µl) of the standard working solution (gizzerosine 5 µg/ml) or sample solution was injected into the chromatograph and developed by the elution program described below. The starting mobile phase was 100% of solvent A and kept for 16 min; then it was changed to 100% of solvent B during 14 min with linear gradient elution. After 30 min the mobile phase was changed to 100% of solvent C and was held for 15 min. After elution the column was equilibrated with starting solvent A for 30 min. The temperature of the analytical column and the post-column reaction coil was kept at 60°C. The flow rate of the mobile phases was set at 0.4 ml/min. The OPA reagent was mixed at a flow rate of 1.0 ml/min.

The excitation and emission wavelengths of the fluoromonitor were set at 350 nm and 450 nm, respectively.

**RESULTS AND DISCUSSION**

**Optimization of hydrolysis**

In our study, the optimum time and evacuated sealing which were generally used on amino acid analysis were investigated. Purification after hydrolysis was undertaken by the proposed manner (see MATERIALS AND METHODS section). When high gizzerosine fish meal (containing 30 ppm, prepared by dry heating in our laboratory) was hydrolyzed at 110°C for 16 to 48 hr in sealed tubes without evacuating, gizzerosine values were not variable after 20 to 48 hr. And acid hydrolysis in vacuum reaction tubes (Pierce Chem. Co.) did not show any significant differences from these values. In addition, recoveries from 0 hr to 48 hr using synthetic DL-gizzerosine added to fish meal showed almost unvariable values (92 ~ 94%). It was concluded that the hydrolysis in the sealed tubes without evacuating at 110°C for 24 hr was satisfactory.

**Pretreatment for Amberlite IR CG-50 column chromatography**

Pretreatment using active charcoal for the hydrolyzate was found to be unnecessary in this procedure. After the hydrolyzate was filtered and evaporated to remove HCl, the pH was adjusted to 7.0 before column chromatography. At this time considerable amounts of precipitate were formed and they were removed by filtration through filter paper.
表1. 比较过滤和离心法去除水解物形成沉淀的效果

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Filtration</th>
<th>Centrifugation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(1)</td>
<td>(2)</td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td>(4)</td>
</tr>
<tr>
<td></td>
<td>(1)</td>
<td>(2)</td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td>(4)</td>
</tr>
<tr>
<td>Gizz. added (µg)</td>
<td>0</td>
<td>12.5</td>
</tr>
<tr>
<td>Gizz. found (µg)</td>
<td>0.2</td>
<td>11.4</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>91</td>
<td>95</td>
</tr>
</tbody>
</table>

a Toyo No. 5B filter paper was used and washed with 50 ml of water.
b Extracted twice at 2000 × g with 25 ml of water.
c Added to 0.5 g of sardine meal hydrolyzate.

Removal by centrifugation (2000 × g) gave about 10% lower recovery and more variable data than filtration (Table 1). We thought that this was due to adsorption by precipitates during centrifugation.

**Amberlite IR CG-50 column chromatography**

1. **Elimination of amino acids.** As the gizzerosine content was much lower than the amino acid content which prevented the measurement of gizzerosine by HPLC, removal of amino acids from hydrolyzate was the most important step in this procedure. A few ion exchange resins and solvent systems were used in preliminary experiments. To remove amino acids from the hydrolyzate, the Amberlite IR CG-50 column using borate solution of pH 8.25 as an eluent was shown to be the most efficient. After the sample solution was put on an Amberlite column (A), elution profiles of amino acids and gizzerosine from the column using borate solution are shown as Fig. 2. Acidic amino acids, neutral amino acids and histidine were quickly eliminated from the column. Lysine was eluted by 200 to 400 ml of eluent and arginine was found in the later fractions. Gizzerosine was found in the latest effluent (over 450 ml). It was accordingly concluded that the best amount of borate solution was 350 ml.

2. **Elution of gizzerosine.** The elution of gizzerosine with HCl (0.5 or 1 n) was inadequate because it caused the elution of Na+ ions from Amberlite column (A) together with gizzerosine, and after evaporation of HCl considerable amount of NaCl (over 1 g) remained, which made the separation on HPLC impossible. On the other hand, the use of 0.5 n NH₄OH could decrease most of the elution of Na⁺ from the Amberlite column (A). But, even in this manner, the elution of Na⁺ was still found to be a little and it made the pH of the eluate higher during evaporation of NH₄OH, and gizzerosine was destroyed. To completely eliminate Na⁺ in the eluate, linking an Amberlite column (B) to the outlet of an
Amberlite column (A) was proved to be efficient. A small amount of Na\(^+\) eluted by 0.5 N NH\(_4\)OH from column (A) was perfectly trapped by column (B) and not found in the eluate through the linked columns.

To recover all the gizzerosine from the linked columns, 40–60 ml of 0.5 N NH\(_4\)OH was used, therefore, 100 ml of elution volume was used in this analytical procedure. Recovery using a gizzerosine standard solution by Amberlite IR CG-50 column chromatography was 98–100\%, and when gizzerosine was added to fish meal hydrolyzate it was 93–95\%, respectively.

**HPLC**

HPLC analysis was done using an OPA post-column detection system for amino acids, of which the elution system was modified. Several solvent systems were used in this experiment and a good result was obtained using gradient elution with two kinds of sodium borate buffer—solvent A and solvent B, prepared as described above. The Na\(^+\) concentration was kept at 0.1 M. The solvents containing 3\% EtOH (v/v) were effective to avoid interfering peaks in the chromatogram, which appeared near the gizzerosine peak and sometimes overlapped with it. Solvent C (0.8\% NaOH) was used for washing so that any residual sample components would be cleaned from the analytical column.

A Shimadzu LC-6A liquid chromatograph, mainly used in this study, was equipped with a high pressure type gradient elution system. A Hitachi 638 liquid chromatograph which was equipped with a low pressure type gradient elution system was also used, but no significant difference was found between the two systems.

The optimum fluorometric detection was done at Ex. 350 nm and Em. 450 nm, which was the same as that generally used for amino acid analysis.

Figure 3 (a) shows the HPLC chromatogram of fish meal. (a) Sardine meal. (b) Sardine meal added with gizzerosine at a level of 5 ppm.

![HPLC Chromatogram of Fish Meal](image)

Fig. 3. HPLC Chromatogram of Fish Meal. (a) Sardine meal. (b) Sardine meal added with gizzerosine at a level of 5 ppm.
Measurement of Gizzerosine

Table II. Recovery of Gizzerosine by Proposed Method

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>(1)</th>
<th>(2)</th>
<th>(3)</th>
<th>(4)</th>
<th>(5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gizz. added (µg)</td>
<td>0</td>
<td>25.0</td>
<td>25.0</td>
<td>25.0</td>
<td>25.0</td>
</tr>
<tr>
<td>Gizz. found (µg)</td>
<td>0.2</td>
<td>22.8</td>
<td>23.1</td>
<td>23.0</td>
<td>23.2</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>—</td>
<td>91</td>
<td>92</td>
<td>92</td>
<td>93</td>
</tr>
</tbody>
</table>

Sample: sardine meal 0.5 g.

Table III. Gizzerosine Content of Commercial Fish Meals

<table>
<thead>
<tr>
<th>Produced region</th>
<th>No. of samples (detected/examined)</th>
<th>Averaged content of detected, ppm (range, ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Japan</td>
<td>4/14</td>
<td>1.5 (1.0~2.2)</td>
</tr>
<tr>
<td>South America</td>
<td>9/16</td>
<td>2.5 (1.0~6.3)</td>
</tr>
<tr>
<td>Other regions</td>
<td>2/3</td>
<td>4.6 (3.8~5.3)</td>
</tr>
</tbody>
</table>

Detection limit: 1.0 ppm.

Gram of sardine meal hydrolyzate in which gizzerosine was not detected. Figure 3 (b) shows the HPLC chromatogram of sardine meal hydrolyzate (Fig. 3 (a)) added with synthetic gizzerosine at a level of 5 ppm. Gizzerosine was eluted between lysine and arginine, and the retention time was 25 min. Relative fluorescence intensity compared to lysine was about one third. Though large amounts of histidine, lysine, arginine, and other unknown peaks were seen in the figure, gizzerosine was clearly separated from them. Figure 4 shows the calibration curve of gizzerosine. The peak area was proportional to the amount of gizzerosine within the range of 0.01 ~ 0.2 µg.

Recovery of gizzerosine by proposed method

A recovery test was done using sardine meal added with a known amount of gizzerosine. The results are shown in Table II, and more than 90% recoveries were obtained. The detection limit was found to be 1 ppm.

Gizzerosine content of commercial fish meals

Table III shows the analytical data of commercial fish meals. Gizzerosine was often detected in the samples imported into Japan within the range of 0 ~ 6.3 ppm, while it was less often detected in the samples produced in Japan.

REFERENCES