Rapid Paper

Gizzerosine, a New Toxic Substance in Fish Meal, Causes Severe Gizzard Erosion in Chicks

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"Black vomit" is a serious disease in chicks and is caused by some kinds of fish meal in the diet. However, the causative compound has not been identified. In the present study, a new substance, 2-amino-9-(4-imidazolyl)-7-azanonanoic acid, has been isolated from mackerel meal and named Gizzerosine. This compound caused severe gizzard erosion in chicks within a week when it was fed to them at the level of 2.2 ppm in the diet. Gizzerosine is produced during fish meal manufacturing by the reaction between histidine and protein in the fish meat. The discovery of Gizzerosine raises a new problem in food chemistry and animal nutrition.

"Black vomit" is a serious disease in chicks and is a big problem in poultry production all over the world.^{1~8)} This disease is accompanied by gizzard erosion and, in severe cases, gizzard ulceration. The causative material in the feed is known to be brown fish meal, especially when overheated, suggesting that the active principle is formed during the heat treatment of manufacturing. One of the active substances in such fish meals has been shown

to be histamine.^{9,10)} However, a relatively large amount of histamine needs to be included in feeds to induce gizzard erosion (usually 0.4~0.5%).^{9,10)} Another line of experiments showed that the active substance was produced by a model system, namely by heating casein with histidine, and that the activity appears in the protein fraction.⁸⁾ This strongly suggests that the major principle is not free histidine and that free histidine in meat would react with some side chain of amino acids in protein to give the active substance. However, the substance which causes the symptoms had not been identified. This paper demonstrates that a new substance isolated from hydrolyzed fish meal induces the disease. The compound, named Gizzerosine, was characterized as a derivative of histamine, 2-amino-9-(4-imidazolyl)-7-azanonanoic acid.

MATERIALS AND METHODS

A special batch of mackerel meal, which induced severe gizzard erosion in chicks in commercial broiler farms and in our laboratory was hydrolyzed in 6N HCl for 24 hr at 110°C. After removing the HCl by evaporation, the hydrolysate was applied to a Dowex 50W×8 column (H form). The column was washed successively with deionized water, 0.5N HCl and 2N HCl to remove impurities and the active substance was recovered by eluting the column with 4N HCl. This fraction was evaporated to dryness and subjected to an Amberlite IR CG-50 column (*N*-ethylmorpholine form, equilibrated with 1M *N*-ethylmorpholine). After subsequently washing the column with 1M and 2M *N*-ethylmorpholine, the active substance was eluted with 3M aqueous ammonia. The eluate was evaporated to dryness and subjected to a LiChroprep RP-8 column (Merck). The active compound eluted from the column with 0.05N triethylammonium acetate (pH 7.0)¹¹⁾ was lyophilized and subjected to cellulose column chromatography. Elution with isopropanol-aqueous ammonia (28%)-water (16:1:4) gave an active fraction which was lyophilized and further purified by cellulose plate-thin layer chromatography. The plate was developed with a solvent of isopropanol-aqueous ammonia (28%)-water (16:3.5:4). The *R_f* of the active compound was 0.56. The substance was extracted from the plate with water and finally purified by high performance liquid chromatography, employing an octadecylsilica gel column (Nucleosil NC-18). The active compound was obtained by eluting with 0.05N triethylammonium acetate (pH 7.0).¹¹⁾ We finally isolated approximately 2 mg of the active compound from 10 kg of

the original fish meal.

The activity to induce gizzard erosion in chicks was determined as described previously.⁵⁾ Briefly, preparations obtained during the purification procedure were mixed with a commercial broiler starter diet and fed to 3-day-old broiler chicks (male and female). After 7 days of feeding, the chicks were killed under chloroform anesthesia and the symptoms of gizzard erosion were inspected. The symptoms were classified into 4 scores¹²⁾: *i.e.*, 0, completely normal gizzards; 1, healthy gizzards but with small local aberrations; 2, gizzards with evident signs of erosion; and

3, severely affected gizzards with hemorrhaging and/or with the linings partially destroyed or chicks dead from gizzard erosion and/or ulceration. Five chicks were usually employed for a sample to assay the activity. The scores were summed and compared with those of the groups of chicks fed with the active fish meal (positive control) and those fed with the inactive one (negative control). The positive control chicks showed a score of over 12 and the negative control chicks less than 2. We defined "one unit" tentatively as the activity to induce the symptoms scoring at least 10 by this procedure. For example, 1 unit/mg means that 5 chicks will show the summed score of more than 10 within 7 days when 1 mg of the preparation is given to the chicks every day. Except for the Gizzerosine fraction, we could not find any other active fractions during the purification procedure. The recovery of activity at every purification step is summarized in Table I.

TABLE I. RECOVERY OF ACTIVITY DURING THE PURIFICATION PROCEDURE

Purification steps	Total dry weight (mg)	Total activity (unit) ^a	Specific activity (unit/mg)
Fish meal	10,000,000	4,400	0.00044
Dowex 50W × 8	50,000	3,800	0.076
Amberlite IR CG-50	12,000	1,100	0.092
LiChroprep RP-8	2,000	550	0.28
Cellulose plate	100	500	5.0
Octadecylsilica gel	7	280	40

^a The activity in a ration to produce the symptoms of gizzard erosion in more than 80% of chicks within 7 days was defined tentatively as 1 units.

RESULTS AND DISCUSSION

Figure 1 shows the elution profile of the octadecylsilica gel column chromatography, the final step of the purification procedure. The peak 2 was the active fraction.

The chemical structure of the active compound, Gizzerosine, was elucidated as follows. The molecular weight of Gizzerosine was determined to be 240 by FD mass spectrometry.

FIG. 1. The Elution Profile of the Octadecylsilica Gel Column Chromatography of Partially Purified Fish Meal Hydrolysate.

Peak 2 was the active compound, Gizzerosine, which caused severe gizzard erosion in chicks.

FIG. 2. Structure and 400 MHz ^1H -NMR Spectra of Natural and Synthetic Gizzerosine, a New Compound Which Causes Gizzard Erosion in Chicks.

(a) natural Gizzerosine isolated from mackerel fish meal which caused gizzard erosion in chicks. (b) synthetic Gizzerosine.

Gizzerosine was converted to a volatile derivative by treating it with acetic anhydride and methanol at room temperature for 48 hr. The EI high resolution mass spectrometry of the derivative gave the molecular ion at m/z 338.19518 ($\text{C}_{16}\text{H}_{26}\text{O}_4\text{N}_4$, calculated 338.19540) showing the presence of two acetylatable amino and/or imino groups and one free carboxyl residue in the original compound. Thus, the molecular formula of Gizzerosine was determined to be $\text{C}_{11}\text{H}_{20}\text{N}_4\text{O}_2$. The 400 MHz ^1H -NMR spectrum of Gizzerosine (Fig. 2a) taken in D_2O showed two heteroaromatic protons at δ_{H} 7.32 and 8.43 due to an imidazole nucleus. Spin decoupling experiments revealed the two additional groups, $-\text{N}-(\text{CH}_2)_4-\text{CH}-\text{N}-$ and $=\text{C}-\text{CH}_2-\text{CH}_2-\text{N}-$, suggesting the presence of lysine and histamine residues in the molecule. The close similarity of the ^1H -NMR spectral data of Gizzerosine and those of authentic amino acids supports this assumption and suggests that the ϵ -methylene in lysine is not connected to the imidazole nitrogen and that the amino methylene in histamine is not involved in the amide bond formation. With additional data by the linked scan method of

EI mass spectrometry of the above-mentioned derivative of Gizzerosine and by means of chemical synthesis,¹³⁾ the structure was finally confirmed as that shown in Fig. 2.

A feed with 2.2 ppm of purified natural Gizzerosine caused severe gizzard erosion in chicks (score 12) within 7 days. A feed containing 6.25 ppm of synthetic (racemic) Gizzerosine also induced severe gizzard erosion in more than 80% of chicks (score 12) within a week. It can be reasonably assumed that the natural Gizzerosine is L-form. Differential synthesis of D- and L-form Gizzerosine is now under study and the activity of each form will be reported soon.

Although many substances are known to be formed in foods and feedstuffs during heating, few biologically active compounds are reported to have been isolated. Gizzerosine showed a far stronger activity than histamine in inducing gizzard erosion or ulceration in chicks. The discovery of Gizzerosine will contribute very much to the poultry industry and to fish meal manufacturing in the world. In particular, overheating should be carefully avoided when producing fish meals.

The effect of Gizzerosine on mammals re-

mains to be elucidated. We could not find any visible effects on rat stomach after 6 months feeding of the fish meal¹⁴⁾ which caused severe gizzard erosion in chicks. However, this does not necessarily rule out the possibility of the induction of some physiological effects on mammals, including man, when a larger amount of Gizzerosine than its natural content in foodstuffs is administered. Dried and heated (or broiled) fish is consumed all over the world. It will be very important to study the distribution of Gizzerosine in foodstuffs. The discovery of Gizzerosine raises a new problem in food chemistry and animal nutrition.

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