Procedure for Radiolabeling Gizzerosine and Basis for a Radioimmunoassay

Marcela Torres,[†] Headdy Manosalva,[†] Isabel Carrasco,[†] Alfredo E. De Ioannes,[†,‡] and María Inés Becker*,[†,‡]

Laboratorio de Inmunología, Departamento de Investigación y Desarrollo, Biosonda S.A., Eduardo Castillo Velasco 2902, Santiago, Chile, and Laboratorio de Inmunología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Alameda 340, Santiago Chile

A method for the labeling of gizzerosine (GZ), a biogenic amine found in fish meal, is described. The labeling procedure with ^{125}I using a water-soluble Bolton−Hunter reagent and a mild water-insoluble oxidant (Iodogen) reagent is rapid and reproducible. The ^{125}I-GZ hapten was demonstrated to be immunologically active in a radioimmunoassay developed with polyclonal antibodies to GZ absorbed with a histamine−Sepharose column. The curves were linear in the range of 0.0001 and 0.1 μg/mL. Samples of fish meal previously extracted of histamine with methanol and submitted to acid hydrolysis were contaminated with known amounts of GZ and submitted to the assay. The fish meal samples contaminated with GZ show a dose−response effect similar to the standard curve, and apparently the other component present in the sample did not interfere with the binding of the antibodies to ^{125}I-GZ. These data indicate the suitability of the radioimmunoassay to determine specifically GZ in fish meal.

INTRODUCTION

Gizzerosine [2-amino-9-(4-imidazolyl)-7-azanonanoic acid; GZ) is a biogenic amine found in brown fish meal. This substance causes the disease called black vomit or gizzard erosion, observed in chicks after feeding (Okasaki et al., 1983; Mori et al., 1983, 1985; Toyama et al., 1985), which causes important losses in poultry production (Wessels and Post, 1989; Fossum et al., 1988). Fairgrieve et al. (1994) reported that salmon fed with a diet rich in fish meal contaminated with GZ presented a stomach with serious alterations.

The formation of GZ has been related to high histamine (H) levels and overheating in the processing of red fish meal rich in lysine. It is assumed that GZ would be produced by a reaction at high temperature between the ϵ-amino group of lysine with the H ethylimidazole group (Okasaki et al., 1983). Histamine is another bioactive substance found in fish meal, but a relatively large amount of this compound in feed (usually 400−500 ppm) is needed to induce gizzard erosion (Harry et al., 1975; Kazama et al., 1980; Kuba et al., 1983; Masumura et al., 1985; Sugahara et al., 1987, 1988). Although GZ has proven to be a histamine H2-receptor agonist, this compound is ~1000 times stronger and has a longer lasting effect than H (Masumura et al., 1985; Hino et al., 1987; Ito et al., 1987). Sugahara et al. (1987) reported that GZ causes chicken mortality at concentrations >1 ppm.

At present, no fast and sensitive in vitro specific assay is available for the determination of GZ in fish meal.

The method currently available for determining its presence is a bioassay using 4-day-old chickens fed with the test meals, and it takes ~7 days to get the scores. Semiquantitative scores are assigned after inspection of the gizzard in comparison to a reference or control fish meal (Horaguchi et al., 1981; Castro, 1991). Although the bioassay is sensitive, it lacks the efficiency, accuracy, and reproducibility of other analytical methods currently used for toxin detection in food. Some procedures for GZ determination using high-performance liquid chromatography (HPLC) have been reported, but they lack the sensitivity and reproducibility required (Ito et al., 1985; Murakita et al., 1990). Wagener et al. (1991) developed an HPLC method and obtained a sensitivity of 10 mg/kg. Notwithstanding, they analyzed 17 samples of fish meal hydrolyzed with HCl—some of which had caused severe erosion in the chicken gizzard—and did not find GZ in any of them. This suggests that the fish meal causing gizzard erosion would have GZ levels <10 mg/kg.

Immunochemical methods have gained wide application for toxin detection in food, and hence there is a great demand for specific antibodies for the assay (Hsu and Chu, 1994). Radioimmunoassay (RIA) and competitive enzyme-linked immunosorbent assay (ELISA) may reach adequate sensitivity and specificity for GZ detection in fish meal. However, the production of antibodies to GZ faces serious difficulties, because, on the one hand, GZ is a hapten (M_r = 240) and, besides, the immunochemical reagent must be free of cross-reaction to histamine, a structurally related compound present in fish meal at higher concentrations than GZ. Rosselot et al. (1996) developed an RIA to evaluate GZ in fish meal and reported a high degree of cross-reaction of the anti-GZ rabbit polyclonal sera with histamine; they found that GZ would be present in a range of 1.6−26

* Author to whom correspondence should be addressed [telephone (56-2) 209-6770; fax (56-2) 274-5462; e-mail biosond@ibm.net].

† Biosonda S.A.

‡ Pontifica Universidad Católica de Chile.

4232 *J. Agric. Food Chem.,* Vol. 47, No. 10, 1999

Torres et al.

μg/mL in fish meal with a score between 0 (no erosions) and 3 (severe erosions), respectively.

Another problem to be solved when an RIA is developed for small molecules such as GZ is radiolabeling, because the hapten lacks a tyrosine residue. To add this group, should be kept the immunodominant group without significant modifications and a mild ^{125}I radiolabeling must be used to avoid oxidation of the gizzerosine domain.

The present paper describes an alternative method for labeling GZ with ^{125}I, using a water-soluble Bolton–Hunter reagent (Bolton and Hunter, 1973) and a mild water-insoluble oxidant (Iodogen) reagent (Fraker and Speck, 1978). Also, we describe the basis for development of an RIA using rabbit polyclonal serum to GZ absorbed with histamine. A preliminary evaluation of the assay was tested with synthetic GZ added to samples of fish meal.

MATERIALS AND METHODS

Development of Anti-GZ Polyclonal Antibodies. *1. GZ Coupling to Carrier Protein.* GZ chemically synthesized (Ajinomoto, Japan) was coupled to hemocyanin from *Concholepas concholepas* (CCH; Blue Carrier developed by Biosonda S.A., Santiago, Chile) using the protocol described by Coligan et al. (1992) with minor modifications by Becker et al. (1998). Briefly, 5 mg of the carrier protein was dissolved in 2 mL of 0.1 M borate buffer (pH 10), and 1 mg of GZ in bidistilled water was slowly added. Glutaraldehyde (Polysciences) was then added to a final concentration of 0.1%. The mixture was incubated for 2 h in darkness at 25 °C and then dialyzed against phosphate buffer saline (PBS; 0.1 M NaCl, 2.5 mM KCl, 1.5 mM KH_2PO_4, 7 mM Na_2HPO_4, pH 7.2) at 4 °C. This antigen was named GZ–CCH.

To determine the specificity of the antibodies, the following adducts were prepared: GZ coupled to bovine serum albumin (Sigma Chemical Co., St. Louis, MO) (GZ–BSA). Histamine (Sigma) was coupled to BSA (H–BSA) and BSA without hapten but treated with glutaraldehyde, to detect antibodies reacting with neoepitopes generated by the coupling agent.

2. Rabbit Immunization. Two female New Zealand rabbits were immunized with GZ–CCH. On day 1, they received a subcutaneous and intradermal injection of 500 μL of antigen in complete Freund's adjuvant (Pierce); on days 17, 37, and 57, the same immunization was carried out, but with incomplete Freund's adjuvant (IFA) (Pierce). At day 87, 1 mg of the antigen in PBS and emulsified in IFA was given intramuscularly and 0.5 mg subcutaneously. Prior to the immunizations, the rabbits were bled by the ear to obtain preimmune control serum. Ten days after each injection, the animals were bled for antiserum collection. A direct ELISA (Crowther et al., 1980) was used to determine the presence of antibodies against GZ.

3. GZ–BSA ELISA. The ELISA was developed as described by Becker et al. (1998). Briefly, 96-well polystyrene plates (Pierce) were activated overnight at 4 °C with 50 μL of a solution of 10 μg/mL GZ–BSA or H–BSA as control and blocked with 250 μL/well of 1% PBS–OVO for 1 h at room temperature. Two-fold serial dilutions (50 μL) of immune serum in PBS–OVO solution were added to the plates followed by incubation for 1.5 h at room temperature. The plates were washed three times for 2 min with 0.02% PBS–Tween. Fifty microliters of goat serum to anti-rabbit IgG conjugated to ALP (Sigma Chemical Co.) diluted 1:1000 in PBS–OVO solution was then added to the wells and incubated for 30 min at room temperature. The plates were washed as described above and revealed during 30 min at 37 °C with 50 μL/well of pNPP 1 mg/mL in a buffer solution containing 0.1 M Tris, 0.1 M NaCl, and 5 mM $MgCl_2$ (pH 9.5). The reaction was stopped with 50 μL of 3 N NaOH, and absorbance was read at 405 nm. Preimmune serum from the experimental animals was used as control.

4. Purification of Rabbit Anti-GZ Serum by Affinity Chromatography. One milliliter of rabbit immune serum centrifuged at 12000 rpm for 5 min was applied on a 3 mL histamine–Sepharose column (Sigma Chemical Co.). The sample was left overnight in the column at 4 °C and eluted from the column; sodium azide, at a final concentration of 0.1%, was added to the fractions. Protein concentration was determined by a Coomasie Plus assay (Pierce). Determinations were performed according to the manufacturer's instructions.

Radioiodination of GZ. All solutions were freshly prepared with grade pro analysis reagents. Ten milligrams of water-soluble Bolton–Hunter reagent [(sulfosuccinimidyl-3-(4-hydroxyphenyl) propionate] (Pierce) was dissolved in 100 μL of DMSO (Merck, Darmstadt, Germany) in an Ependorff tube; 36 μL of 0.1 M sodium borate buffer (pH 9.0) was added to 4 μL of Bolton–Hunter solution, and it was labeled BH*. At the same time, in a Khan tube, 100 μg of GZ was dissolved in 300 μL of sodium borate buffer (pH 9.0) and—in a new Ependorff tube—400 μCi of [125]INa (Comisión Chilena de Energía Nuclear, Santiago, Chile) with 10 μL of sodium borate buffer were added. Radiolabeling was started by adding 80 μL of 0.1 M sodium borate buffer (pH 9.0) to a precoated Iodogen–[1,3,4,6-tetrachoro-3α,6α-diphenylglycouril] tube (Pierce), and then 20 μL of solution BH* and 10 μL of ^{125}I solution were added. The mixture was incubated for 1 min at room temperature. To stop the iodination reaction, the solution was transferred to the test tube with GZ and 7 μL of 0.1 M sodium metabisulfite was added. The solution was incubated for 30 min at room temperature, and then the mixture was applied on a Sep-Pak C_{18} column preactivated with 3 mL of methanol (Merck). The column was eluted stepwise with increments of 2% in acetonitrile (0–24%) in 0.1% trifluoroacetic acid (TFA) (Merck). The ^{125}I-GZ was collected from 10 to 16% acetonitrile fractions in a total volume of 9 mL.

Development of RIA. Sodium borate buffer (0.1 M, pH 8,9) containing 1% BSA was used for dilutions of the RIA components: anti-GZ serum, samples, ^{125}I-GZ, and GZ standards.

1. Binding Studies. The buffer (200 μL) was added to polypropylene RIA tubes (Falcon), and then 100 μL of the ^{125}I-GZ containing ~10000 cpm and 100 μL of the diluted anti-GZ antibody were added; the contents of the tubes were stirred and incubated overnight at 4 °C. The immune complexes were precipitated by adding 100 μL of 1:2 diluted goat anti-rabbit IgG serum (Nuclear Medicine Laboratory, Pontificia Universidad Católica, Santiago, Chile), 100 μL of 2% normal rabbit serum, and 500 μL of 8% (w/v) poly(ethylene glycol) 8000 (PEG) (Sigma). Samples were incubated for 2 h at 4 °C and centrifuged at 2700 rpm for 45 min. The supernatant was discarded, and the walls were carefully dried with filter paper. The bound radioactivity was determined in a γ-counter. Nonspecific binding was determined using normal rabbit serum instead of anti-GZ antibody. The working dilution of the rabbit polyclonal was determined by immunoprecipitation assays of ^{125}I GZ with serial dilutions of the antibodies.

2. Displacement Studies. The specificity and sensitivity performance of the anti-GZ antibodies in a competitive RIA were determined by displacement. The standard curve was prepared by serial dilutions in 0.1 M sodium borate buffer/1% BSA of a stock solution of 1 mg/mL GZ. Displacement experiments were done with antibody dilutions binding ~30% of the tracer. The RIA was developed as follows: 100 μL of buffer borate, 100 μL of the first antibody in the appropriate dilution, 100 μL of the tracer (~10000 cpm), and 100 μL of cold GZ at growing concentrations (0–10 μg/mL) were placed into polypropylene tubes. The mixture was stirred and incubated at 4 °C, and the immune complexes were precipitated as described above.

Similar assays were conducted for specificity studies, with increasing concentrations of histamine in PBS.

3. RIA with Fish Meals. Three samples of fish meal were processed as described by Okasaki et al. (1983) with modifications. Five milliliters of methanol was added to 50 mg of fish meal in a centrifuge tube with vigorous shaking, and the excess of methanol was eliminated by centrifugation at 2500 rpm for 20 min and drying at 37 °C for 24 h. Proteins present in the
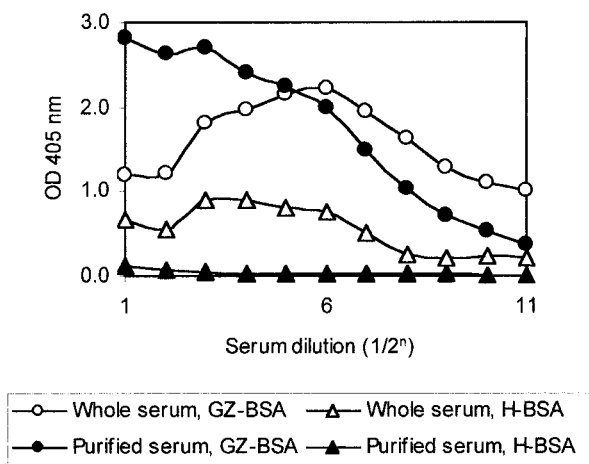
Gizzerosine Radioimmunoassay

J. Agric. Food Chem., Vol. 47, No. 10, 1999 **4233**

Figure 1. Rabbit immune humoral response to GZ; titration of serum from rabbit immunized with GZ–CCH, using a direct ELISA: nonabsorbed serum (open circles) and adsorbed serum on a Sepharose–histamine column (solid circles). The plates were activated with GZ–BSA. The binding of the antibodies to the plate was determined using an anti-rabbit IgG labeled with FAL. Due to the dilution of the serum resulting from the affinity chromatography, normal serum was diluted to the same protein concentration of the nonadsorbed serum before titration. The first dilution corresponds to 1:100 and, then, continuous serially.

samples were acid hydrolyzed as follows: 10 mg of extracted fish meal was hydrolyzed by adding 1 mL of 6 N HCl in an N_2 atmosphere for 24 h at 110 °C. The final solution was centrifuged at 10000 rpm and was kept at 4 °C until used. Samples were neutralized by dilution (1:40) in 0.1 M sodium borate (pH 9) buffer.

Synthetic GZ was added to neutralized samples at final concentrations of 0, 0.001, 0.01, 0.1, and 1 μg/mL. The RIA was developed as follows: 100 μL of borate buffer (pH 9) 1% BSA, 100 μL of the first antibody at the appropriate dilution, 100 μL of the tracer (~10000 cpm), and 100 μL of sample containing synthetic GZ added to fish meal meals were placed into polypropylene tubes. The mixture was stirred and incubated at 4 °C and the immune complexes were precipitated as described before.

RESULTS

Development of Anti-GZ Polyclonal Antibodies. The best results were obtained with GZ linked to hemocyanin from *C. concholepas* with glutaraldehyde in borate buffer (pH 10). The same procedure was used to generate monoclonal antibodies to GZ (Becker et al., 1998). The presence of rabbit antibodies to GZ in rabbit serum was determined by a direct ELISA on plates activated with GZ–BSA, H–BSA, and BSA treated with glutaraldehyde. Results presented in Figure 1 showed a high titer of antibodies to GZ (~2^{14} dilutions), both rabbits exhibiting similar titers. However, as expected, the antiserum showed strong cross-reaction to histamine, because both molecules share the imidazole domain.

The development of a specific immunoassay for GZ in the presence of histamine is a difficult task because the concentration of GZ is much lower than that of histamine in fish meal; hence, a small cross-reaction of the antibodies with histamine yields unreliable results. To tackle this problem, the anti-GZ rabbit antiserum was adsorbed on a histamine–Sepharose column and re-evaluated by ELISA. Figure 1 shows that this procedure was effective for the removal of anti-histamine antibodies.
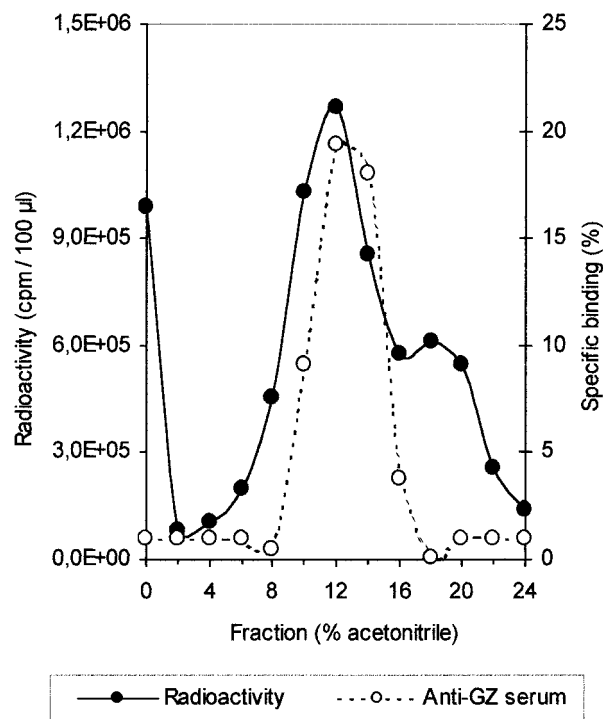

Figure 2. Elution profile of ^{125}I-labeled GZ. Radioiodination was carried out using a water-soluble Bolton–Hunter reagent and a mild water-insoluble oxidant (Iodogen) as described under Materials and Methods. Elution of the ^{125}I in the C_{18} Sep-Pak cartridges was performed with 2% increments of an acetonitrile gradient containing 0.1%TFA. Fractions were screened for specific immunoprecipitation using rabbit antisera to GZ diluted 1:1000 (open circles). Radioactivity in each fraction was measured in 100 μL aliquots using a Gamma counter.

Radioiodination of GZ. Figure 2 shows the elution profile of ^{125}I-GZ from the column; the first peak elutes in fractions containing acetonitrile from 10 to 16%. The immunoprecipitation analysis with anti-GZ rabbit serum indicated that fractions eluting from 12 to 14% acetonitrile presented immunoreactivity. These results were similar in three independent labeling experiments and fluctuated ~30% with GZ antiserum diluted 1:1000. In all experiments, a second nonantigenic radioactive peak eluting between 18 and 20% of acetonitrile was observed. It may correspond to the ^{125}I-Bolton–Hunter free reagent. The labeled hapten was still immunoreactive after 3 months of storage at 4 °C.

GZ RIA. Figure 3 shows the results of an RIA using free GZ and ^{125}I-GZ with the rabbit antiserum to GZ. Experiments were carried out under two different conditions: (1) at equilibrium, for example, co-incubating the antibodies with the labeled and nonlabeled hapten; (2) nonequilibrium, for example, incubating overnight with GZ, then adding ^{125}I-GZ for 1 h at 4 °C. Similar displacement curves were observed for both assays, with a linear range of displacement between 0.0001 and 0.1 μg.

To determine the specificity of the RIA, experiments were run in the presence of increasing concentrations of GZ and histamine with the anti-GZ rabbit antiserum normal and adsorbed by a histamine–Sepharose column. Results presented in Figure 4 show that histamine concentrations >1 μg/mL lightly displaced the curves. These results make it necessary, anyway, to eliminate histamine of fish meal samples before it is submitted to acid hydrolysis for GZ extraction.
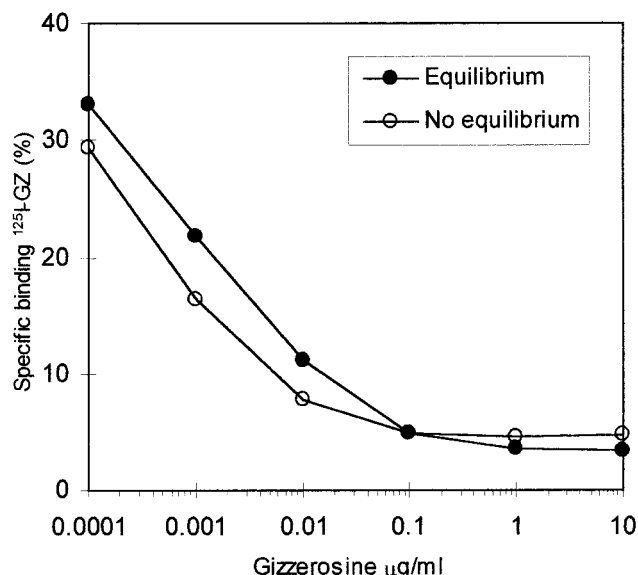
4234 *J. Agric. Food Chem.,* Vol. 47, No. 10, 1999

Torres et al.

Figure 3. Radioimmunoassay to synthetic GZ with polyclonal antibodies to GZ: (solid circles) experiments under equilibrium conditions, e.g., co-incubating the antibodies with GZ and ^{125}I; (open circles) experiments under nonequilibrium conditions, e.g., first, the rabbit polyclonal antibodies to GZ were incubated overnight at 4 °C with GZ and, then, by adding ^{125}I for 1 h at 4 °C. Each point in the plots is the mean of duplicate measurements. The experimental error was <10%.
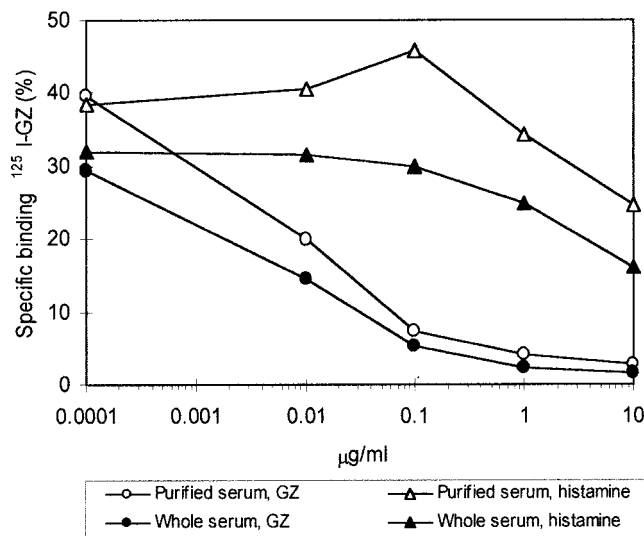

Figure 4. Specificity of GZ RIA. The assay was developed by incubating normal serum diluted to the same protein concentration of the adsorbed serum (titer near 1:1000) with ^{125}I-GZ and increasing amounts of GZ (solid circles) or histamine (open circles), a biogenic amine structurally related to GZ present in brown fish meal. Each experimental point in the plots is the mean of duplicate measurements. The experimental error was <10%.

A critical point in the development of a reliable RIA for GZ was to determine the effect of other compounds of the fish meal and, as mentioned above, the extraction procedure for the samples. GZ is mostly incorporated into proteins; consequently, it is necessary to submit the samples to the procedure described by Okasaki et al. (1983). To this end, samples of fish meal were first extracted of histamine with methanol, then submitted to acid hydrolysis, neutralized, and finally contaminated with known amounts of synthetic GZ (in the range of 0–1 µg/mL). Although the preliminary results of Table 1 obtained in this RIA are promising, because a dose–

Table 1. Gizzerosine RIA: Effect of Fish Meal Samples Previously Extracted of Histamine and Submitted to Acid Hydrolysis and Then Contaminated with Gizzerosine[a]

standard curve		% binding in fish meals		
GZ (µg/mL)	% binding	sample 1	sample 2	sample 3
0	100	100	100	100
0.001	85.1	100	72.7	73.4
0.01	18.8	24.5	37.5	25.4
0.1	6.1	1.8	5.5	4.3
1	0	0	1.0	1.4

[a] The experiments were run in duplicate with an experimental error of 10%.

response effect was observed in the antibodies binding to ^{125}I-GZ in the presence of GZ added to fish meal, they show that the treatment procedure of the sample needs to be improved. We think that such variations may largely be explained by the complexity and heterogeneity of the fish meal samples. Nonetheless, the results showed that 6 N HCl neither destroys nor modifies the GZ and, apparently, no other compounds of the fish meal would interfere with the binding of the antibodies to ^{125}I-GZ.

DISCUSSION

Producers as well as consumers of fish meal have outlined the need of controlling the presence of biogenic amines, that is, toxic compounds generated during the degradation of proteins present in fish meal by the action of microorganisms on amino acids along the production process (Kimata, 1960). Among the biogenic amines, GZ causes important losses in poultry production. It is the main cause of black vomit, a chicken disease with severe ulceration of the gizzard (Okasaki et al., 1983; Sugahara et al., 1987). On the other hand, little is known of its effect on other animal species. Therefore, its determination remains as an important issue in the quality control of fish meal.

In this work, we describe a procedure to develop rabbit polyclonal antibodies against GZ, their characterization, and their application to the development of an RIA. Also, a reliable and mild iodination method of the compound is described that does not affect the antigenicity of the hapten. The radiolabeled hapten has been used for setting up an RIA and to screen a panel of anti-idiotypic monoclonal antibodies directed to the monoclonal GZ−3H4 specific for GZ (H. Manosalva, A. E. De Ioannes, M. I. Becker, unpublished data).

The Bolton−Hunter method (Bolton and Hunter, 1973) for radiolabeling proteins has been used extensively, and various modifications to improve the procedure have been developed as to incubation time, temperature, nature of the oxidizing agent for converting ^{125}I into the reactive species ^{125}I$_2$ or ^{125}I$^+$ (Langone, 1980; Bailey, 1996a,b). In the present work, we report a two-step method that uses an Iodogen coated tube to label the water-soluble Bolton−Hunter reagent. Simply removing the labeled reagent from the tube can control the extent of the iodination. The Bolton−Hunter labeled reagent is incubated with GZ, avoiding possible harmful effects arising from the direct contact of the hapten with the oxidant. This method is a simple procedure that does not impair the subsequent binding of the ^{125}I-GZ to the antibodies. Recently, Rosselot et al. (1996) labeled GZ with a water-insoluble Bolton−Hunter reagent in the presence of a strong oxidant. In addition to the concerns
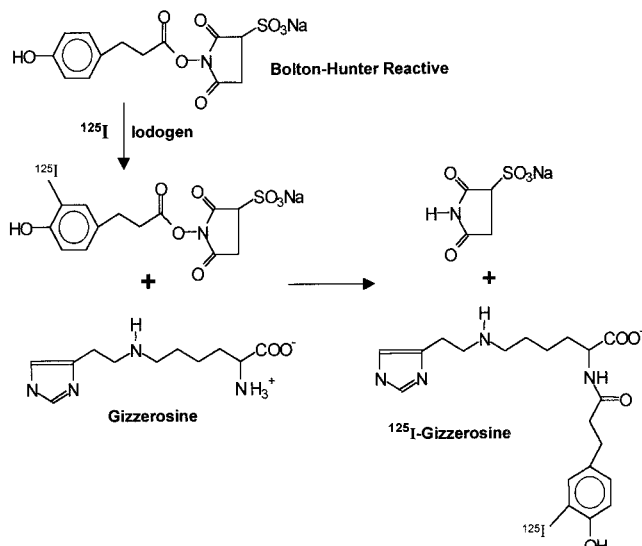
Figure 5. Diagrams showing the labeling reactions of GZ with ^{125}I using a water-soluble Bolton−Hunter reagent and a mild water-insoluble oxidant reagent (Iodogen) and subsequent generation of ^{125}I-GZ.

arising from the use of choramine T, the small reaction volumes make it difficult to handle the organic phases that contain the ^{125}I-GZ for further purification.

As shown in Figure 5, the labeling method described here introduces the Bolton−Hunter molecule, already labeled, on the α-amino group of the lysine moiety of GZ at sufficient distance from the imidazole group, thus minimizing steric effects that may impair the antibody binding to the tracer. Also, the two-step labeling procedure may avoid losses of antigenicity due to oxidation.

The lack of an appropriate immunoassay for GZ determination in fish meal may be attributed to its being a hapten and, therefore, the production of antibodies against it implies its coupling to carrier proteins. Moreover, the recognition of antibodies may be independent of the modification induced on the hapten by the coupling agent. Previous works of our research group to obtain anti-GZ monoclonal antibodies indicate that when GZ is coupled to hemocyanin with carbodiimide, the humoral response against the hapten is poor (Becker et al., 1998). This is confirmed by Rosselot et al. (1996), who used carbodiimide (CDI) to couple GZ to keyhole limpet hemocyanin (KLH) and obtained a low-titer serum. Although this method for coupling peptides creates a well-characterized and stable amide bond (i.e., those peptides bearing lysine residues), CDI may react at more than one site, possibly affecting antigenicity (Carter, 1996). To overcome this problem, we used glutaraldehyde, a homobifunctional cross-linker. In addition to the imidazole moiety, GZ exhibits the α-amino and α-carboxy group from lysine and a secondary amine group found only in GZ; perhaps these features make glutaraldehyde a better choice than CDI to produce polyclonal and monoclonal antibodies to gizzerosine.

Finally, we have already devised a method to label GZ and demonstrated the suitability of the assay to dose GZ. Nonetheless, the development of an assay attractive to laboratories that assess quality control of fish meal would need a more simple and efficient protocol to solubilize GZ from the protein fraction of fish meal. In this respect, experiments were run using enzymatic treatment with proteases—Pronase, trypsin, and pepsin or a mixture of them—on fish meal pretreated with methanol to extract histamine. However, the results (not shown) were not conclusive. We believe that enzymatic treatments may be an interesting approach, but a systematic work is required to determine the better enzyme(s) and the extraction conditions. Rosselot et al. (1996) reported that GZ extraction can be achieved by heating the fish meal in PBS at 37 °C for 2 h, without previous extraction of histamine. These results suggest that GZ would be water soluble, which is not in keeping with the current literature on the formation of the compound (Okasaki et al., 1983; Masumura et al., 1985; Sugahara et al., 1987). Furthermore, because histamine is not extracted and considering the degree of cross-reaction of the antiserum to GZ with histamine, the presence of this biogenic amine should strongly interfere with GZ in this polyclonal antibody based RIA.

The problem of cross-reaction may be avoided by using monoclonal antibodies (Köhler and Milstein, 1975; Becker et al., 1994, 1996; Becker and De Ioannes, 1998b). Our group has developed highly specific monoclonal antibodies to GZ that may be useful in the formulation of a competitive ELISA (Becker et al., 1998). In addition, they should be useful in a GZ RIA determination, and studies are in progress. However, at present our polyclonal-based RIA has shown to have better sensitivity than our monoclonal-based ELISA to gizzerosine.

ABBREVIATIONS USED

ALP, alkaline phosphatase; PBS, phosphate-buffered saline; BSA, bovine serum albumin; CCH, *Concholepas concholepas* hemocyanin; CDI, carbodiimide; ELISA, enzyme-linked immunosorbent assays; GZ, gizzerosine; H, histamine; HPLC, high-performance liquid chromatography; IFA, incomplete Freund's adjuvant; KLH, keyhole limpet hemocyanin; OVO, ovoalbumin; p-NPP, *p*-nitrophenyl phosphate; PEG, poly(ethylene glycol); RIA, radioimmunoassay; TFA, trifluoroacetic acid.

ACKNOWLEDGMENT

LITERATURE CITED

Bailey, G. S. The Bolton and Hunter method for radiolabeling protein. In *The Protein Protocols Handbook*; Walker, J. M., Ed.; Humana Press: Totowa, NJ, 1996a; pp 671−672.

Bailey, G. S. The Iodogen method for radiolabeling protein. In *The Protein Protocols Handbook*; Walker, J. M., Ed.; Humana Press: Totowa, NJ, 1996b; pp 673−674.

Becker, M. I.; De Ioannes, A. E. Anticuerpos monoclonales. In *Fundamentos de Inmunología*; Palomo, I., Ferreira, A., Sepúlveda, C., Rosemblatt, M., Vergara, U., Eds.; Universidad de Talca MIXM: Talca, Chile, 1998; pp 607−629.

Becker, M. I.; Juica, F.; Jamett, A.; Tzichinovsky, S.; Barros, S.; Aguayo, J.; De Ioannes, A. E. Development of anti-human B blood group monoclonal antibodies suitable for blood typing reagent. *Hybridoma* **1994**, *13*, 303−310.

Becker, M. I.; Aguayo, J.; Jamett, A.; Juica, F.; Yudelevich, A.; Foradori, A.; De Ioannes, A. E. An alternative ELISA for T4 determination based on idiotype anti-idiotype interaction and a latex method for anti-idiotype monoclonal antibody selection. *J. Immunol. Methods* **1996**, *192*, 73−85.

Becker, M. I.; Carrasco, I.; Beltrán, C.; Torres M.; Jaureguiberry, B.; De Ioannes, A. E. Development of monoclonal antibodies to gizzerosine, a toxic component present in fish meal. *Hybridoma* **1998**, *17*, 373−381.

Bolton, A. E.; Hunter, W. M. The labeling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. *Biochem. J.* **1973**, *133*, 529−538.

Carter, J. M. Conjugation of peptides to carrier protein via carbodiimide. In *The Protein Protocols Handbook*; Walker, J. M., Ed.; Humana Press: Totowa, NJ, 1996; pp 693−694.

Castro, E. Biotoxicología de harina de pescado. In *Tecnología Avipecuaria*; Publicaciones Media Relaciones S.A.: México City, Mexico, 1991; Vol. 36, pp 21−23.

Coligan, J.; Kruisbeek, A.; Margulies, D.; Shevach, E.; Strober, W. Peptides. In *Current Protocols in Immunology*; National Institutes of Health: Bethesday, MD, 1991; Vol. 1, Suppl. 1, p 9.4.3.

Crowther, J. R.; Abu-Elzein, E. M. Detection of antibodies against foot-and-mouth-disease virus using purified *Staphylococcus A* protein conjugated with alkaline phosphatase. *J. Immunol. Methods* **1980**, *34*, 261−267.

Fairgrieve, W.; Myers, M. S.; Hardy, R. W.; Dong, F. M. Gastric abnormalities in rainbow trout (*Oncorhynchus mykiss*) fed amine-supplemented diets or chicken gizzard-erosion-positive fish meal. *Aquaculture* **1994**, *127*, 219−232.

Fossum, O.; Sandstedt, K.; Engstrom, B. E. Gizzard erosions as a cause of mortality in white leghorn chickens. *Avian Pathol.* **1988**, *17*, 519−525.

Fraker, P. J.; Speck, J. C. Protein and cell membrane iodinations with a sparingly soluble chloramide 1,3,4,6-tetrachloro-3α-diphenylglycoluril. *Biochem. Biophys. Res. Commun.* **1978**, *280*, 849−857.

Harry, E. G.; Tucker, J. F.; Laursen-Jones, A. P. The role of histamine and fish meal in the incidence of gizzard erosions and pro-ventricular abnormalities in the fowl. *Br. Poult. Sci.* **1975**, *16*, 69−78.

Hino, T.; Noguchi, T.; Naito, H. Effect of gizzerosine on acid secretion by isolated mucosal cells of chicken proventriculus. *Poult. Sci.* **1987**, *66*, 548−551.

Horaguchi, H.; Masumura, T.; Horikawa, H.; Sugahara, M. Gizzard erosion and ulceration in broiler chicks. 2. Effects of fish meal. *Poult. Sci.* **1981**, *16*, 351−357.

Ito, Y.; Noguchi, T.; Naito, H. Fluorometric determination of gizzerosine, a Histamine H2-receptor agonist discovered in foodstuffs, employing high-performance liquid chromatography. *Anal. Biochem.* **1985**, *151*, 28−31.

Ito, Y.; Terao, H.; Noguchi, T.; Naito, H. Gizzerosine raises the intracellular cyclic adenosine-3′,5′-monophosphate level in isolated chicken proventriculus. *Poult. Sci.* **1987**, *67*, 1290−1294.

Kazama, T.; Makino, A.; Abe, M.; Akaike, Y.; Sugahara, M.; Taniguchi, T. Gizzard erosion and ulceration in broiler chicks. *Poult. Sci.* **1980**, *17*, 344−350.

Kimata, M. The Histamine problem. In *Fish as Food*; Borgstrom, G., Ed.; Academic Press: New York, 1960; pp 329−352.

Köhler, G.; Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **1975**, *256*, 495−498.

Kuba, K.; Miyazaki, S.; Umemura, Y. Contents of free histidine and histamine in fish meals and in the model compounds and their toxicities to induce gizzard erosion. *Natl. Inst. Anim. Health* **1983**, *23*, 69−70.

Langone, J. J. Radioiodination by use of the Bolton-Hunter and related reagents. *Methods Enzymol.* **1980**, *70*, 221−247.

Masumura, T.; Sugahara, M.; Noguchi, T.; Mori, K.; Naito, H. The effect of gizzerosine, a recently discovered compound in overheated fish meal, on the gastric acid secretion in chicken. *Poult. Sci.* **1985**, *64*, 356−361.

Mori, K.; Okazaki, T.; Noguchi, T.; Naito, H. Synthesis of (±)-gizzerosine, as inducer of gizzard erosion in broiler chicks. *Agric. Biol. Chem.* **1983**, *47*, 2131−2132.

Mori, K.; Sugai, T.; Maeda, Y.; Okasaki, T.; Noguchi, T.; Naito, H. Synthesis of the racemic and optically active forms of gizzerosine, the inducer of gizzard erosion in chicks. *Tetrahedron* **1985**, *41*, 5307−5311.

Murakita, H.; Gotoh, T. *o*-Phthalaldehyde postcolumn derivatization for the determination of gizzerosine in fish meal by high-performance liquid chromatography. *J. Chromatogr.* **1990**, *515*, 527−530.

Okazaki, T.; Noguchi, T.; Igarashi, K.; Sakagami, Y.; Seto, H.; Mori, K.; Naito, H.; Masumura, T.; Sugahara, M. Gizzerosine, a new toxic substance in fish meal, causes severe gizzard erosion in chicks. *Agric. Biol. Chem.* **1983**, *47*, 2949−2852.

Rosselot, G.; Lopez-Lastra, M.; McMurtry, J. P. Determination of gizzerosine activity in fish meal with a homologous radioimmunoassay. *Poult. Sci.* **1996**, *75*, 873−880.

Sugahara, M.; Hattori, T.; Nakajima, T. Lethal toxicity of gizzerosine for broiler chicks. *Agric. Biol. Chem.* **1987**, *51*, 3423−3424.

Sugahara, M.; Hattori, T.; Nakajima, T. Effect of synthetic gizzerosine on growth, mortality and gizzard erosion in broiler chicks. *Poult. Sci.* **1988**, *67*, 1580−1584.

Toyama, K.; Hoshi, M.; Kaizukaq, M. Gizzard erosion in poultry in the U.K. B. *Jpn. Soc. Sci. Fish.* **1985**, *51*, 985−993.

Wagener, W.; Koch, K.; Wessels, J.; Post, B. An investigation into the occurrence of the toxic amino acid gizzerosine in fish meal. *J. Sci. Food Agric.* **1991**, *54*, 147−152.

Wessels, J.; Post, B. Effect of heat treatment of fish meals, fines and the addition of lysine as related to gizzard erosion in chickens. *J. Sci. Food Agric.* **1989**, *46*, 393−406.