

PROGRESS ON THE DEVELOPMENT OF SPECIES IDENTIFICATION KITS FOR ATLANTIC ISTIOPHORIDAE

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INTRODUCTION

Accurate catch statistics for various billfish species cannot be obtained at present because dockside identification of butchered carcasses is difficult. The purpose of the present study is to determine the specificity and sensitivity of polyclonal antisera produced against white muscle proteins from three species of western Atlantic billfishes (blue marlin, *Makaira nigricans*; white marlin, *Tetrapturus albidus*; sailfish, *Istiophorus platypterus*). Developing a method for producing specific antisera would allow construction of field identification kits. The present paper also evaluates the use of albumin as a biomarker for billfish muscle. Since albumin is one of the most rapidly evolving proteins, it has been used by a number of workers in taxonomic, systematic, and evolutionary studies (Lowenstein, 1985; Sarich, 1969). Our paper also addresses the effects of pH on the solubility of billfish muscle proteins, and the tolerization of mice to billfish muscle proteins.

MATERIALS AND METHODS

Production of Rabbit Antisera:

Rabbits were tolerized with a mixture of water extracted white muscle proteins and adjuvant to two of three species of billfish, respectively, to eliminate any antibodies produced against those species. The tolerization regimen included injecting 1 ml of tolerogen containing 1 mg of protein at birth. Repeated injections of tolerogen followed by cyclophosphamide (0.2 mg/kg body weight, 3 times; 200 mg/kg, 2 times) were given to maintain tolerance (Matthew and Sandrock, 1987). Rabbits thus tolerized to two of the three species were then immunized by repeated intramuscular injection of white muscle extract from the third billfish species (5 mg/ml protein, 0.5 ml, injected at 6 sites in adjuvant).

Following each immunization, the rabbits were bled and the titer of antiserum they produced was determined by a precipitin test (Burrell and Lewis, 1987). The specificity of the antiserum obtained was evaluated using double diffusion analysis (Campbell et al., 1970; Ouchterlony and Nilsson, 1986).

Absorption of Rabbit Antisera with Cross-Reacting Antigens:

In order to remove the unwanted cross-reacting antibodies from rabbit serum that showed reactivity to one of the species, either of two methods was employed. Equal volumes of antiserum and the two heterologous antigens were mixed and incubated overnight at 4°C. The mixture was centrifuged at 10,000 x G to remove the antibody-antigen precipitate. Alternatively, the heterologous antigens were applied to the center of a double diffusion well pattern and allowed to diffuse into the agar prior to the addition of antiserum to the center well and antigen solutions to the peripheral wells (Irving Stone, pers. comm.).

Purification of Sailfish Albumin:

The method of Hoch and Chanutin (1954) with modifications suggested by Ray Simon (pers. comm.) and the use of a lipid extraction step were employed. Fourteen ml of sailfish serum or 28 ml of sailfish muscle homogenate containing 5 mg/ml protein was mixed with an equal volume of water and then centrifuged for 20 min at 10,000 x G. Caprylic acid, 81 ul, was then added to the supernatant and the pH adjusted to 5.9. After heating for 90 min at 63°C, the pH was adjusted to 4.2 with 2 N HCl and the precipitate that formed was removed by centrifuging for 30 min, 10,000 x G at 4°C. The supernatant fluid was re-adjusted to pH 5.9 with 1 N HCl and an equal volume of saturated NH₄SO₄ added. This solution was stirred overnight, centrifuged at 10,000 x G to

remove the precipitate, and the supernatant was dialyzed against 0.08 M tris-HCl buffer pH 6.0. The dialysate was then extracted with Seroclear (Calbiochem, San Diego, CA) to remove lipids, and the resulting fluid was placed under vacuum for 30 min.

High Pressure Liquid Chromatography and Polyacrylamide Gel Electrophoresis of Sailfish Serum and Muscle Albumins:

A Bio-Sil TSK 400 HPLC gel filtration column fitted to a Bio-Rad model 401T, Acer 710-controlled gradient module was loaded with 200 ul from the purification described above. The mobile phase consisted of 0.05 M Na₂SO₄, 0.02 M NaH₂PO₄ pH 6.8. A flow rate of 1.0 ml/min was used to effect separation. A Bio-Rad variable wavelength ultraviolet detector (model 1705) set at 260 nm was used to screen the column effluent.

The peaks of protein obtained from HPLC were analyzed by density gradient native polyacrylamide gel electrophoresis (PAGE) using the Pharmacia Phastsystem and Pharmacia 8-25 Phastsystem gradient gels, which were subsequently stained with silver according to the manufacturer's instructions (Pharmacia, Piscataway, NJ). Bovine serum albumin and Pharmacia low molecular weight standards were separated simultaneously to estimate the molecular weight of the billfish albumin.

Tolerization of Mice to Billfish Proteins:

Female BALB/c mice (Harlan Sprague Dawley, Inc., Ind., IN), 8 weeks old, were injected with 1.0 ml of a mixture of tolerogen (two of the three species) intraperitoneally. They were then injected with cyclophosphamide (100 mg/kg in sterile saline) 30 min, 24 hr and 48 hr following each tolerogen injection. This tolerization regime was repeated every two weeks for a total of four tolerization treatments. Two weeks after the last treatment blood was obtained from the ophthalmic venous plexus and the serum was tested for antibody activity by double diffusion analysis (Matthew and Sancrook, 1987).

Determination of the Osmolarity and pH of Billfish Muscle Homogenate:

In order to perform immunological analyses of antisera produced against billfish muscle proteins, these two reagents must be reacted under identical conditions. After performing the first double diffusion analysis, it was noticed that much of the billfish muscle protein failed to migrate under conditions that were optimal for rabbit antibody activity. Therefore, a water extract of billfish muscle which was previously found ideal for isoelectric focusing (Hartmann and Waldner, 1989) was evaluated for pH using a Corning model 125 pH meter, and for osmolarity using a Wescor vapor pressure osmometer (Wescor, Logan, VT).

Results

Rabbit Antisera Specificity:

For the first 6 months of tolerization and immunization, no specific anti-billfish antibodies were obtained. Only when the level of tolerogen and immunogen were raised to 5 mg/ml and the cyclophosphamide dosage was increased to 200 mg/kg were species-specific antibodies obtained after 8 months of immunization.

In Fig. 1, two specific lines of precipitation representing antibody activity against blue marlin are visible between the center well containing rabbit anti-blue marlin antiserum and the peripheral wells containing blue marlin muscle protein (bands BM). However, additional lines of precipitation were evident between blue marlin and white marlin (bands BM-WM). A faint precipitant also formed between anti-marlin antibody and sailfish muscle protein (band S).

A nearly identical pattern showing one line of specific activity against sailfish but with additional lines exhibiting cross reactivity to the other two species was obtained.

Fig. 2 shows a heavy precipitant forming between anti-white marlin serum and white marlin antigen (band WM). This line of precipitation was contiguous with precipitants which formed for

blue marlin and sailfish, even though the latter two were not as heavy. An additional line of precipitant occurred with all three species when using this antiserum.

Absorption of Cross-Reacting Antisera:

Following absorption of antisera with heterologous antigens, double diffusion analysis yielded very weak precipitin arcs against sailfish, but none were obtained for blue marlin and white marlin.

Purification of Sailfish Albumin:

Separation of sailfish albumin by HPLC yielded two peaks with retention times of 6.67 min and 9.32 min on a 300 mm x 7.5 mm Bio-Sil TSK-400 column (Fig. 3). Analysis of the peaks collected from the HPLC separation by native PAGE is shown in Fig. 4. The material from peak two exhibited two bands of molecular weights 67,000 and 25,000. The bovine serum albumin band showed two bands of 67,000 and 30,000 molecular weight.

The yield of albumin from sailfish serum was approximately 0.5 mg/ml. However, no detectable level of albumin was obtained from sailfish white muscle on two attempts, starting with either 1 ng/ml or 5mg/ml of protein in a muscle homogenate.

Determination of pH and Osmolarity of Billfish Muscle Extract:

A water extract of billfish white muscle, previously found ideal for isoelectric focusing analysis, was found to have a pH of 6.0 and an osmolarity of 100 mOs. Raising the pH to 7.3 in order to conduct antigen-antibody reactions caused a considerable portion of the solubilized muscle protein to precipitate out of solution without the presence of antibody. Therefore, we employed a buffer with a pH of 6.0 for all serological reactions.

Tolerization of Mice:

Following four tolerization regimens over an eight week period, the BALB/c mice exhibited complete tolerance. No antibody-antigen precipitate formed when the serum from the 12 mice were reacted with the tolerogen (two of the three species).

DISCUSSION

Polyclonal antibodies which produced lines of precipitation specific for blue marlin and sailfish were obtained after 8 months of immunization (Figs. 1 and 2). The incorporation of these antibodies into a latex agglutination format could provide a field kit for the identification of billfish carcasses. The cross reactivity obtained with the antiserum against white marlin demonstrates the close systematic relationship between the three

species under study and the need for extensive tolerization treatment in order to obtain species specific antisera. The current limitation on the construction of a field kit employing antisera from the present study is the marked drop in titer following absorption of the antisera with heterologous antigen. One way to avoid this loss of specific antibody would be to couple the heterologous antigens to cyanogen activated sepharose and construct an affinity column (Fuchs and Sela, 1986). The use of an affinity column would overcome the nonspecific co-precipitation which occurred with the methods described in this paper.

The use of a readily purified and well-characterized protein such as albumin would be ideal for the immunization of animals, screening of antibodies, absorption of antibodies and the construction of kits. A number of previous studies have demonstrated the utility of albumin for taxonomic purposes. The albumin obtained from the sailfish in the present study (Figs. 3 and 4) would be invaluable as a species biomarker. However, albumin was not recovered from white muscle in detectable levels by the chemical extraction method employed. It is possible that it is present in levels which could be detected by the more sensitive immunological methods. Even if albumin is not detectable in white muscle, an antibody specific to the albumin of a species could be used for the identification of that species if red muscle were present on the carcass.

Since the polyclonal antisera produced by the rabbits showed considerable cross-reactivity, the authors have investigated using mice for antibody production. Following an eight week tolerization regime, groups of mice were rendered tolerant to two of the three billfish species, respectively. We are currently immunizing mice with the sailfish albumin described above, and are purifying albumin from the red muscle of blue marlin and white marlin. The mice thus immunized represent a source of polyclonal antisera from ascites fluid (Bradshaw, 1988). In addition, mice which produce specific polyclonal antisera could be sacrificed and their spleen cells fused to myeloma cells to produce an immortalized hybridoma cell line which would serve as a source of monoclonal antibodies.

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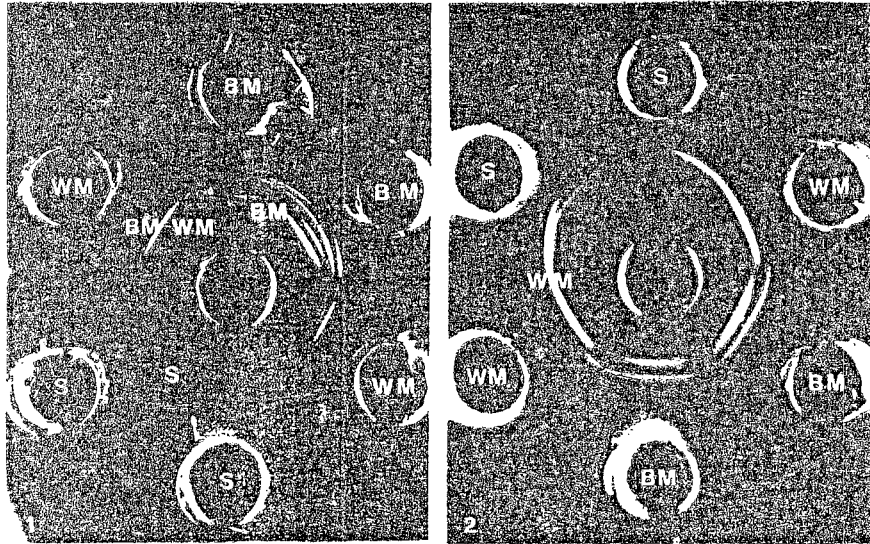


Figure 1. Gel Diffusion analysis of rabbit anti-blue marlin antiserum and blue marlin, white marlin and sailfish muscle homogenate. Center well = anti-blue marlin serum; BM = blue marlin precipitate; BM-WM = line of identity between blue marlin and white marlin

Figure 2. Gel Diffusion analysis of rabbit anti-white marlin antiserum and blue marlin, white marlin and sailfish homogenates. Center well = anti-white marlin; white marlin = heavy precipitate for white marlin, identity with blue marlin and white marlin.

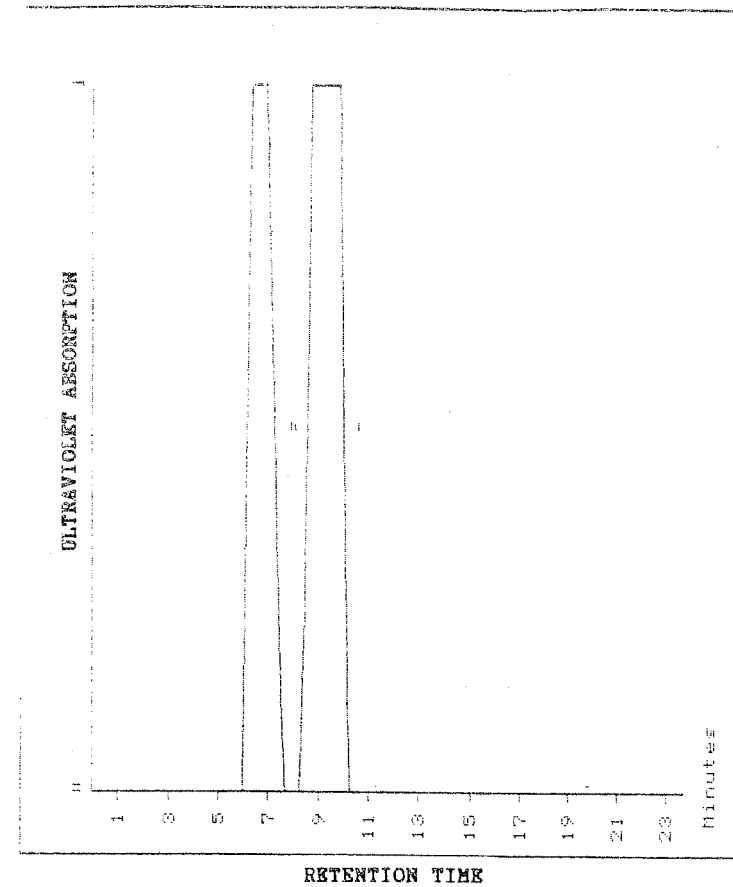


Figure 3. Separation of sailfish albumin by high pressure liquid Chromatography.

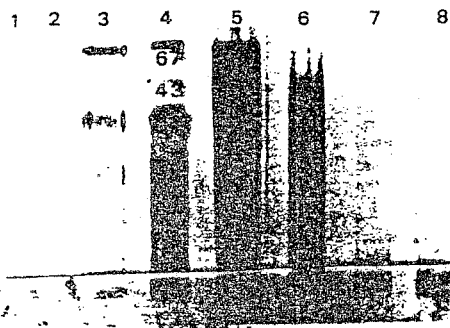


Figure 4. Analysis of the molecular weight of albumin purified by HPLC. Lanes: 1, 2, 7 & 8 = material before HPLC; 3 = bovine albumin; 4 = molecular weight stds; 5 = peak 2 from HPLC; 6 = peak 1 from HPLC. Numbers under the bands on the standard lane indicate their known molecular weights.