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ELECTROPHORETIC SEPARATION OF MUSCLE PROTEINS FROM BLUE MARLIN, WHITE MARLIN, AND SAILFISH FROM THE WESTERN ATLANTIC OCEAN: ISOLATION OF BIOMARKER PROTEINS FOR IDENTIFICATION OF BILLFISH CARCASSES

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SUMMARY

Species specific protein banding patterns were obtained for western North Atlantic blue marlin, white marlin, and sailfish when their muscle tissues were subjected to polyacrylamide gel isoelectric focusing. Sailfish exhibited intraspecific variation in a single band, which may indicate genetic differences within or between stocks, or differences in gender. Biomarker proteins were identified for all three species on pH 5-8 gels. Muscle proteins from Pacific and Atlantic blue marlin were electrophoresed on pH 3-9 gels for comparative purposes; unique bands not present in Atlantic blue marlin were noted for all Pacific specimens. Biomarker proteins from the western Atlantic study species are being used to produce antibody-based field identification kits to allow the rapid identification of butchered billfishes from tissue samples.

RESUME

Les modes de striation protéique spécifiques des espèces ont été obtenus pour le makaire bleu, le makaire blanc et le voilier de l'Atlantique nord-ouest en soumettant leurs tissus musculaires à un foyer isoélectrique au moyen d'un gel polyacrylamide. Les voiliers montraient des variations intraspécifiques dans une même strie, ce qui peut indiquer des différences génétiques entre stocks ou dans l'intérieur d'un même stock, ou des différences dues au sexe. Les protéines qui servent de marqueurs biologiques ont été définies pour les trois espèces au moyen de gels pH 5-8. Les protéines musculaires de makaires bleus du Pacifique et de l'Atlantique ont été étudiées par électrophorèse aves des gels pH 3-9 à des fins de comparaison; les stries uniques absentes chez le makaire bleu de l'Atlantique ont été observées chez tous les spécimens du Pacifique. Les protéines qui servent de marqueurs biologiques pour l'étude des espèces ouest-atlantiques servent à élaborer des équipements de terrain basés sur les anti-corps, et destinés à l'identification rapide des istiophoridés endommagés à partir d'échantillons de tissus.

RESUMEN

Se obtuvieron distribuciones de bandas de proteínas características de las especies aguja azul, aguja blanca y pez vela del Atlántico Noroeste, sometiendo sus tejidos musculares a un tratamiento isoeléctrico en gel de policrilamida. En el pez vela se observó una variación intraespecífica en una banda simple, lo cual puede ser un indicio de diferencias genéticas entre stocks o dentro de los mismos, o de diferencias en el género. Se identificaron proteínas biomarcadoras de las tres especies, en geles de PH 5-8. Las proteínas de los músculos de aguja azul del Atlántico fueron tratadas con electroforesis en geles de PH 3-9 con fines de comparación; las bandas únicas, que no se encontraron en la aguja azul del Atlántico, se observaron en todos los ejemplares del Pacifico. Las proteínas biomarcadores de las especies del Atlántico Oeste se emplean para obtener equipos de identificación sobre el terreno basados en anticuerpos, con el fin de identificar con rapidez los marlines despedazados, por medio de muestras de tejido.

Introduction

Fish species can be readily identified by the characteristic banding patterns produced when their muscle proteins are separated in an electric field (Bremner and Vail, 1983; Durand and Landrein, 1982; Lundstrom, 1980, 1981a, 1981b, 1983; Lundstrom and Roderic, 1979; Mathews, 1984; Yamada and Suzuki, 1982). Similarly, electrophoresis

of eye lens tissues can allow both species identification and the evaluation of polymorphism within a species (Al-Hassan, 1985; Menezes, 1985; Smith, 1962; Smith and Gilman, 1982). In addition, fish stock identification and an analysis of genetic variation as indicated by biochemical differences have been performed by studying polymorphism in muscle enzymes which were separated electrophoretically (Anderson, 1982; Grant, 1985).

Conser (1985) suggests that the future of billfish stocks may depend largely on our ability to determine the status of the stocks with greater accuracy and precision. At present, data on the Atlantic stocks of certain billfishes such as sailfish (Istiophorus platypterus) and longbill spearfish (Tetrapturus pfluegeri) are difficult to interpret, due to the lumping together of data from different species (Conser, 1980; Limouzy and Cayre, 1981). Part of this problem results from the inability to accurately identify billfishes once they have been butchered and frozen. In addition, recent legislation prohibits the sale of most white marlin (Tetrapturus albidus), Atlantic blue marlin (Makaira nigricans), longbill spearfish, and Atlantic sailfish in the United States, and imposes minimum size limits on members of these species if they are kept for any reason. However, once butchered and frozen, these species are not only difficult to identify, but it is also difficult to determine whether a carcass is from a billfish taken in the Atlantic Ocean or caught elsewhere. Since present legislation centers around Atlantic billfishes, determination of where a particular fish was caught is important if these regulations are to be enforced.

The goal of the present study is to determine if white muscle tissues from Western North Atlantic blue marlin, white marlin, and sailfish contain biomarker proteins - proteins with electrophoretic mobility unique to each species. During the evolution of each of these species, advantageous mutations in the enzymatic and/or structural proteins of their muscles have been selected for. Such changes in the primary structure of a protein may affect the protein's net charge. This, in turn, will result in a change in its isoelectric point and thus its position on a gel subjected to isoelectric focusing.

The biomarker proteins will be used to produce antibodies which will be employed in the construction of portable identification kits. These kits will allow the dockside identification of butchered, frozen billfish carcasses lacking key meristic and morphometric characteristics, and may also prove useful in distinguishing between different stocks. Thus, accurate catch statistics for the various billfishes could be obtained, and current regulations pertaining to billfish conservation could be enforced. The gathering of accurate billfish catch statistics has been identified as a major goal by the International Commission for the Conservation of Atlantic Tunas.

Materials and Methods

Sources of Fish Samples

White muscle samples from four blue marlin, two white marlin, and six sailfish caught off southeast Florida and the Bahama Islands were used in this study. An additional four blue marlin caught off Hawaii were sampled to see if noticeable differences in muscle proteins exist between these fish and their Atlantic counterparts. Muscle samples were received either fresh or frozen and were then maintained at -70°C until needed. Fresh samples were frozen prior to protein extraction so that no observed differences could be attributed to using fresh versus frozen tissues. Samples were then thawed at room temperature, and identical techniques were used to extract the proteins from each sample.

Muscle Tissue Extraction

After testing a number of protein extracting solutions containing a variety of solubilizing, stabilizing, and preservative agents, distilled/demineralized water was found to produce the most consistent and reproducible electrophoretic banding patterns for marine teleost white muscle. In this study, white muscle from each billfish was homogenized at a ratio of 0.6 g of muscle to 3 ml of water using a glass homogenizer. The homogenates were decanted into 1.5 ml screw-top centrifuge vials. Each sample was then centrifuged for 30 min in a Savant Speed Vac Concentrator, Model SVC-200H (Savant, Farmingdale, NY) at 1725 rpm (650xg), after which the supernatant fluid was drawn off with a pipette and further diluted with distilled/demineralized water.

Assay of Extracted Proteins

After collecting the supernatant fluids, a two-fold dilution series was prepared from each sample in a 96 well microplate. Each dilution was then mixed with 30 microliters of Bio-Rad Protein Assay Solution (Bio-Rad, Richmond, CA.), as described by Bradford (1976). A series of blank wells and standards was added to the microplate as described in the Bio-Tek Automated Microplate Reader Model 309 Owner's Manual (Anonymous, 1986), and the relative optical density of each well in the microplate was read in a Bio-Tek Microplate Autoreader model EL309 (Bio-Tek Instruments, Winooski, VT), at a wavelength of 600 nm. The dilutions were then applied to pH 5-8 or 3-9 Pharmacia PhastSystem IEF gels and underwent PGIEF and silver staining as indicated below. Protein extracts having relative optical densities of 0.500 to 0.600 were found to be optimal for PGIEF and silver staining using the Pharmacia PhastSystem. Subsequent testing showed that this concentration could be approximated by diluting the supernatant solutions 1:16 with distilled water.

Electrophoresis

The programmable Pharmacia PhastSystem electrophoretic unit (Pharmacia, Piscataway, NJ) was used for the separation of muscle proteins. Electrophoresis was performed on precast Pharmacia isoelectric focusing gels having pH gradients of 5-8 and 3-9. Polyacrylamide gel isoelectric focusing (PGIEF) and subsequent silver staining followed the procedures outlined in the PhastSystem Owner's Manual (Anonymous, 1987).

Results

When separated on pH 3-9 gradient gels, the majority of the study species' white muscle proteins migrated towards the cathode (Fig. 1). Separating the proteins on pH 5-8 gradient gels allowed for higher resolution of a portion of a species' white muscle protein spectrum, which proved useful in distinguishing biomarker proteins.

Blue marlin can be distinguished from sailfish and white marlin by the presence of protein bands 15.8 and 10.5 mm from the cathode on 36.2 mm pH 5-8 gradient gels (Fig. 2, bands a & b). In addition, using pH 3-9 gradient gels, Atlantic and Pacific blue marlin can be distinguished by the presence of anodic bands present in Pacific blue marlin but lacking in their Atlantic counterparts (Fig. 3, band a).

Sailfish have one or, in some individuals, two very darkly stained bands located 14.2 and 14.9 mm from the cathodic end of the 36.2 mm pH 5-8 gradient gels (Fig. 4, bands a & b). Although the other study species also exhibited bands at this approximate location, the proteins from sailfish white muscle consistently stained much darker when the same protein concentration was used for all species. The fact that all six sailfish exhibited band a while only two exhibited band b indicates intraspecific genetic variation. An additional band 13.3 mm from the cathode can be clearly seen in four of the six sailfish (Fig. 4, band c). All of the sailfish tested exhibited a unique band 11.3 mm from the cathode (Fig. 4, band d). The two white marlin displayed a band slightly closer to the cathode (11.1 mm) (Fig. 4, band e), indicating a higher pI. This band appears to be unique to white marlin, but there is an indication of a very lightly stained protein in this area in sailfish. Thus, this protein may prove to be shared by sailfish and white marlin.

The gels shown in Fig. 2 & 4 are representative of over ten electrophoretic separations using pH 5-8 gradient gels. Numerous additional separations have been made on pH 3-9 gradient gels, as represented by Fig. 1 & 3.

Discussion

This is the first comparative study of muscle proteins from these billfish species. Previous electrophoretic studies on billfishes have focused on single species, such as Shaklee *et al.* (1983), who studied isozyme patterns of Pacific blue marlin as a means of identifying stocks. We appear to have identified unique biomarker proteins consistently associated with all samples of a given species, for all species we examined. However, due to the intraspecific variation observed in sailfish and the fact that only two white marlin were sampled, the results for white marlin should be regarded as tentative.

Because of their common evolutionary ancestry, considerable similarity exists in the electrophoretic mobility/isoelectric points for a majority of the white muscle proteins from blue marlin and Western North Atlantic white marlin and sailfish. Intraspecific variation was observed between some sailfish, but the overall banding pattern was still very distinct from that of blue and white marlin. The two sailfish phenotypes could be due to factors such as genetic differences between or within stocks, or to gender. Muscle protein polymorphism has previously been observed in monkfish, *Lophius americanus* (Lundstrom, 1981a). If such variations prove to be stock specific they could provide a means to identify stocks. Fish stock identification has also been accomplished by examining muscle isozyme polymorphism in other species (Anderson, 1982; Grant, 1985). All sailfish muscle samples used in this study were from mature fish taken off the southeast coast of Florida, but the point of origin of these fish remains undetermined. Intraspecific variations indicate a need to examine several individuals of a species in order to electrophoretically characterize that species. As only two white marlin were utilized in this study, the results for this species should be regarded as tentative.

The unique bands present in the PGIEF white muscle banding patterns of Pacific blue marlin on pH 3-9 gradient gels allows these individuals to be biochemically distinguished from Atlantic blue marlin. This apparently consistent difference in blue marlin muscle proteins may represent intraspecific polymorphism. However, as consistent variations in morphological characteristics have been noted by other workers (E. Irby, pers. comm.), the protein differences may indicate that Atlantic and Pacific blue marlin represent two distinct species. Additional blue marlin from a variety of locations in the Atlantic, Pacific, and Indian Oceans should be electrophoretically analyzed before any definite conclusions are drawn on the basis of biochemical differences.

The biomarker proteins observed in each of the study species provide an additional means of distinguishing between these billfishes. Although biomarker proteins were observed in all individuals of a given species used in this

study, further studies using muscle samples from representatives of each species collected at a number of geographic locations are necessary to determine if variations exist in these proteins.

We are currently involved in the production of polyclonal antisera against the biomarker proteins (antigens) for each of the Western Atlantic species. Antibodies specific to each of the biomarker proteins will be coupled to latex spheres and used in the construction of user-friendly field kits. The procedure for using each kit will involve stirring a small sample of meat juice or meat drip with latex-coupled antibodies specific for a particular billfish species. If an antigen that corresponds to the antibodies is present in the muscle extract, agglutination will occur. The polyclonal antisera will be absorbed with muscle tissue from two of the three species of billfish prior to the preparation of the identification kits, and an evaluation of cross-reactivity will be made.

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Figure 1. Silver stained pH 3-9 gradient gel showing protein patterns for Atlantic sailfish, blue marlin, and white marlin (lanes 2, 4, and 6, respectively), and Pharmacia IEF standards (lanes 1, 3, and 5).

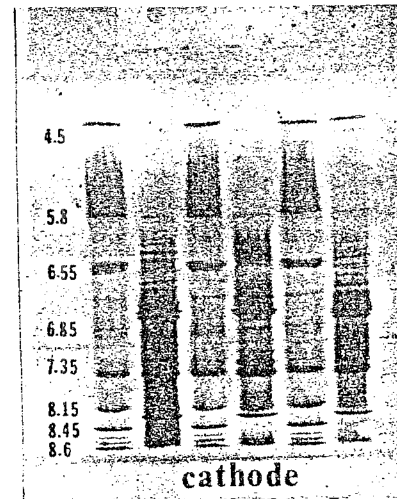

Figure 2. Silver stained pH 5-8 gradient gel showing protein banding patterns of four Atlantic sailfish (lanes 1-4) and four Atlantic blue marlin (lanes 5-8). a & b = blue marlin biomarker proteins

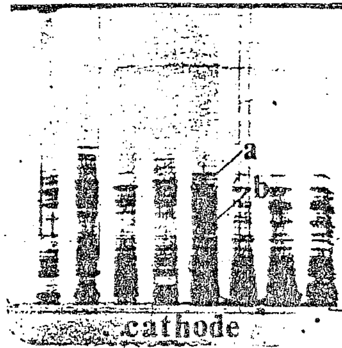

Figure 3. Silver stained pH 3-9 gradient gel showing protein banding patterns of four Pacific blue marlin (lanes 1-4) and four Atlantic blue marlin (lanes 5-8). a = protein band unique to Pacific blue marlin

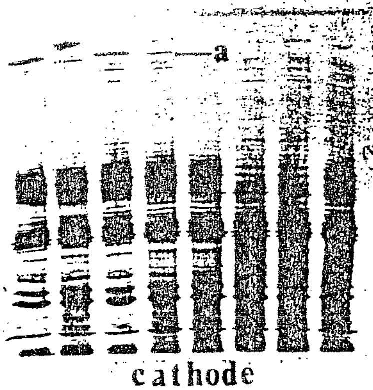

Fig. 4. Silver stained pH 5-8 gradient gel showing protein banding patterns of six Atlantic sailfish (lanes 1-6) and two white marlin (lanes 7 & 8). a = darkly stained band in all sailfish; b = darkly stained band in 2 of 6 sailfish; c = polymorphic sailfish band; d = sailfish biomarker protein; e = white marlin biomarker protein