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A COMPARISON OF ELECTROPHORETIC BANDING PATTERNS OBSERVED FOR PROTEINS FROM TWO SPECIES OF THE GENUS *DOROSOMA*

THOMAS E. BYRNE[1]
AND
JOHN W. HARRIS

Tennessee Technological University
Cookeville, Tennessee 38501

ABSTRACT

Polyacrylamide gel electrophoresis was used for the taxonomic comparison of lactate dehydrogenase, esterase, and general proteins of *Dorosoma petenense* Gunther and *Dorosoma cepedianum* LeSueur in Center Hill Lake. The fish were separated by sex and grouped by age into one, two, and three year old classes. The zymograms for the two species were exactly the same for the general muscle protein and similar for esterase and lactate dehydrogenase. No sex differences were noted in the banding patterns. The banding patterns for esterase and lactate dehydrogenase were found to vary with age. Some electrophoretic differences that may be of taxonomic value in distinguishing the two species were noted for esterase and lactate dehydrogenase.

INTRODUCTION

Specimens of gizzard shad, *Dorosoma cepedianum* LeSueur, and threadfin shad, *Dorosoma petenense* Gunther, are sometimes difficult to distinguish from each other by conventional taxonomic methods, particularly when fry are used. This difficulty is compounded by the fact that there is evidence for natural hybridization between the two species (Minckley and Krumholz, 1960; Shelton and Grinstead, 1972). The position of the mouth is very helpful in identifying the species when the anal fin rays are not fully developed. The mouth of threadfin shad is at the anterior end of the head. This position is considered to be terminal. In gizzard shad, a portion of the snout extends before the mouth, which is in a subterminal position. Upon maturation, gizzard shad has 30-33 anal fin rays while threadfin shad has 20-25 anal fin rays.

In recent years, analysis of the electrophoretic banding patterns of isozymes has been shown to be quite useful as an aid in making taxonomic distinctions between various groups of fish (Avise, 1974; Smith et al., 1976; Utter et al., 1974). The purpose of this study was to assess the utility of this parameter for distinguishing between the two species and to compare electrophoretic banding patterns observed for proteins from gizzard shad with those of threadfin shad in order to obtain additional information concerning the taxonomic relationship between them. Esterase, lactate dehydrogenase, and general muscle proteins were examined.

Esterase

The esterases are a very complex family of enzymes with overlapping specificities, multiple tissue sources, and genetic variations in some species. Various classes of esterases may be distinguished on the basis of differential substrate and inhibitor specificities. Four main types of soluble esterases have been observed in vertebrate tissue extracts. They are carboxylesterase (E.C. 3.1.1.1), arylesterase (E.C. 3.1.1.2), acetylesterase (E.C. 3.1.1.6), and cholinesterase (E.C. 3.1.1.8) (Aldridge, 1954; Bergmann et al., 1957; Augustinsson, 1960; Holmes et al., 1968).

Lactate dehydrogenase

The enzyme lactate dehydrogenase (E.C. 1.1.1.27) exists in many organisms in isozymic form (Markert and Moller, 1959). It is a tetramer with a molecular weight of 140,000 (Markert, 1963). Two different subunits, A and B, join to form the tetramer. The subunits are specified by two different genes (the A locus and the B locus), and have five arrangements designated LDH-1 (B_4), LDH-2 (A_1B_3), LDH-3 (A_2B_2), LDH-4 (A_3B_1) and LDH-5 (A_4). The most anodal band is LDH-1, while LDH-5 is the most cathodal band. The LDH-1 and LDH-5 isozymes are often termed homotetramers, and the heterogenous combinations of subunits (LDH-2, LDH-3 and LDH-4) are referred to as allotetramers. The LDH subunits are not found in equal quantities in all tissues. The A subunit is produced in large amounts in white skeletal muscle, while the B subunit predominates in heart and brain tissue (Wilson et al., 1964).

A third LDH gene (the E locus) has also been found to be expressed in higher teleosts (Markert and Faulhaber, 1965). Originally postulated to be active only in retinal tissue and the eye lens, it has subsequently been observed in liver and other tissues (Shaklee et al., 1973).

General muscle protein

Muscle tissue contains a heterogeneous mixture of proteins. The basic structural proteins of muscle are actin and myosin. Also present are smaller and more soluble proteins such as parvalbumins (Pechere and Pantel, 1974). Thick filaments of muscle are composed almost exclusively of myosin, and this protein constitutes about 50 percent of myofibrillar protein. Myosin is a long slender molecule which consists of two helically twisted polypeptide chains with a molecular weight of 225,000 each. At regular intervals along the filament, globular head proteins are present (Lehninger, 1973). Red and white skeletal muscle proteins have been found to show tissue variations (Sarkar et al., 1971). On the basis of amino acid differences, it has

[1]Present address: Roane State Community College
Harriman, Tennessee 37748

been concluded that the myosin of red muscle and that of white muscle are under separate genetic control (Hazar and Elzinger, 1972).

Actin, a monomeric protein with a molecular weight of approximately 46,000, comprises about 25 percent of the muscle proteins. In some organisms, including fish, actin and myosin are tightly bound and it is difficult to separate them. Alpha actin is another protein of muscle tissue. It is found mostly in Z bands of muscle and has a molecular weight of 100,000 (Prosser, 1973).

MATERIALS AND METHODS

Collection of samples

All fish were collected by Tennessee Cooperative Fishery Research Unit personnel from the area between Tech Aqua Biological Station and the Cookeville Boat Dock on Center Hill Lake. Most of the fish were collected by electrofishing, but nets were used on several occasions to obtain the desired specimens. Collected fish were immediately packed in ice for transport to to the laboratory where they were stored in a freezer.

Preparation for electrophoresis

Several scales were collected from each fish and impressed in plastic, using a modification of the method described by Greenbank and O'Donnell (1950). Scale impressions were observed on an Eberback projector, using Cating's (1953) method for determining the age of shad. In addition to age and total length, the sex of each fish was recorded whenever an accurate determination could be made by examination of gonads.

Skeletal muscle from each fish was used for extraction of protein. Heart and optic nerve tissues were also used in some cases. With very small specimens, the digestive tract of the fish was removed to avoid interference by enzymes from the digested food particles. A 1:1 mixture (V/W) of 0.18 M tris-Na_2EDTA-boric acid buffer, pH 8.4, and body tissue was homogenized using an insonator, model 1000, manufactured by Ultrasonic Systems Incorporated. Each extract was packed in ice and insonated for a total of two minutes with the instrument set at ⅓ power. The homogenates were then centrifuged at 10,000 × g for 10 minutes. The resulting supernatants were adjusted to 0.5 M with sucrose and frozen for later use.

Electrophoresis

Electrophoresis was accomplished utilizing a vertical slab polyacrylamide gel apparatus, manufactured by the E-C Apparatus Corporation. Two 0.1 ml aliquots of each extract were subjected to electrophoresis using 7.5 percent gels with a constant current of 220 volts for a three-hour period. Each gel was composed of 200 ml of 0.18 M tris-Na_2EDTA-boric acid buffer, pH 8.4, and 12.5 g of cyanogum 41, with 0.2 ml of N, N, N', N'-tetramethylethylenediamine and 0.2 g of ammonium persulfate added to catalyze the polymerization. A continuous buffer system utilizing 0.18 M tris-Na_2EDTA-boric acid buffer, pH 8.4, was used for all separations. Following electrophoresis, each gel was stained for the appropriate enzyme. Only those isozymes migrating to the anode of the electrophoretic cell were examined.

Staining procedures

The esterase solution was composed of 138 ml of distilled water, 9 ml of 0.2 M sodium phosphate buffer, pH 7.7, 1 ml of 1 percent alpha-naphthyl acetate in 50 percent acetone (W/V), and approximately 150 mg of fast blue RR salt. The solution was filtered through glass wool onto the gels, which were incubated at room temperature for five hours. The gels were then placed in distilled water and scored the next day.

The lactate dehydrogenase stain was composed of 50 mg of NAD, 30 mg of nitro blue tetrazolium, 2 mg of phenazine methosulfate, 15 ml of 0.5 M tris-HCL buffer, pH 7.1, 10 ml of sodium DL-lactate substrate, pH 7.0, 5 ml of 0.1 M sodium cyanide, and 70 ml of distilled water. The lactate substrate was prepared by combining 10.6 ml of 85 percent DL-lactic acid with 49 ml of 1 M sodium carbonate, adjusted to 100 ml with distilled water. The gels were incubated at 37°C for one hour in the dark after the stain was poured onto them.

Amido black was used to stain for general muscle proteins. The stain was composed of 0.2 g of amido black, 50 ml of distilled water, 50 ml of absolute methanol, and 10 ml of glacial acetic acid. The gel was placed in this stain for 10 minutes, after which it was placed in a destaining solution composed of 50 ml of distilled water, 50 ml of absolute methanol and 10 ml of glacial acetic acid. The destaining solution was changed several times over a period of two days until the bands were distinct.

RESULTS

All fish examined in this study were obtained from the same area of Center Hill Lake. For both species, four females and four males in each age class (one, two, and three year old) were sampled. Except for the esterases, the isozyme banding patterns differed very little from one individual to another within the same species. Therefore, it was quite simple to classify the isozymes by taking the banding patterns and numbering the bands consecutively with respect to distance of migration from the origin.

The general muscle protein banding patterns observed for the gizzard and the threadfin shad were the same. The pattern did not vary with the sex or age of the organism and was found to be uniform throughout the study.

Examination of the lactate dehydrogenase isozyme banding patterns observed for gizzard and threadfin shad muscle tissue (Figure 1) reveals several cases where the bands overlap. A comparison of isozyme banding patterns for one year old fish demonstrates that band 1 observed for gizzard shad overlaps with band 1 observed for threadfin shad and band 3 of gizzard shad overlaps bands 2 and 3 of the threadfin shad. A comparison of banding patterns for two year old fish shows that band 2 of the gizzard shad overlaps the threadfin shad band 2 and band 3 of the gizzard shad overlaps the threadfin shad band 3. The only LDH bands for the three year olds that overlap are the gizzard shad band 3 and the threadfin shad band 3. The patterns did not vary with sex of the organism, but migrational distance did vary with age of the organism. It is of interest to note that all of the fish examined had three lactate dehydrogenase bands. The only differences observed were in the comparative migration distances of the bands.

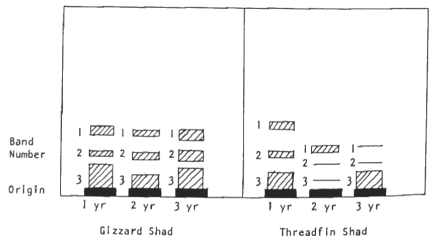

FIG. 1. *Diagrammatic Representation of Lactate Dehydrogenase Banding Patterns Observed for Gizzard and Threadfin Shad.*

The fewest number of overlaps and greatest number of differences in the banding patterns observed for muscle tissue samples occurred with the esterase isozyme patterns (Figure 2). A comparison of the banding patterns for one year old fish reveals that band 1 of the threadfin shad overlaps band 2 of the gizzard shad; other bands do not overlap. A comparison of esterase banding patterns for two year olds shows that band 2 of the threadfin shad overlaps band 3 of grizzard shad; other bands do not overlap. An examination of the banding patterns for three year old fish demonstrates that band 1 of the threadfin shad overlaps gizzard shad band 2; also, band 2 of the threadfin overlaps gizzard shad band 3. The banding patterns did not differ with the sex of the organism, but the migrational distance did vary with the age of organism.

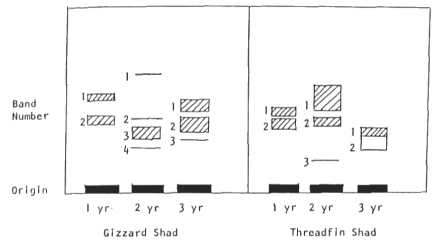

FIG 2. *Diagrammatic Representation of Esterase Banding Patterns Observed for Gizzard and Threadfin Shad.*

Figure 3 is a diagrammatic representation of lactate dehydrogenase banding patterns observed for heart, eye and muscle tissue from gizzard and threadfin shad. Several gels were done in this fashion in an attempt to demonstrate the presence of a lactate dehydrogenase isozyme specified by the E locus in the genus *Dorosoma.*

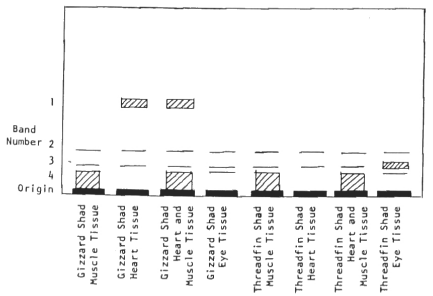

FIG. 3. *Diagrammatic Representation of Typical Lactate Dehydrogenase Banding Patterns Observed for Heart, Muscle and Eye Tissue from Gizzard and Threadfin Shad.*

In comparing gizzard shad muscle tissue and gizzard shad heart tissue, one notices band 1 in heart tissue samples which is not seen in muscle tissue, and band 4 of muscle tissue which is not found in heart tissue samples. When the two tissue extracts were mixed and electrophoresed, the gizzard shad heart tissue band 1 and the gizzard shad muscle tissue band 4 appeared along with bands 2 and 3 which have the same electrophoretic mobility in both tissues. The gizzard shad eye tissue has three bands which have migrated the same distance as bands 2, 3, and 4 of gizzard shad muscle tissue.

Upon examination of the threadfin shad muscle tissue and threadfin shad heart tissue, one finds band 4 in threadfin shad muscle tissue samples which is not observed for threadfin shad heart tissue. When the two tissues were mixed and electrophoresed, the threadfin shad muscle tissue band 4 appeared along with bands 2 and 3 which have the same electrophoretic mobility in both heart and muscle tissue. The threadfin shad eye tissue has three bands which have migrated the same distance as bands 2, 3, and 4 of the threadfin shad muscle tissue. Thus, there is no evidence for the occurrence of a unique isozyme specified by the E locus in the genus *Dorosoma.*

DISCUSSION

The most striking difference in banding patterns observed for the two species is in the heart muscle lactate dehydrogenase banding patterns. The pattern for gizzard shad heart muscle tissue (Figure 3) has a fast migrating anodal band which is not present in threadfin shad heart muscle tissue. This difference may be useful in differentiating these two species, and should be studied further.

Although the esterase banding patterns were the most irregular of the three proteins examined, some consistent differences between the two species were observed. The one year old gizzard shad esterase banding pattern has a band which migrates to the anode faster than any esterase band for the one year old threadfin shad. The two year old gizzard shad esterase banding pattern has four bands, one of which migrates to the anode faster than any of the three bands for the two year old threadfin. The three year old gizzard shad esterase banding pattern has three bands, one of which migrates to the anode faster than either of the two bands for the three year old threadfin shad. Thus, each of the gizzard shad esterase banding patterns has one band that migrates to the anode faster than any of the threadfin shad esterase bands observed for individuals of the same age. This shows promise as a means for differentiating these species; however, it should be noted that the age differences observed absolutely require that individuals of comparable age be used. For example, bands 1 and 2 observed for the three year old gizzard shad overlap bands 1 and 2 for one year old threadfin shad.

When comparing the banding patterns, a number of similarities may be observed. General muscle protein rates of migration for gizzard shad and threadfin shad were identical in every respect. Lactate dehydrogenase skeletal muscle tissue banding patterns did not vary greatly either within or between species. As noted, all of the fish examined had three lactate dehydrogenase bands, with the differences involving the comparative migration distances of the bands.

Even for the esterase skeletal muscle tissue banding

patterns, several similarities were noted. For example, in comparing three year old individuals of the two species, two bands were observed to overlap. As noted above, when banding patterns for different age groups are compared, a number of similarities between the bands for the two species are observed. In comparing the three year old gizzard shad esterase banding pattern with the two year old threadfin shad esterase banding pattern, one notes that the gizzard shad bands 1 and 2 overlap the threadfin shad bands 1 and 2. Also, when one compares the two year old gizzard shad esterase banding pattern with the three year old threadfin shad esterase banding pattern, one notes that the gizzard shad bands 3 and 4 overlap the threadfin shad bands 1 and 2. From examination of the electrophoretic banding patterns in this study, the similarities suggest that the two species are closely related on a taxonomic basis.

Additional studies are needed in order to clearly establish the utility of electrophoresis to taxonomically distinguish between these two species. The studies could include the following areas of research: use of different proteins or isozymes, use of cathodal banding patterns (in this study only anodal patterns were examined), use of different buffer systems, and use of two dimensional electrophoresis.

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