IDENTIFICATION OF FISH SPECIES BY THIN-LAYER POLYACRYLAMIDE GEL ISOELECTRIC FOCUSING

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ABSTRACT

Conventional electrophoretic techniques for the identification of fish species are limited in the resolution and reproducibility needed for the reliable identification of fish species. This paper describes the potential of a high resolution protein separation technique, thin-layer polyacrylamide gel isoelectric focusing (IEF), as a new means of identifying fish species. Sarcoplasmic protein patterns are shown for 11 species of commercially important New England fishes under both low resolution (pH 3.5–10 gradient) and high resolution (pH 3.5–5 gradient) conditions. The reproducibility of the protein patterns and pH gradients from day to day is also shown. The inherent high resolution and excellent reproducibility of IEF should allow the positive identification of fish species without the costly procedure of maintaining a supply of known species for use as standards.

Many different electrophoretic techniques have been used for the identification of fish species. Protein extracts from several species of fishes were first compared using moving boundary electrophoresis (Connell 1953). Differences in the electrophoretic protein patterns between species formed a "fingerprint" for each. In an effort to obtain higher resolution and reproducibility of the protein patterns, starch gel zone electrophoresis was applied as a method for diffentiating fish species (Thomson 1960). Subsequent attempts to further improve species identification techniques centered on the investigation of new stabilizing media. The use of polyacrylamide gels (Payne 1963; Cowie 1968) and agar gels (Hill et al. 1966) resulted in shortened analysis times, increased resolution, and easier handling and storage of gels. A rapid identification technique based on cellulose acetate electrophoresis (Lane et al. 1966) has found widespread use in quality control.

Each of these electrophoretic techniques (except moving boundary electrophoresis) is still in common use and has contributed much towards eliminating problems of species substitution. Unfortunately, each of these techniques is subject to one or more limitations that lessen its effectiveness as a routine species identification test. Variations in stabilizing media composition, sample application technique, separation time, applied voltage or current, and the technician's skill indicated the need for simultaneously running known species along with unknown samples to obtain a reliable identification. Collaborative studies of the two most widely used species identification procedures, disc electrophoresis (Thomson 1967) and cellulose acetate electrophoresis (Learson 1969, 1970), showed that reproducibility of specific protein patterns from analysis to analysis was a major problem.

This paper describes the potential of a high resolution protein separation technique, thin-layer polyacrylamide gel isoelectric focusing (IEF), as a new means of identifying fish species. IEF is an equilibrium technique in which proteins are separated according to their isoelectric points in a reproducible natural pH gradient. The pH gradient is formed in the gel by the electrolysis of amphoteric buffer substances called carrier ampholytes. Protein molecules migrate in the electric field along the pH gradient until they reach the pH equal to their isoelectric point. Here the protein has a net charge of zero, and no further migration can take place. The proteins become concentrated into very sharp bands and molecules whose isoelectric points differ by 0.07 pH units (pH 3.5–10 gradient) or 0.02 pH units (pH 3.5–5 gradient) may be resolved.

PROCEDURE

Isolation of Sarcoplasmic Proteins

Fresh iced fish was obtained from various Glou-

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Manuscript accepted February 1977.
FISHERY BULLETIN: VOL. 75, NO. 3, 1977.

cester fish processors. Four specimens of each species were examined except for cod and haddock where 15 individuals each were examined. All fish were held on ice from purchase to filleting. Fillets were held at 8°C until extraction of sarcoplasmic proteins.

Sarcoplasmic protein extracts were prepared by blending 100 g of muscle tissue with 200 ml of distilled water in a 500-ml Waring[2] blender jar. A Teflon baffle shaped to fit the inside contour of the blender jar about 1 cm below the water level was used to prevent the incorporation of air bubbles during the blending operation. The distilled water, blender jar, and baffle were chilled to 8°C prior to use to prevent protein denaturation from heat generated during blending. The resulting mixture was centrifuged at 1,400 *g* for 30 min at 4°C in an International PR-2 Refrigerated Centrifuge. The resulting supernatant was used for analysis without any further purification.

Preparation of Polyacrylamide Gel Slab

The polyacrylamide gel slab was chemically polymerized between a glass plate and an acrylic template. The glass plate and acrylic template were separated by a 0.75-mm acrylic spacer that extended around three sides leaving the top open. The template had embedded teeth that formed sample wells in the gel surface. The gel slabs used in these experiments were 175 mm × 90 mm × 0.75 mm and contained 12 sample wells, each capable of holding up to 5 μl.

A 4% (wt/vol) polyacrylamide gel containing 2% (wt/vol) carrier ampholytes was prepared as follows:

Into a 25-ml Erlenmeyer flask was pipetted
- 8.2 ml distilled water
- 3.0 ml 50% (vol/vol) glycerol (final concentration 10% [vol/vol])
- 3.0 ml 20% (wt/vol) acrylamide (final concentration 4% [wt/vol]) plus 0.8% (wt/vol) bisacrylamide (final concentration 0.16% [wt/vol])
- 5.0 μl tetramethylethylenediamine (final concentration 0.03% [wt/vol])
- 0.75 ml 40% (wt/vol) ampholine of appropriate pH range (final concentration 2% [wt/vol]).

This solution was degassed under vacuum for 4 min. Polymerization was started with the addition of 50 μl 10% (wt/vol) ammonium persulfate (final concentration 0.03% [wt/vol]). After a final degassing under vacuum for one more minute, the solution was immediately pipetted into the gel mold. The top of the gel solution was layered with water to form an even surface. Polymerization was complete in 20 min at room temperature. The open top of the gel mold was then sealed with masking tape, and the whole assembly was placed in a refrigerator (8°C) overnight before use. A supply of gel slabs may be prepared and stored for 2 wk in this manner. After the gel had polymerized, the template and spacer were removed leaving the gel adhering to the glass plate.

Electrofocusing Procedure

Electrofocusing was carried out using a Medical Research Apparatus Slab Electrofocusing Apparatus, Model M-150. The gel slab was placed on the cooling platform and cooled to −2°C prior to sample application. To insure good thermal contact, a layer of light paraffin oil was used between the glass plate and the cooling platform. After the gel slab had cooled, 5 μl of the protein extract was pipetted into a sample well with a micropipette. Up to 12 samples may be compared in a single gel slab. Felt strips soaked in 1M NaOH (catholyte) and 1M H_3PO_4 (anolyte) were applied to the edges of the gel to provide electrical contact with the platinum wire electrodes. A power supply was connected to the electrodes, and power was applied until equilibrium focusing was attained. Both constant-power and constant-voltage power supplies were used in these experiments. In isoelectric focusing, a power supply capable of delivering constant power is preferred. Using a constant power of 10 W, equilibrium focusing was complete in 1.5–2.0 h. Using constant voltage, the voltage must be manually increased to compensate for increased resistance through the gel as the pH gradient forms. Separation times are longer (5–6 h) and resolution suffers due to joule heating within the gel. With either type of power supply, equilibrium focusing is attained and the reproducibility of the protein patterns is not affected. After electrofocusing is complete, the pH gradient may be measured as a check on reproducibility or to determine the isoelectric points of the separated proteins. The plate is warmed to room temperature and the pH gradient

[2]Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

is measured using a 3-mm diameter Ingold microcombination surface pH electrode and Corning Model 101 digital pH meter. The electrode was calibrated with standard pH buffer solutions at room temperature.

The protein patterns were stained with Coomassie Blue R-250 and destained in 10% ethanol–10% acetic acid (Righetti and Drysdale 1974). After destaining, the gels may be air dried and stored indefinitely.

RESULTS AND DISCUSSION

Figure 1 shows typical protein patterns for 11 species of commercially important New England fishes. The pH gradient in this gel runs from pH 3.5 at the top (anode) to pH 10.0 at the bottom (cathode). The pattern for each species appeared to be unique and demonstrated resolution not normally attained by conventional electrophoretic techniques. Closely related species such as cod and haddock or red hake and white hake show similarities in overall patterns, but enough differences are present to permit a positive identification.

Due to the large number of protein bands resolved in the pH 3.5–10.0 gradient, many of which have the same isoelectric point, it is sometimes advantageous to look at only a small portion of the pattern under increased resolution. Figure 2 shows the same 11 species compared in a pH 3.5–5.0 gradient. The resolution is much greater and identification is not complicated by the presence of as many proteins with the same isoelectric point from species to species.

Figures 3 and 4 illustrate the reproducibility of the protein patterns through a time interval. The proteins in Figure 3 were focused in 2.5 h using a constant power of 10W. The proteins in Figure 4 were focused in 5.5 h using a constant-voltage power supply. The voltage was manually increased from 100 V to 300 V in hourly 100-V intervals. The voltage was then held constant at 300 V for 3.5 h. The proteins in both plates have been focused to equilibrium, and the pattern for each species is reproducible.

The protein patterns one obtains in isoelectric focusing are dependent on the pH gradient formed in the gel. Commercially prepared carrier ampholytes form pH gradients that remain stable and reproducible during the time necessary for the complete equilibrium focusing of sarcoplasmic proteins. Figure 5 shows the pH gradients formed in the previous two figures. The pH gradient curve labeled "A" corresponds to the plate in Figure 3, and the one labeled "B" corresponds to the plate in Figure 4. The slightly lower position of pH gradient A is also seen by the displacement of the patterns in Figure 3 toward the lower end of the gel (cathode). This slight shift of the pH gradient, however, was not enough to affect the reproducibility of the protein patterns.

Isoelectric focusing offers several advantages over electrophoretic techniques for the identification of fish species. Isoelectric focusing is an equilibrium technique where the proteins are limited in how far they can travel by the pH gradient. Since proteins have a net charge of zero at their isoelectric point, no migration beyond that point can take place. Diffusion of the isoelectric proteins is prevented by the electric field. During the course of a normal electrofocusing experiment, as long as the pH gradient remains stable, the protein patterns will not vary. In contrast, protein patterns from conventional electrophoretic techniques are time dependent and may suffer loss of resolution due to diffusion.

Another advantage of isoelectric focusing over conventional electrophoretic techniques is the ease of sample application. Samples were applied directly from micropipettes into molded sample wells. Samples may also be applied as a drop or streak on the gel surface or by placing a small rectangle of filter paper saturated with the sample directly on the gel. The position of sample application may be at any point on the gel slab. While some of these sample application techniques may be common to other electrophoretic procedures, only in IEF may these techniques be used interchangeably without affecting the protein patterns. This versatility is an important asset. Dilute extracts (e.g., when the amount of muscle tissue available is unavoidably small) may be applied in a large volume to obtain a protein pattern comparable to that obtained with a small volume of a concentrated extract (e.g., a drip fluid sample from a recently frozen fish). Large sample volumes may also be applied so that minor components may be detected and compared between species. The ability to vary the position of sample application without affecting the protein pattern eliminates one more possibility for human error. Sample application technique in conventional electrophoretic methods affects the protein pattern. Samples must be carefully applied as a thin

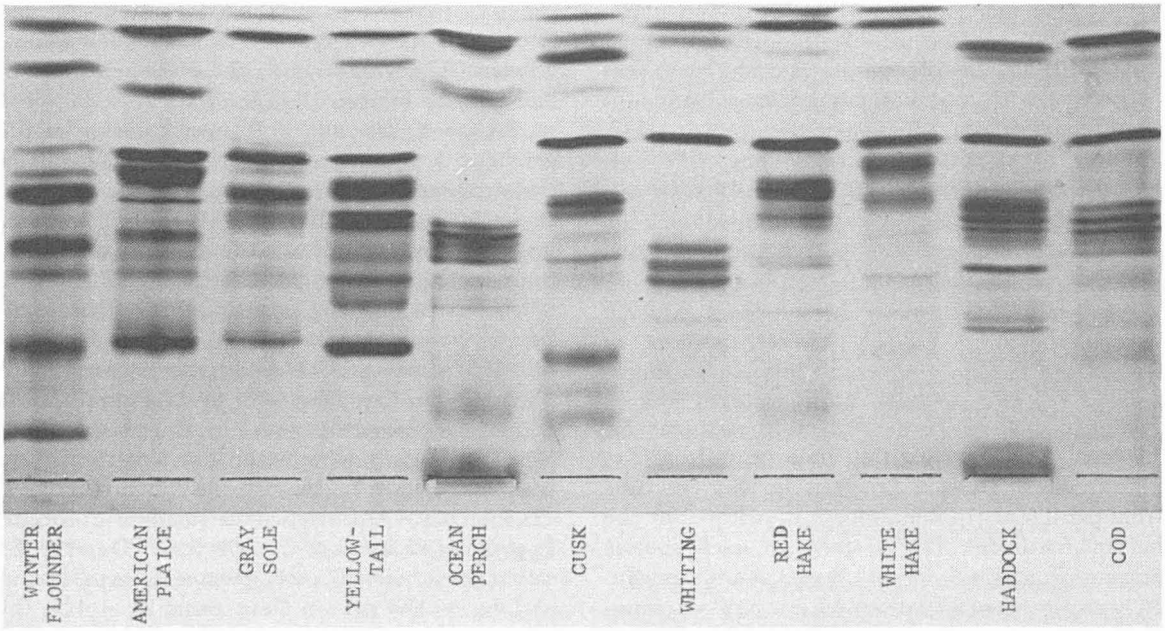

FIGURE 1.—Sarcoplasmic protein patterns from 11 species of fishes focused in a pH 3.5–10 gradient. The species are from left to right: winter flounder, *Pseudopleuronectes americanus*; American plaice, *Hippoglossoides platessoides*; gray sole, *Glyptocephalus cynoglossus*; yellowtail, *Limanda ferruginea*; ocean perch, *Sebastes marinus*; cusk, *Brosme brosme*; whiting, *Merluccius bilinearis*; red hake, *Urophycis chuss*; white hake, *Urophycis tenuis*; haddock, *Melanogrammus aeglefinus*; and cod, *Gadus morhua*.

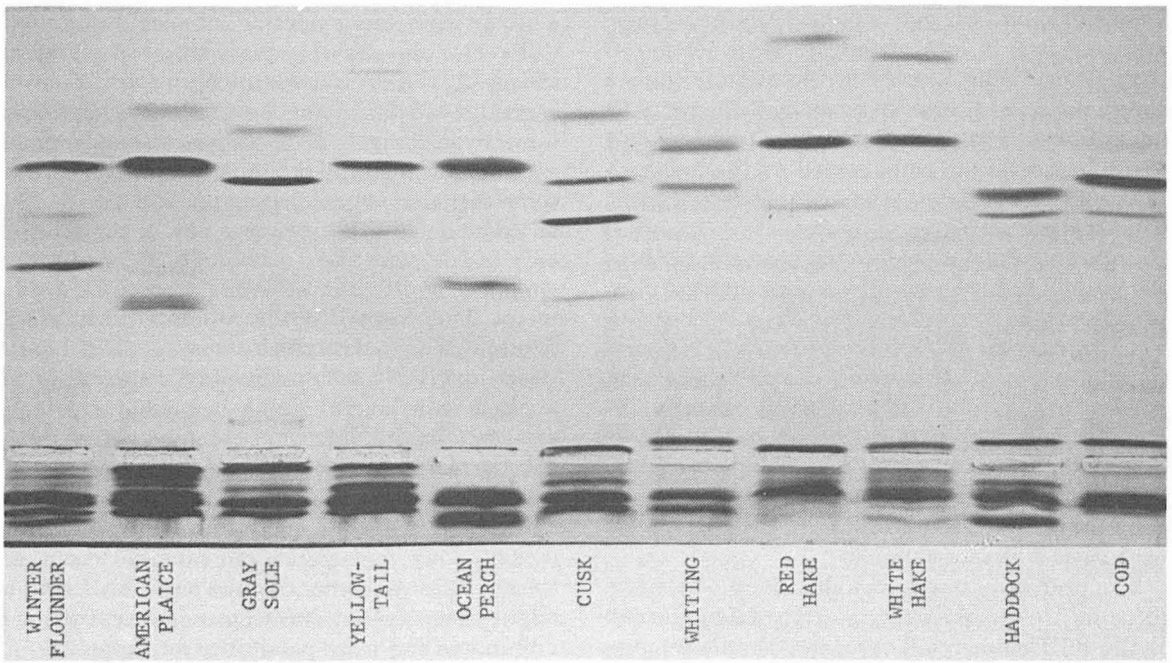

FIGURE 2.—Sarcoplasmic protein patterns from 11 species of fishes focused in a pH 3.5–5 gradient. The species arrangement is the same as shown in Figure 1. Note that the bands separated in Figure 2 correspond to the bands shown in the upper portion of the gel in Figure 1.

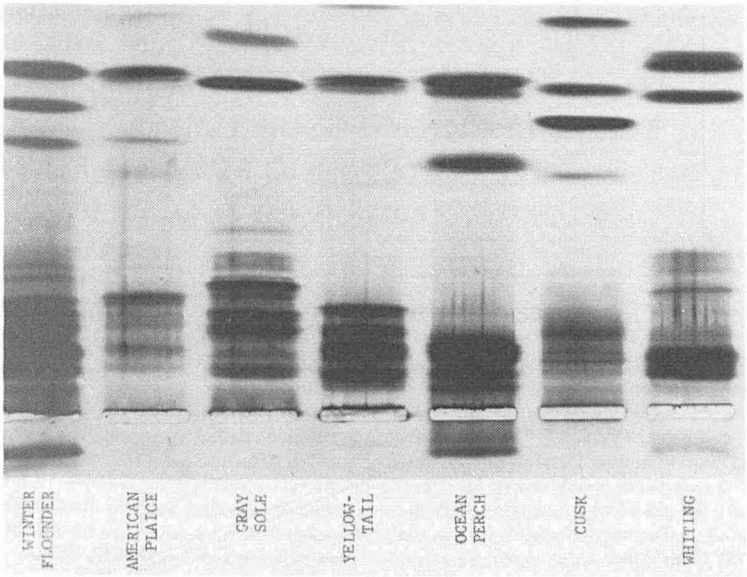

FIGURE 3.—Sarcoplasmic protein patterns from seven species of fishes focused in a pH 3.5–5 gradient under constant power conditions. The species are from left to right: winter flounder, *Pseudopleuronectes americanus*; American plaice, *Hippoglossoides platessoides*; gray sole, *Glyptocephalus cynoglossus*; yellowtail, *Limanda ferruginea*; ocean perch, *Sebastes marinus*; cusk, *Brosme brosme*; and whiting, *Merluccius bilinearis*.

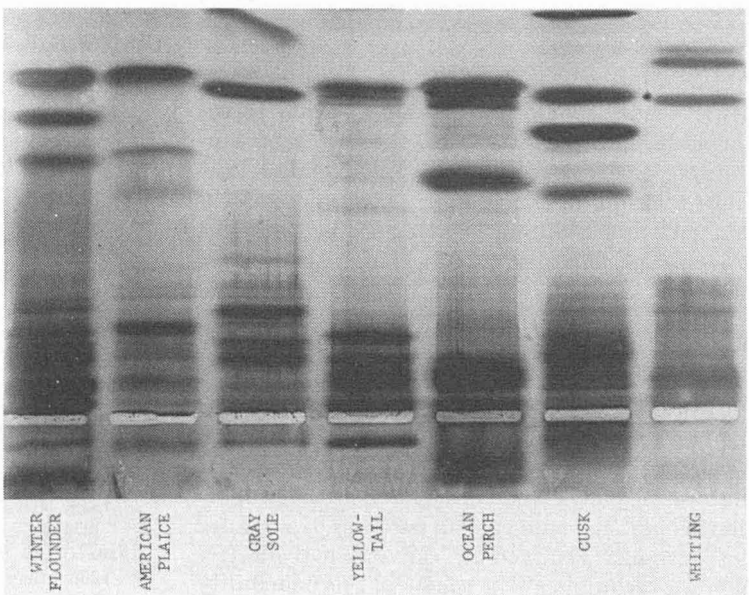

FIGURE 4.—Sacroplasmic protein patterns from seven species of fishes focused in a pH 3.5–5 gradient under constant voltage conditions. The species arrangement is the same as shown in Figure 3. Figures 3 and 4 illustrate the reproducibility of the protein patterns for seven species of fishes on two successive days.

zone at a particular position to obtain a satisfactory separation. Isoelectric focusing is actually less demanding in experimental technique when compared to electrophoresis, yet still offers increased resolution and reproducibility.

Due to the limited number of individuals and species studied, additional work is underway to increase the reliability and potential of IEF as a species identification test. Additional species will be compared. Their protein patterns will be added to a library of standard IEF protein patterns. Additional individuals from each species will be tested for variations in protein patterns due to size, sex, season, or geographical origin. Variations in some minor components of the protein patterns for some species after frozen storage have been observed. Work is planned to examine this in greater detail. The use of commercially prepared polyacrylamide gel slabs will reduce variations in stabilizing media composition and eliminate gel preparation time. These ready prepared gels used with a high-voltage constant-power

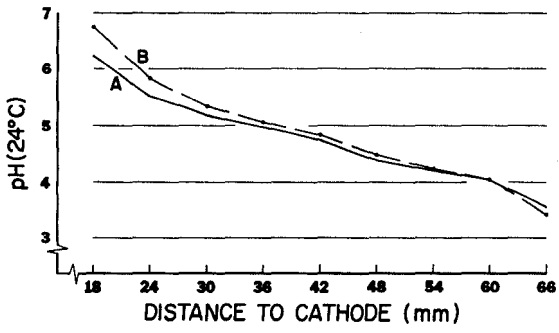

FIGURE 5.—Reproducibility of pH gradients. Measurements of pH were taken after focusing the gels shown in Figures 3 and 4. The pH gradient A corresponds to the pH measurements taken from the gel in Figure 3. The pH gradient B corresponds to the pH measurements taken from the gel in Figure 4. (The pH gradients do not match exactly because the platinum electrodes were not placed with the same relative sample well to cathode distance. The only effect this has on the protein patterns is to shift them either up or down. Relative distances between the various proteins in the pattern remain essentially the same.) The similarity of these two pH gradients may be correlated with the reproducibility of the protein banding patterns shown in Figures 3 and 4.

power supply should produce high quality sarcoplasmic protein patterns in 1.0–1.5 h. New protein staining methods have been investigated that allow staining of the protein patterns in 15–30 min with no destaining required. Using these improvements, samples may be identified in less than 2 h.

CONCLUSIONS

Thin-layer polyacrylamide gel isoelectric focusing has been shown to be a promising technique for the identification of fish species. The inherent high resolution of this method allows the production of characteristic protein patterns of a quality not normally attained by conventional electrophoretic techniques. The excellent reproducibility of this technique should allow the positive identification of fish species without maintaining a supply of known species for use as standards. Investigations utilizing commercially prepared gel slabs, high-voltage constant-power power supplies, and rapid staining techniques promise to produce an extremely reliable procedure for the routine identification of fish species.

ACKNOWLEDGMENT

I thank James Drysdale and Wendy Otavsky of Tufts University Medical School, Boston, Mass., for their valuable assistance in the early stages of this work.

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