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 differentiating 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 (Larsen 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-
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® 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 BOC 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 x 90 mm x 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 H₃PO₄ (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

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is measured using a 3-mm diameter Ingold micro-
combination 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 Coo-
massie 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 En-
gland 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 resolu-
tion not normally attained by conventional
electrophoretic techniques. Closely related spe-
cies 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 re-
solved 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 in-
creased 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 ampho-
lytes 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 identifica-
tion of fish species. Isoelectric focusing is an equi-
librium 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 tech-
niques 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 appli-
cation 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 inter-
changeably without affecting the protein pat-
terns. 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 com-
ponents 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 pat-
tern. Samples must be carefully applied as a thin
FIGURE 1.—Sarcoplastic 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.—Sarcoplastic 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.
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
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.

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