CSIRO
Marine Laboratories

REPORT 177

# A Practical Laboratory Guide to the Techniques and Methodology of Electrophoresis and its Application to Fish Fillet Identification

James B. Shaklee and Clive P. Keenan

1986

COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANIZATION
MARINE RESEARCH LABORATORIES
GPO BOX 1538, HOBART, TAS. 7001, AUSTRALIA

**National Library of Australia Cataloguing-in-Publication Entry**

Shaklee, James B.
A practical laboratory guide to techniques and methodology of electrophoresis and its application to fish fillet identification.

Bibliography.

ISBN 0 643 03649 0.

1. Electrophoresis—Laboratory manuals. 2. Fish fillets —Identification. I. Keenan, Clive P. II. Commonwealth Scientific and Industrial Research Organization, Australia. Marine Laboratories. III. Title: (Series: Report (Commonwealth Scientific and Industrial Research Organization, Australia. Marine Laboratories); no. 177).

541.3'7



*86.171-850*

## CONTENTS

# A PRACTICAL LABORATORY GUIDE TO THE TECHNIQUES AND METHODOLOGY OF ELECTROPHORESIS AND ITS APPLICATION TO FISH FILLET IDENTIFICATION

*James B. Shaklee[1] and Clive P. Keenan[2]*

Division of Fisheries Research
CSIRO Marine Laboratories
PO Box 120, Cleveland, Qld 4163

[1]Present address: Washington State Department of Fisheries, 115 General Administration Building, Olympia, Washington 98504 USA

[2]Department of Zoology, University of Queensland, St Lucia, Qld 4067

Aust. CSIRO Mar. Lab. Rep. 177 (1986)

***ABSTRACT***

Gel electrophoresis is a simple, fast and powerful method of separating proteins in a complex mixture. When combined with sensitive histochemical methods of visualizing general proteins and specific enzymes, electrophoretic techniques can be used to provide a qualitative characterization of the protein composition of cells, tissues and organs. Because organisms generally exhibit species-specific protein profiles, electrophoresis is an ideal technique for the identification of the species of origin of many different biological products. In this report, we present a description of specific methodologies for both starch and polyacrylamide gel electrophoresis and for subsequent staining. Although the procedures have a variety of applications, the emphasis throughout this guide is on species discrimination and fish fillet identification. We also provide detailed documentation of the results of these procedures for over 150 different species. Our goal in this research is to encourage the development and adoption of a reliable standardized experimental protocol for such investigations that will strengthen and facilitate the entire process of fish fillet identification.

## INTRODUCTION

There is a clear and growing need to be able to identify processed fishery products to: 1) monitor and regulate fishing activities in the field, 2) inspect and certify products in the wholesale and retail trade, and 3) protect the consumers of both uncooked and cooked fishery products. This need is a consequence of the steadily increasing trend of marketing processed products instead of intact fish. Such marketing practices make it difficult to identify the species involved because the external features by which species are usually identified are commonly removed or obscured by processing. As a result, the frequency and extent of substitution of one species for another has increased, both in the red meat industry and in the marketing of fishery products. The problems faced in these two fields are fundamentally different in that few "red meat species" can be substituted for one another, while in fisheries, the number of potential substitute species is frequently in the tens or even hundreds.

We have recently published the results of our investigations employing slab polyacrylamide gel electrophoresis (followed by non-specific protein staining) together with starch gel electrophoresis (followed by specific enzyme staining) as a two-part methodology for identifying fish fillets and other fishery products (Keenan and Shaklee, 1985). The present report is a detailed, practical guide outlining the specific reagents, equipment and procedures of this methodology, and is intended to facilitate the use of these techniques by others.

## APPARATUS AND REAGENTS

### A. Starch gel electrophoresis

Horizontal starch gel electrophoresis has been used throughout our investigations for reasons of simplicity, efficiency and economy. Many of the basic features of the apparatus and techniques we use are derived from those of Selander

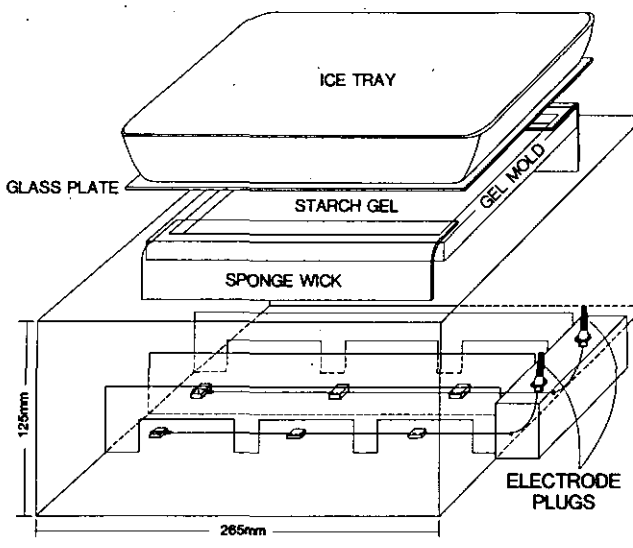


**Figure 1.** Starch gel apparatus. exploded view (lid not shown).

*et al.*, 1971 (see also, Siciliano and Shaw, 1976). The gel molds and electrophoresis chambers (Figure 1) are made of ethylmethacrylate (Perspex®, Plexiglas®), require no machining of parts, and can be readily made in any laboratory. The particular dimensions of the gel molds (internal dimensions: 178 mm x 178 mm x 5.5 mm) allow up to 28 samples to be run on a gel and up to four different slices to be cut (allowing for the staining of four different enzymes on the same gel) using the specific procedures outlined below. The electrophoresis apparatus (chamber and lid) contains the two electrode buffer reservoirs, supports the gel mold, encloses the entire experiment during the electrophoretic run, and prevents evaporation of the electrode buffers between runs. The glass plate supports a tray of crushed ice on top of the starch gel during electrophoresis. The ice helps to maintain the temperature of the gel near 5°C during the electrophoretic run to minimize protein denaturation due to the heating that occurs.

Detailed listings of required reagents (including abbreviations and sources) as well as recipes for buffers and other solutions used in starch gel electrophoresis are presented in Appendixes 1-3. Recipes for enzyme stains are in Appendix 4.

## B. Polyacrylamide gel electrophoresis

The analysis of general muscle proteins by horizontal slab polyacrylamide electrophoresis has proven to be a simple and powerful method of species identification. The gel casting method of Gahne *et al.*, 1977 was modified slightly to yield large (200 x 260 mm), thin (0.8mm) polyacrylamide gels (Figure 2) in order to improve staining and resolution. The LIOH buffer described in Appendix 5 was used for all polyacrylamide gels because of its superior resolving power and its relatively low current-carrying properties which enabled high voltage runs of relatively short duration to be used. Three concentrations of acrylamide were employed in the gel to allow efficient sample loading and produce a "stacking" effect resulting in increased resolution. All necessary equipment can be built in the laboratory from readily available supplies. The gels may be made up to a month prior to use if they are wrapped in polyethylene film, e.g. Glad Wrap® (or placed in a moist chamber) to prevent desiccation, and kept in a refrigerator.

The details of all solutions used in polyacrylamide gel electrophoresis and subsequent staining of general proteins are included in Appendix 5.

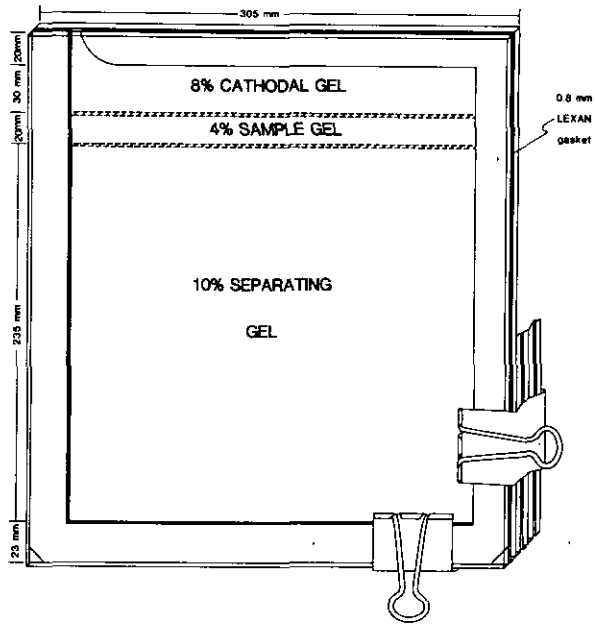


**Figure 2.** Polyacrylamide gel molds (3) assembled for pouring.

# PROCEDURES

## A. Sample preparation and storage

### *(i) Reference samples and specimens*

It is necessary to utilize reference or "control" samples on each gel in order to identify unknown samples unambiguously by electrophoretic techniques (see Learson, 1970; Lundstrom, 1983). This is primarily because of differences between gels resulting from variability in: the electrophoretic medium (due to differences among lots of chemicals and/or conditions during the making of the gels; see Rodbard and Chrambach, 1971) and/or the conditions of electrophoresis (due to differences in temperature, duration of the run, voltage and/or current applied to the gels, and/or buffer conditions).

Not only should the reference tissue sample or extract be representative of the species and in good condition, but it should also be supported by a voucher museum specimen (the preserved carcass of the animal from which the control sample was taken) if possible. The existence of such museum voucher specimens ensures that the species identity of the reference sample could be established independently, if necessary, at any time in the future. Thus, voucher specimens provide an important form of documentation in the event of any doubt of the authenticity of the reference sample and protect the entire process in the case of litigation over

any contentious species identifications. Museum specimens should be accompanied by as much collection data as possible, both to provide documentation that might be of relevance to fillet identification studies, per se (e.g. in cases of geographically localized polymorphisms in electrophoretic characteristics; see Lundstrom, 1981; Keenan and Shaklee, 1985; Shaklee and Salini, 1985), and to ensure that the preserved specimen itself is of maximal utility to other users of the museum's collection.

We have retained a number of such voucher museum specimens from species used in our research (see Appendix 7). These specimens are lodged in the fish collection of the Australian Museum (6-8 College Street, Sydney, NSW 2000, Australia). The specimens from Tasmania and southern Australia were identified by Mr K. Evans (Tasmanian Fisheries Development Authority), and all other specimens were identified by Dr J. Paxton (Australian Museum). At the same time, we have established a "frozen tissue bank" of fillet samples from many of these same fishes. These frozen muscle samples are currently being stored at -70°C by the CSIRO Division of Fisheries Research at its laboratory in Cleveland. Individuals wishing to obtain small portions of some of these frozen tissue samples should contact the officer-in-charge of this laboratory.

### *(ii) Tissues*

All tissue samples should either be dissected from fresh specimens as soon as possible or from specimens that have been stored frozen at -20°C or colder. Frozen specimens should be wrapped in plastic to reduce desiccation until they are dissected. To minimize potential artifacts due to protein denaturation during handling, it is best to conduct all steps of sample processing at 5°C.

Most fish species have both "white" and "red" types of skeletal muscle; these two types of tissue have major physiological and biochemical differences (Bone, 1966; Crabtree and Newsholme, 1972; Johnston and Goldspink, 1973a, b; Tsuyuki, 1974; Sharp and Pirages, 1978; Love, 1980). Because the enzyme and general muscle protein profiles of these two types of muscle can be substantially different (Hamoir *et al.*, 1972; Clayton *et al.*, 1973; Sharp and Pirages, 1978; Totland *et al.*, 1978), it is important to dissect the appropriate muscle tissue from the specimen. In general, white skeletal muscle fibers predominate in the body musculature of most fishes, while the red muscle fibers are restricted to a small, superficial band of fibers immediately internal to the lateral line and a second minor band surrounding the vertebral column. As a result, most fish products (fillets, steaks, mince, etc.) also consist of predominantly white muscle tissue. Red muscle fibers are more widespread in the body musculature of salmonid (trout and salmon) and scombrid (tunas and mackerels) fishes. Thus, products from these fishes generally have a high proportion of red muscle fibers.

Reference or control muscle extracts should have a similar muscle-type composition to that expected for the unknown samples being tested. Throughout our experiments with fish we have intentionally prepared and analyzed muscle samples consisting of essentially only white muscle fibers.

The muscles of many invertebrates are similarly heterogeneous in their physiology and biochemical composition. Indeed, major quantitative and/or qualitative differences in protein (enzyme) content of various invertebrate muscles have been documented in the literature Thus, the composition of muscle in the tail of decapod crustaceans can be significantly different from that in walking leg or claw; similarly, the muscle in squid fins has a substantially different protein composition from that of squid mantle (Zammit and Newsholme, 1976; Dando *et al.*, 1981). For this reason, it is important to use appropriate 'control' muscle samples as reference material for identifying unknowns.

### *(iii) Homogenates*

The most versatile type of sample for analysis consists of a supernatant extract derived from homogenizing a piece of tissue (muscle) in a buffer solution and then subjecting the homogenate to centrifugation to remove all insoluble cellular debris. Such tissue extracts have the advantage of generally superior resolution during electrophoretic analysis and are easily stored for long periods in a freezer, which permits their repeated analysis if desired. For these reasons, tissue homogenates are particularly appropriate for reference samples of known species against which unknowns will be tested.

The homogenization procedure we recommend is to mix a piece of tissue with an equal volume of "homogenizing" buffer in a polycarbonate centrifuge tube and macerate the mixture, using a loose-fitting, motor-driven, stainless steel pestle for approximately 15-30 sec. Following homogenization, the slurry is clarified by centrifugation at 5°C. We routinely centrifuge homogenates at approximately 24,000 x g for 30 min, but shorter runs at lower g forces are adequate to remove most of the cellular debris and unhomogenized tissue fragments.

Following centrifugation, the supernatant solution is pipetted off and sealed in a small, labelled glass or plastic vial, which is subsequently stored frozen. Such homogenate samples remain usable for several weeks or even months when stored at -20°C, and for 6 to more than 18 months if stored at -70°C (see below),. Repeated thawing and refreezing of such samples has little effect on the resulting protein or enzyme profiles (see below), provided such samples remain on ice (at 5°C) for the short time that they are thawed.

### *(iv) Heated samples*

Ordinary cooking of fish fillets results in the denaturation of most water-soluble proteins present in the tissue. However, one family of proteins, the parvalbumins, is largely unaffected by normal cooking. Cooked fish products can, therefore, be identified by electrophoresis, using these proteins as species-specific markers (Keenan and Shaklee, 1985).

As with the electrophoretic identification of raw fishery products, the identification of cooked products requires the preparation of suitable reference samples. This can be done simply by heating a portion of the normal muscle extract from the reference species. An aliquot of muscle extract (about 1 ml) in a 4 ml polycarbonate centrifuge tube should be heated by immersion in a boiling water bath for 5 min. After centrifugation, the resulting heat-treated supernatant is pipetted into vials and stored frozen until required. This heat treatment usually denatures all soluble proteins present in the extract except the parvalbumins, of which there are usually 1-4 different forms present in any given species. As they seem to be unaffected by these heating conditions, they serve as ideal species-specific markers for the identification of cooked fish products.

### *(v) Tissue "drip" samples*

For the routine screening of large numbers of unknown samples, the preparation of homogenates is a time-consuming and largely unnecessary process. An alternative approach is to use some of the intercellular fluid or tissue "drip" that is present in virtually all tissue samples (Numachi, 1971). This procedure simply involves making a cut in the muscle tissue sample and touching a "sample wick" (a small piece of filter paper, see below) to the tissue. The sample wick will absorb a small amount of the intercellular fluid present in the tissue. Because the intercellular fluid contains dissolved proteins characteristic of the tissue, it can be used in place of a tissue homogenate for electrophoresis. If appropriate, the remaining tissue sample can be stored refrigerated at 5°C until the electrophoretic analyses are completed, or it can be wrapped in aluminium foil or plastic and stored frozen at -20°C indefinitely.

## B. Starch gel electrophoresis

### *(i) Making the gel*

All the starch gels used in our investigations consist of 12% (w/v) starch. The dry starch is weighed into a tared 1,000 ml heavy-walled Erlenmeyer flask and the electrophoresis gel buffer added. The mixture of starch and buffer is then immediately swirled to generate a uniform suspension and heated to boiling over a Mekar burner. It is very important to swirl the starch suspension actively as the gel is cooked to ensure that it is heated evenly throughout and that none of it burns.

After the solution has gently boiled for about 30 sec, it is degassed for approximately 15 sec using a water aspirator or a vacuum pump to remove dissolved air, which will otherwise form bubbles in the gel as it cools. The hot starch solution is then poured into the gel mold. Any bubbles that form during this process should be removed immediately from the molten gel with a Pasteur pipette.

The hot gel is allowed to cool at room temperature for 20-40 min. Once the gel has cooled to room temperature it is covered with a piece of Glad Wrap® (or similar product) to prevent desiccation. Care should be taken to avoid trapping air bubbles between the surface of the gel and the Glad Wrap®, as these lead to localized desiccation of the surface of the starch gel. The gel can either be cooled in a refrigerator to 5°C and then used immediately, or it can be stored at **room temperature** for up to 24 h before being cooled to 5°C for use.

Because the properties of the gel change slightly as it ages, all gels should be treated in a uniform manner. We find that cooking the gels on the afternoon of the day preceding an experiment is convenient, as it allows one to begin an experiment first thing in the morning.

Many different buffer systems can be used in starch gel electrophoresis (cf. Appendix 2 and associated references). Because the final resolution of enzyme banding patterns is strongly influenced by the buffer conditions during electrophoresis and because different enzymes of the same species and the same enzyme in different species respond uniquely, the choice of appropriate buffer systems is difficult, and must be determined empirically. As a starting point, we recommend the TC-1 and CAEA buffers, which give good resolution for most enzymes tested. These two buffers were used to generate the data in Appendix 6 and most of the starch gel figures throughout the text.

Many of the characteristics of starch gels are dependent upon the properties of the starch from which they are made. Because starches from different manufacturers and even different starch lots from the same manufacturer differ significantly, it is essential that each new lot of starch be thoroughly tested using "known" samples in order to calibrate it before it is used for the identification of unknown samples. Once a starch lot has been characterized adequately, it can then be used for routine testing. We have used Connaught starch (lots # 380-2 and # 396-1) throughout the present study.

### *(ii) Gel loading and running*

Once the starch gel has been cooled to 5°C, it is ready to be loaded with samples. The samples to be tested should be made ready for loading before the gel is removed from the cold. Frozen samples should be thawed and placed on ice, tissues from which "drip" samples will be taken should be readied, etc. The Glad Wrap® covering should be removed from the cooled starch gel and the gel cut into two pieces along a straight line parallel to the cathodal edge of the mold, using a scalpel or knife to create the sample origin. It is important that the origin be cut straight, and that it be parallel to the cathodal end of the gel and vertical through the thickness of the gel. The precise position of the sample origin cut can be altered to suit the particular tests being run, although we find that for most species, buffers and enzymes, a sample origin placed between 40 and 60 mm from the cathodal (negative) end of the gel is optimal.

Because the actual position of the sample origin often affects the resulting relative mobilities, it is important to standardize the position of the sample origin so that results from different gels will be comparable.

Portions of the samples to be analyzed, whether from tissue homogenates or "drip" samples, are "loaded" onto the gel using (5 mm wide x 6.5 mm high) filter paper wicks (made from Whatman #3 paper). The wick is dipped into the sample, allowing it to absorb some of the sample fluid by capillarity. When the wick is saturated with sample solution for about four-fifths of its length, it is removed from the sample and placed on a large piece of filter paper. Tissue fluid originally saturating the lower four-fifths of the sample wick will be drawn up into the unwetted portion of the wick, thereby ensuring that the wick is not too wet. The passive blotting that occurs while the sample wick is on the filter paper serves to remove any excess fluid from the sample wick and thereby reduces the chance of sample leakage from a wick overly saturated with fluid.

The procedure is then repeated until wicks have been dipped into all samples to be loaded onto one gel. The saturated sample wicks are then individually inserted side-by-side into the cut in the gel forming the sample origin. Twenty-eight sample wicks can be loaded per gel. The handling of sample wicks is best accomplished using watchmaker's forceps. Wick insertion into the gel is facilitated by carefully spreading apart the two cut edges of the gel at the sample origin, using the thumb and forefinger of one hand while holding the sample wick in the forceps in the other hand. Care must be taken to ensure that each wick is positioned vertically in the gel and that adjacent wicks are separated from each other by approximately 1 mm. The gap between adjacent wicks is very important as it reduces the chance that a sample will "leak" and contaminate a neighboring one. It also makes interpretation of the final enzyme patterns easier because adjacent channels are well separated from one another. In order to minimize distortion of the first and last samples due to retarded migration at the edges of the gel, a gap of approximately 3-4 mm is left between the edges of the gel and the first and the last sample wicks.

In order to monitor the progress of the electrophoretic run, we routinely load a half-width sample wick with 0.1% bromophenol blue tracking dye and insert it as the first wick in the gel. This dye has a large negative charge and migrates anodally through the gel ahead of all proteins. To standardize each gel, it is necessary to apply one or more control or reference sample(s) to the gel. This allows the comparison of the banding patterns of unknown samples with that of the standard and compensates for any gel-to-gel variation in actual electrophoretic conditions.

We also routinely load a reference sample at the seventh and twenty-first positions on the gel. In this way, no unknown is more than 7 positions away from a standard, and a determination of the relative mobilities of all bands in the unknowns compared to those of the reference sample(s) is easily and reliably made. We have used an extract of barramundi muscle as a general reference sample in all of our fish fillet identification studies, but almost any well-characterized sample would serve the intended purpose of standardizing the results of each individual gel. If one is analyzing a large number of samples all presumed to be a single species, one of the best controls would be a tissue sample from a verified specimen of the same species. In such a case, any samples of other species should be readily distinguished by the fact that they exhibit a different banding pattern from that of the reference sample.

Once all sample wicks have been inserted into the gel, the cathodal portion of the gel should be pushed gently toward the anodal portion to expel any air bubbles trapped at the sample origin during sample loading. The covering of Glad Wrap® is then replaced on top of the gel surface. At the extreme anodal and cathodal ends of the gel, the Glad Wrap® is folded back to expose a 10 mm wide strip of the gel

surface. The gel mold is then placed in the electrophoresis chamber (which was previously filled with the appropriate electrode buffer) and the sponge wicks (Wettex®, Sweden) in each electrode chamber are placed so that one end of the sponge is in contact with the exposed strip of the gel surface and the other end of the sponge wick is in the electrode buffer solution. (Previously unused sponge wicks should be extensively washed in tap water and then rinsed in distilled water before they are used for electrophoresis.)

When both sponge wicks are in place, the ends of the Glad Wrap® are folded back over the exposed ends of the sponge wicks and a glass plate is placed over the gel, supported slightly above the gel surface by the sponge wicks (cf. Figure 1). A metal tray (approx. 23 cm x 23 cm x 5 cm deep) filled with crushed ice is then placed on top of the glass plate and the lid placed on the electrophoresis chamber.

Once the electrophoresis chamber is covered, the electrode leads from the power supply can be connected to the chamber. The assembled apparatus is then cooled in a refrigerator at 5°C for about 15 min before the power supply is turned on at half power for 10 min to "run in" the samples. After this initial 10 min period, the power is turned off, the apparatus partially disassembled and the sample wicks removed from the gel. The cathodal end of the gel is again pressed gently against the anodal piece to expel any air bubbles trapped at the sample origin.

The apparatus is then reassembled, returned to the refrigerator, and electrophoresis commenced at full power. Most buffer systems work best under conditions of **constant voltage** but a few, such as LIOH and POULIK, are optimally run at **constant current**. The duration of each electrophoretic run will be determined by the particular species, buffer and enzyme(s) being analyzed, but in general, electrophoretic runs of 4 to 6 hours are optimal. During long electrophoretic runs (e.g. >5 h) it may be necessary to replenish the ice in the ice tray to ensure that the temperature of the gel remains close to 5°C.

### *(iii) Gel slicing and staining*

At the end of the electrophoretic run, the power supply must be turned off **before** the apparatus is disassembled. After the Glad Wrap® is removed from the surface of the gel, the edges of the starch gel are trimmed off, using a scalpel or sharp knife. A cut is made along each lateral edge of the gel (perpendicular to the sample origin) about 1-2 mm in from the edge of the gel. Similarly, the anodal and cathodal extremes of the starch gel are cut off. The exact placement of these cuts is determined by the probable position of proteins in the gel (in general a piece of 10-30 mm is removed from each end of the gel). This trimming is done to remove the edges of the gel (where desiccation during the electrophoretic run is greatest) to facilitate the cutting of uniform slices for staining and to remove unnecessary portions of the gel so that subsequent slices will fit into the staining boxes easily.

Small diagonal notches are then cut into the extreme anodal and cathodal edges of the anodal and cathodal pieces of the gel (respectively) at the edge where the first sample (position #1) was loaded. These notches serve to mark the position of the first sample in all subsequent slices of the gel.

The pieces of the gel are then carefully pushed horizontally in the mold to loosen the bottom surface of the gel. Once the gel slides freely in the mold, each piece can be carefully removed by hand and placed on the slicing template (the surface of the gel that was in contact with the gel mold should be in contact with the surface of the slicing template). The slicing template we use is a sheet of Perspex® (185 mm wide x 450 mm long) with raised edges (15 mm wide x 1.4 mm) running along its length. For gels made with electrophoresis buffers of pH < 7.5, it is a good idea to remove excess fluid on the bottom surface of the gel by lightly blotting it before

placing it on the slicing template (this prevents the gel from sliding on the template during slicing). Any air bubbles trapped between the template and the bottom surface of the gel should be expelled by carefully pressing downward and laterally with moistened fingertips. A piece of plate glass (approx. 6 mm thick) is then placed on top of the gel to ensure that the gel remains flat against the template during slicing.

A thin piece of the gel is then sliced off the bottom. We recommend using a 0.28 mm (= 0.011 inch) diameter stainless steel wire tightly stretched on a hacksaw frame as a slicer. This apparatus (like a giant cheese slicer) gives a clean, uniform cut and will generally work for many weeks or even months before the wire breaks and needs to be replaced. Alternatively, it is possible to use lightweight monofilament fishing line (e.g. 2 lb or 4 lb test) tightly stretched between the hands to cut the gel. The stainless steel wire is cleaned between subsequent cuts by wiping it with a damp cloth to remove any starch sticking to its surface, while a new length of fishing monofilament line is used for each new cut of the gel.

The precise thickness of each gel slice is not absolutely critical, although it is quite important that the thickness is uniform across the entire slice. In practice, we usually use a template with guides set to give slices of about 1.5 mm thickness. Thinner slices are difficult to handle and often result in inadequate staining intensity for many enzymes. Thicker slices, while being easier to handle and often yielding stronger enzyme staining, mean that fewer slices can be obtained from a given gel and thus fewer enzymes stained in any one experiment.

Once a slice has been cut through the gel, the glass plate is removed and the top (thick) piece of the gel is carefully lifted by hand off the bottom slice (which remains in contact with the slicing template) and then repositioned on the template (in its original orientation) for the next cut.

The slicing process is repeated as many times as necessary, each time cutting off a slice from the bottom surface of the starch gel. Each cut slice is then carefully picked up and placed in a clear plastic staining box. We use boxes (with hinged lids) measuring 18.6 cm x 13.5 cm x 4.8 cm deep (box A-401 obtained from Flambeau). A few drops of distilled water in the bottom of the staining box facilitate placing of the gel slice in the box, as the water prevents the gel from sticking to the surface of the box. The cut surface of the slice should always face up, since subsequent enzyme staining depends upon reactions largely restricted to the surface of the gel. Any large air bubbles trapped under the gel slice should be expelled before staining to ensure a flat gel surface and to avoid subsequent oxidation problems in any staining reactions that are dependent upon reduction of staining components for enzyme localization (e.g. dehydrogenases).

The enzyme stains are usually applied to the gel surface as either agar overlays or as solutions. The former technique is preferable, as it often yields better resolution (particularly for enzymes that are visualized by staining employing multiple linked reaction steps); it is usually slightly more sensitive; and it requires smaller volumes of staining reagents (and is, therefore, less expensive).

In the agar overlay technique, the staining reagents are dissolved in one volume (approx. 12-15 ml) of staining buffer and then mixed with an equal volume of 2% agar (dissolved in distilled water) at 55-60°C and immediately poured uniformly over the surface of the gel. If the agar solution is too hot it can inactivate the enzymes, and if it is too cold it will begin to solidify before the overlay has been completely poured over the gel surface. With the few enzyme stains that are not optimal with the agar overlay technique, staining is accomplished using a solution of staining components and staining buffer to a final volume of about 50 ml. Whatever volume is used, it is important that the staining solution completely covers the surface of the gel.

The pH of the staining buffer used for each enzyme stain is determined by the pH optimum of the enzyme and/or of the staining reaction and the pH of the electrophoresis buffer used. The staining reactions for most enzymes have pH optima near neutrality, so generally the pH of the electrophoresis buffer determines which staining buffer should be used. Thus, pH 8 staining buffer is usually used for staining enzymes when electrophoresis has been accomplished in a gel buffer of pH $< 8$, while pH 7 staining buffer is used for staining enzymes on gels having a pH $> 8$. This ensures that the final pH of staining is about 7.5 to 8. Exceptions to these general guidelines include esterases (which are routinely stained using a pH 4.5 staining buffer), aspartate aminotransferase and peptidases (which are always stained using pH 8 buffer), and superoxide dismutase (which is stained using pH 8 buffer when electrophoresis has been at a pH of 7.5 or greater, or using pH 9 staining buffer when electrophoresis has been at a pH of less than 7.5).

All staining is routinely accomplished in an incubator set at 37-43°C. Because many of the enzyme staining reactions utilize light-sensitive components, staining must be accomplished in the dark. An incubator provides such a dark environment and the elevated temperature inside the incubator increases the rate of enzyme staining.

## C. Polyacrylamide gel electrophoresis

### *(i) Making the gels*

A complete gel mold consists of two glass plates of the same size (300 x 330 x 2 mm with bevelled edges) separated by a polycarbonate (Lexan®) gasket (0.8 mm thick) as illustrated in Figure 2. The glass plates and gaskets used to make the gels must be washed thoroughly with detergent, rinsed with tap and then distilled water, and dried and stored in a dust-free environment e.g. on a rack in a drying oven. Three complete glass-gasket-glass gel molds can be assembled and held together by strong paper clamps (Esco #5 fold back clip) placed side-by-side around all four edges of the molds. This allows the pouring of three gels at once. Care should be taken when assembling the molds, as both finger prints and dust particles can cause problems in casting the gel.

Different concentrations of acrylamide solution are used to produce three regions of the gel: a large separating gel of 10% acrylamide, a small sample loading gel of 4% acrylamide, and a small cathode end gel of 8% acrylamide. Quantities of the four stock solutions necessary to pour **one set of three gels** (consisting of 10%, 4% and 8% layers) are indicated below:

| **Components (ml)** | **Gel Concentration** | | |
|---|---|---|---|
| | **10%** | **4%** | **8%** |
| Acrylamide stock | 50 | 2 | 8 |
| Gel buffer stock | 40 | 4 | 8 |
| Ammonium persulphate | 30 | 4 | 8 |
| Distilled water | 40 | 6 | 8 |

The 10% layer is poured first, after mixing the required volumes of acrylamide stock, gel buffer stock and water together in a 500 ml Erlenmeyer flask. This mixture is degassed using a water aspirator (while tapping the flask on the counter) until all small bubbles disappear from the mixture. The ammonium persulphate solution is then added with a minimum of splashing and the mixture degassed quickly to avoid premature polymerization. After degassing, this final solution is poured between the glass plates to the required height (235 mm), using a separatory funnel fitted with a flexible plastic tube and a fine tip (a shortened 19 gauge needle is satisfactory).

After each mold is filled, 0.5 ml of AR grade isobutanol (2 methylpropan-1-ol) is carefully pipetted onto the top surface of the acrylamide to facilitate polymerization of the gel. (The funnel must be emptied and rinsed well with distilled water before the solution polymerizes in the fine tubing.) After about 20 minutes a straight boundary curving down at the edges of

the mold forms near the top of the gel solution, indicating that the polyacrylamide has polymerized.

The next (4%) solution may now be mixed, degassed and poured. The original isobutanol will rise over the new solution. Finally, after the 4% layer has polymerized, the alcohol (and unpolymerized acrylamide solution) is poured off to waste. The molds are inclined so they can be filled to the top (without trapping air bubbles) with the final 8% solution. After this final layer has polymerized, the gels are ready to use.

If the gels are not going to be used immediately, they should be wrapped to prevent desiccation, and stored at 5°C. Gels can be successfully stored in this manner for up to 30 days prior to use.

### *(ii) Gel loading and running*

Before samples can be loaded on a gel, one of the glass plates and the Lexan® gasket must first be removed. A scalpel or spatula is inserted between the two glass plates in one corner at the 10% end of the gel and slowly twisted to separate the two pieces of glass. The gel adheres to one or the other of the plates and it is often difficult to see if it is the top or bottom plate. If necessary the mold is turned over so the gel remains adhering to the lower glass plate. Once the two pieces of glass begin to separate from one another in the corner, the scalpel (or spatula) is run down the edge of the gel mold toward the 4% gel. If the gel has desiccated during storage it is often difficult to separate the two glass plates cleanly.

Once one of the glass plates and the Lexan® gasket have been removed, a clean piece of polyethylene (about 4 mil thickness) is laid over the top surface of the gel and any trapped air bubbles gently removed. The sheet of plastic prevents drying of the gel during electrophoresis. The covering plastic is folded back to expose the surface of the 8 and 4% layers. The gel is now ready for loading.

Samples are prepared in the same way as for starch gel electrophoresis (with or without heating; see EXAMPLES AND DISCUSSION section B (ii) Parvalbumins). However, unlike for starch gels, the samples are applied to the **surface** of the 4% gel in a straight line down the middle of this section. Wicks made from Whatman #3 filter paper (8 mm x 3 mm) are dipped into the sample, lightly blotted to remove excess liquid, and laid directly on the gel surface, separated by a 1 mm space from adjoining wicks. For maximum resolution, 24-27 samples can be loaded on a gel with the wicks positioned so that their short axis (3 mm) is parallel to the direction of protein migration. For routine screening, the number of samples loaded on a gel can be increased to approximately 50-60 if the wicks are positioned so that their long axis (8 mm) is parallel to the direction of protein migration. Light pressure is applied to the sample wicks as they are placed on the surface of the gel to ensure adequate contact between the wick and the surface of the gel.

If the sample wicks are too dry when they are loaded onto the gel, they will be difficult to remove after the sample has been "run in" to the gel. If not enough protein is being transferred to the gel, Schleicher and Schuell #470 or Whatman #17 chromatography paper can be used for the sample wicks. These thicker sample wicks are usually necessary when loading 50-60 samples per gel, using wicks turned so that their short axes are parallel to the sample origin.

Standards of BSA (1 mg/ml) are loaded at both edges and in the center of the gel (i.e. positions #1, #14, and #27) to facilitate determination of the relative mobilities of the protein bands in the unknown samples on the gel.

Electrophoresis can be accomplished with the gel in a refrigerated cabinet at 5°C, on a bench top at room temperature with the gel resting on a cooling plate (temperature about 5°C), or with the gel on a cooling plate in a refrigerated cabinet. The use of a cooling plate allows electrophoresis to be conducted at higher

voltages and, therefore, allows shorter run times. However, all methods produce comparable results.

The cathodal and anodal electrode buffer tanks are each filled with 250 ml of LIOH A buffer. Bromophenol blue (4 drops of 1% aqueous solution) is added to the cathodal buffer tank to serve as a tracking dye. The gel plate rests on the edge of the buffer tanks (or directly on the cooling plate, if used) and the gel connected to them with sponge wicks (Wettex®) soaked in the electrode buffer. It is important to place the wicks correctly on the gel. Sponge wicks generally have one smooth side (the other side usually has a somewhat irregular surface). The smooth side should be in contact with the gel to ensure a uniform electrical current throughout the gel. The edges of the wick in contact with the gel should be parallel to the sample origin (and parallel to one another). The wicks should overlap the distal 2 cm of both the 8% cathode gel and the 10% anodal (separation) gel and are covered by the plastic gel covering.

After loading the sample wicks on the gel, the gel is allowed to cool on the cooling plate or in the refrigerator for 10 min before any electrical current is applied. Samples are "run in" the gel for 10 minutes at the normal power setting. The "run in" time can be varied to increase or decrease the amount of protein passing into the gel and hence the strength of staining. The power is then switched off and the sample wicks carefully removed from the surface of the gel.

The starting electric potential for a gel run in a refrigerator at 4°C is 500V (current 27 mA and power 14 W). Heating in the gel is a result of the power (wattage) and some power supplies can be adjusted to maintain constant wattage. We recommend an upper limit of 15 W be applied to gels run in a refrigerator. If a cooling plate (circulating water at 5°C) is used to cool the gel, a power limit of 35 W can be set. The starting potentials for such a gel are 510 V, 35 mA (set max.) and 18 W.

After the power is applied to the gel for a few minutes, a discontinuous boundary or buffer front is formed (its position is marked by the bromophenol blue). By the time sample wicks are removed, the boundary should be a fine straight line across the 4% gel. As the boundary passes through the samples, the proteins are "stacked" in groups and then enter the 10% gel, where the major part of the separation occurs. The electrophoretic run is stopped when the boundary reaches the anodal wick. If a 500 V power supply is used, the total electrophoresis time will be about 6-7 hours, and if a 1000 V power supply and cooling plate are used, the run time will be approximately 3 hours.

### *(iii) Gel staining*

*(a) Coomassie blue or Serva violett*

The gel (still on the glass plate) is removed from the apparatus and a note made of the distance the bromophenol blue boundary has moved from the 4% - 10% gel interface. The gel is cut along the 4% - 10% interface and the 4% stacking gel and the 8% cathodal gel are discarded. A second cut is made along the bromophenol blue boundary (marking the buffer front) and the portion of the 10% gel anodal to this cut is discarded. A small diagonal cut is then made across the anodal corner of the gel at the edge where the first sample was loaded to identify the orientation of the gel. Fresh Coomassie blue staining solution is mixed in a suitable container (e.g. 8 x 10 inch photographic tray). The gel (still on the glass plate) is inverted over the container and the diagonally cut corner pulled gently away from the glass plate until the entire gel separates from the glass plate and falls into the staining solution. Staining usually takes 15 - 20 minutes with continuous agitation.

When staining is judged to be adequate, the stain solution is removed (by aspiration) and the gel washed with 300 ml of distilled water to remove residual stain. If necessary, the gel can be subsequently destained in 7% aqueous acetic

acid until the background clears. Serva violett 49 tends to stain and destain faster than Coomassie blue R-250.

*(b) Silver stain*

If more sensitivity is required the gel may be stained with a silver stain based on the method of Merril *et al.*, 1982. Silver staining can be accomplished directly after electrophoresis or after the gel has been first stained with Coomassie blue or Serva violett. Certain steps have been modified or deleted to simplify the procedure of Merril *et al.* without any apparent loss in quality.

Step I - fix in 330 ml of fixing solution overnight (this step will destain the gel if it was previously stained in Coomassie blue or Serva violett).

Step II - 3 washes, 330 ml distilled water, 10 minutes each.

Step III - Sensitize proteins with 330 ml silver nitrate solution for 30 minutes in subdued light. Note: a 30-sec rinse with distilled water following removal of the silver nitrate solution helps to minimize surface staining (formation of a mirror-like coating).

Step IV - Visualize proteins with developer in darkness. Three washes of 330 ml developer: the first wash for only 30-60 seconds, the second wash for 3 minutes, and the last wash until the gel is stained as required (usually 5-10 minutes, depending on the quality of the sodium carbonate).

Step V - Stop staining with a wash of 2% aqueous acetic acid (in subdued light) for 10 minutes.

Step VI - Rinse with distilled water.

Continuous agitation is essential during Step IV and desirable throughout.

The silver stain is relatively permanent, although some fading can occur if the stained gels are not stored in the dark. It is advisable to photograph and score the gel soon after staining to avoid any inadvertent loss of information.

A number of "silver" staining kits available from commercial suppliers are well suited to polyacrylamide gels. Examples include: **Kodavue®** from Eastman Kodak, **Gelcode®** from Pierce Chemical Company (P.O. Box 117, Rockford, Illinois 61105, USA), and **Bio-Rad silver stain** (Bio-Rad, 2200 Wright Ave., Richmond, California 94804, USA).

## D. Data recording and gel interpretation

### *(i) Photography*

It is important to keep a permanent record of the results of each experiment, especially if future litigation is a possibility. Although stained starch gels can be preserved more or less indefinitely (de Ligny, 1968; Numachi, 1981), the procedures involved are time consuming and the results often imperfect. Furthermore, the resulting preserved gels are often bulky and difficult to store. For these reasons we recommend photographing each stained starch gel and using the resulting negatives and/or prints as the permanent record. We also find that photographs of polyacrylamide gels are the best form of permanently recording all aspects of the banding patterns obtained, although these gels can be readily dried (Wallevik and Jensenius, 1982) and apparatus for this purpose is available from several commercial sources.

In the case of polyacrylamide gels, the gel is first photographed after it is stained with Coomassie blue or Serva violett. It is then subsequently stained using the silver stain, if necessary, and then photographed a second time. In this way, the same gel can be used for both Coomassie blue (or Serva violett) and silver staining and the results of both stains can be permanently recorded. The photography is easily accomplished using a standard 35 mm camera (preferably with a macro lens) mounted on a photographic stand above a light box and black and white film (such as Kodak Panatomic-X).

The stained starch gels are photographed with uniform direct lighting from above, while the stained polyacrylamide gels are photographed

on a translucent white background with transmitted light from below the gel. The use of a yellow filter enhances contrast of blue bands and is routinely employed in photographing all polyacrylamide gels stained with Coomassie blue or Serva violett and all starch gels stained with MTT (or NBT).

Enzymes exhibiting fluorescent staining are readily photographed using a UV light box (reflected light) with the same camera, yellow filter, and film as for all other stains (note, however, that the average exposure for "UV-stained" gels is approximately 15-30 sec). An automatic exposure camera greatly facilitates the photographing of such gels. A small identifying number is placed in the corner of each stained gel prior to photography to identify each negative uniquely (the same number is written on the experimental protocol sheet, which contains information on the individual samples tested and the date and conditions of that particular experiment). The identifying number thus appears in the corner of each negative and of any subsequent positive prints from the negative so that there is no ambiguity in identifying any photograph.

We regularly make a small contact print of each gel and attach it to the appropriate protocol sheet so that some visual record is associated with the written record for each experiment. When a larger photograph of the results is needed, it is a simple matter to make a photographic enlargement from the filed negative.

### *(ii) Determination of relative mobilities*

The initial interpretation of the banding patterns resulting from electrophoresis simply involves a determination of the **relative mobility** of each band in the unknown sample(s) compared to that of the band(s) in the reference sample(s) on the same gel. This is readily accomplished by laying a small ruler on the top surface of the stained gel and measuring the distance (to the nearest 0.5 mm) from the sample origin to the center of each band on the gel. The **relative mobility** of each band is then calculated by dividing the measured mobility of the band in the unknown by that of the reference band in the control sample and multiplying the result by 100. Thus, a band in an unknown sample having a measured mobility of 45 mm on a gel where the reference band had a measured mobility of 60 mm would be identified as having a relative mobility of 75. In practice, variation in electrophoretic mobility from experiment to experiment is frequently 1-5 mm in absolute distance or 5-10% in relative mobility (whichever is larger), so small differences in mobility should be carefully assessed in light of the behaviour of the reference sample(s) on the gel.

### *(iii) Interpretation of polymorphisms*

Just as it is incorrect to consider all individuals of a species as identical, it is misleading to assume that all individuals will express identical biochemical phenotypes. Indeed, it is now well established that most species exhibit relatively high levels of genetic polymorphism in their enzymes and general proteins (e.g. Ayala, 1976). Furthermore, variability in biochemical phenotype can also be due to non-genetic factors, such as the post-translational modification of proteins **in vivo** or **in vitro**. It is, thus, of paramount importance to determine the protein phenotype(s) **characteristic** of a species before reliable identification of unknown samples can be accomplished.

Artifactual variations in protein banding patterns can be largely eliminated or controlled by ensuring that all samples are carefully treated prior to analysis (e.g. kept at 5°C for short periods or frozen at -20°C or colder for long periods) and by standardizing the conditions of analysis. Genetic polymorphisms, on the other hand, are inherent characteristics of the species, and must be correctly recognized and interpreted if sample identification is to be reliable. In nearly all cases, the banding pattern of heterozygous individuals can be predicted accurately from information concerning the molecular structure of the protein under study.

Thus, monomeric proteins exhibit two-banded patterns in heterozygotes, dimeric proteins exhibit three-banded patterns in heterozygotes, trimeric proteins (rare) exhibit four-banded patterns in heterozygotes, tetrameric proteins exhibit five-banded patterns in heterozygotes, and hexameric proteins exhibit seven-banded patterns in heterozygotes (assuming that a single gene locus is encoding the protein in each case).

Information is available on the molecular structure of most enzymes commonly studied by electrophoresis in fishes or invertebrates (Darnall and Klotz, 1975, Table 1). Indeed, because the subunit structure of proteins is an evolutionarily conservative property, a given protein commonly exhibits the same subunit structure within large taxonomic groups (e.g. among all fishes, among all vertebrates, or in many cases, even among all eukaryotes, both vertebrates and invertebrates). The subunit structures and general characteristics of the enzymes discussed in this report are summarized in Table 1 to facilitate recognition and correct interpretation of genetic polymorphisms, should they be encountered.

### *(iv) Final identification of unknown samples*

In most cases, unknown samples should be readily identifiable by the congruence in their biochemical phenotype with that of a single reference species. Since most cases of product testing will undoubtedly involve the verification of labelling, it should be a simple matter to determine whether or not the phenotype of the tested sample is identical to that of the appropriate reference sample (from a known specimen of the species that the tested sample is purported or suspected to be). However, in cases of apparent mislabelling, the phenotypes of the tested and reference samples may not be identical. This could even be true for one or two specific proteins if the species involved is polymorphic.

Ambiguities of sample identification are usually readily resolved by repeating the initial analysis (possibly with additional and/or different reference samples) or by conducting a second analysis utilizing another medium. For example, if the initial test were done using general protein staining on a polyacrylamide gel, then the second test could involve the analysis of specific enzymes on starch gels and vice versa. If the biochemical phenotypes of the tested and reference samples still differ significantly from one another, it can be concluded that they represent different species.

Perhaps the ultimate proof of non-identity between two samples is to demonstrate that the unknown tested sample exhibits a biochemical phenotype indistinguishable from that of another species. Such an unequivocal result could presumably be obtained by testing the unknown sample against additional reference samples. Whether or not it is possible to match the biochemical phenotype of an unknown with that of a reference sample, non-identity can nevertheless be reliably established by demonstrating that the phenotype of the unknown significantly exceeds the known range of variation of the reference species. If data exist concerning the range and level of genetic polymorphism in the reference species (for example, see Shaklee and Salini, 1985; Keenan and Shaklee, 1985), then statistical arguments can also be used to support the identification of the unknown as being of a different species.

## EXAMPLES AND DISCUSSION

The primary purpose of this report is to present a detailed methodology for fishery product identification using electrophoresis. Although the methodology is applicable to both fishes and invertebrates, major emphasis throughout this report has been on marine fishes found in Australian waters. In addition to documenting the specific methodology we recommend, we feel it is necessary to provide some actual examples of the results obtained using these techniques, especially results that relate to our generalizations and recommendations on sample handling, electrophoretic conditions and data interpretation. Several of the figures and tables presented in this report are intended to document the results we have obtained using

**Table 1.** Subunit structure, characteristics, and relevant literature citations for enzymes useful in the identification of fishery products.

| Enzyme | Subunit Structure | Characteristics | References |
|---|---|---|---|
| AAT | dimer | 1-2 loci encode cytoplasmic isozymes; usually anodal in fish | (68) (78) (93) |
| mAAT | dimer | usually cathodal in fish | (68) (78) (93) |
| ADA | monomer | usually quite anodal in fish | (30) (46) (82) |
| AK | monomer | 1 locus predominantly expressed in fish muscle<br>2-3 loci expressed in invertebrate muscles | (65) (100)<br>(12) |
| ALPDH | monomer* | only present in some molluscs | (18) |
| ARGK | dimer | only present in invertebrates | (44) |
| CK-A | dimer | 3-banded heterozygotes in elasmobranchs;<br>2-banded heterozygotes in teleosts<br>absent in invertebrates | (23)<br>(23) |
| ENO | dimer | often cathodal in fish | (71) (75) (79) |
| EST-D | dimer | usually very anodal | (85) (86) |
| GPI (fish) | dimer | 2 loci in most fishes; major muscle form ($B_2$) is usually cathodal (often with anodal subbanding); $A_2$ (minor form in muscle) is anodal | (2) (59) (82) (86) |
| GPI (invert.) | dimer | 1 locus; subbands often present | (85) |
| G-3-PDH | dimer | 1 locus encoding the muscle predominant isozyme; 2 or more subbands anodal to primary isozyme often present | (13) (21) (66) (96) |
| IDH | dimer | 1 locus encoding the muscle predominant form (especially strongly expressed in red muscle) | (30) (50) |
| LDH (vert.) | tetramer | specific for L-lactate as substrate; $A_4$ isozyme predominates in white skeletal muscle; (2 additional loci present in teleosts, but usually only expressed at low levels, if at all, in white muscle) | (55) (83) |
| LDH (invert.) | dimer | specific for D-lactate as substrate; present in marine molluscs | (51) |
| | tetramer | specific for L-lactate as substrate; 1-2 loci, one predominant isozyme in tail muscle of decapod crustaceans; (a 2nd locus often expressed in walking leg muscle) | (43) (51) |

Cont d.

| Enzyme | Subunit Structure | Characteristics | References |
|---|---|---|---|
| MDH | dimer | 1-2 loci encoding cytoplasmic isozymes in teleosts<br>subbanding common in invertebrates | (4) (15) (30) (45)<br>(36) |
| mMDH | dimer | usually the least anodal isozyme in teleosts | (30) (45) |
| MDH ($NADP^+$) | tetramer | 1-2 loci in fish muscle;<br>(usually only one isozyme clearly resolved) | (8) (28) (38) (49)<br>(91) (97) |
| MPI | monomer | usually quite anodal | (31) (62) (81) |
| ODH | dimer | present in tail muscle of decapod crustaceans | (61) (62) |
| OPDH | monomer | only present in molluscs (and various other invertebrates) | (5) (7) (99) |
| PARV[1] | monomers | only present in fishes;<br>1-5 forms usually highly anodal (pI $<$ 6.0);<br>heat stabile | (29) (60) (69) (92) |
| PEP-A | dimer | | (24) (25) (50) |
| PEP-B (vert.) | dimer | two-banded "heterozygous" phenotypes reported for some fishes | (24) (25) (82) |
| PEP-D (vert.) | dimer | 1-2 loci expressed in teleosts;<br>specific for dipeptides containing C-terminal proline | (24) (25) (30) |
| PEP (invert.) | ? | not well characterized;<br>both monomers and dimers reported for various species | (56) (85) |
| PGM | monomer | 1 locus invariably expressed at high levels in fish white muscle;<br>a 2nd locus (encoding a highly anodal isozyme = "PGM-1") sometimes expressed at low levels;<br>subbanding common in invertebrates | (35) (82) (95)<br><br><br>(6) (62) (85) |
| STRDH | monomer* | only present in some molluscs | (18) |
| SOD | dimer | inhibited by 1mM cyanide | (1) (26) |
| mSOD | tetramer | | (26) (34) (70) |

1 = parvalbumins bind calcium but do not have any known enzymatic activity
* = probable subunit structure

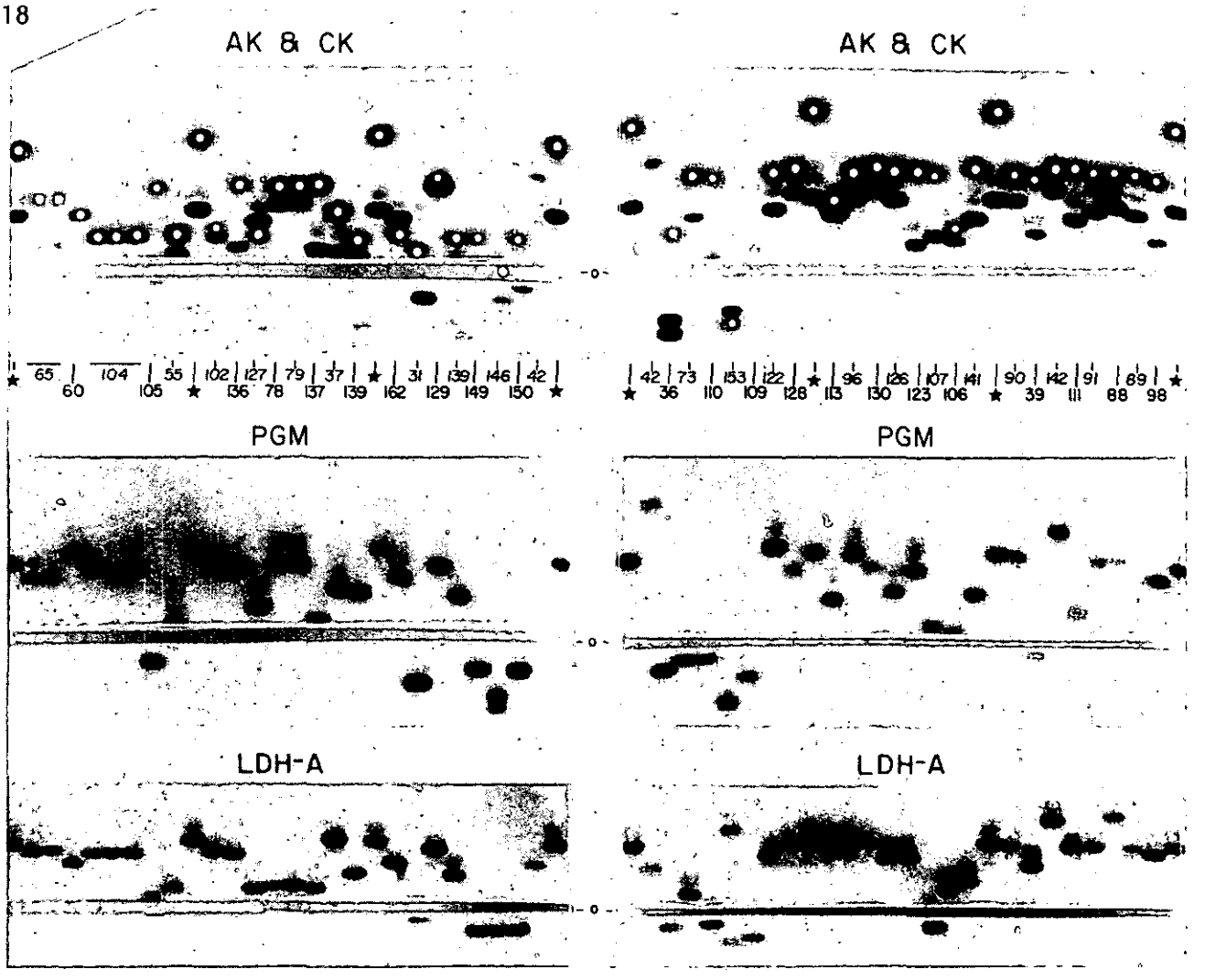


**Figure 3.** Starch gel isozyme patterns for 45 species of fishes. In this and all subsequent figures: enzyme abbreviations follow those used in APPENDIX 4; numbers identify species according to APPENDIX 6 and 7; gel photographs are oriented with the anode toward the top, the cathode toward the bottom and the sample origin (point of sample application) at or near the bottom (unless otherwise noted); and barramundi extracts (★) and/or bovine serum albumin (=BSA; ●) controls have been run on each gel as mobility standards. All starch gels in this study were made using Connaught starch (lot # 380-2 or # 396-1). The AK isozyme of each species has been identified with a solid white circle to distinguish it from the CK-$A_2$ isozyme(s) on the same gel. Note that species 36 is a CK-$A_2$ heterozygote (cf. Table 1), and species 146 is a PGM heterozygote.

the apparatus and techniques described above. These examples are not, however, comprehensive, and the interested reader is referred to the paper by Keenan and Shaklee (1985), which details numerous other results of the techniques and their application.

## A. Starch gel electrophoresis

### *(i) Typical isozyme patterns*

Figure 3 illustrates the type of electrophoretic patterns obtained in analyzing different enzyme systems in numerous fish species. In this and all subsequent starch gel experiments, extracts of barramundi (*Lates calcarifer*) have been

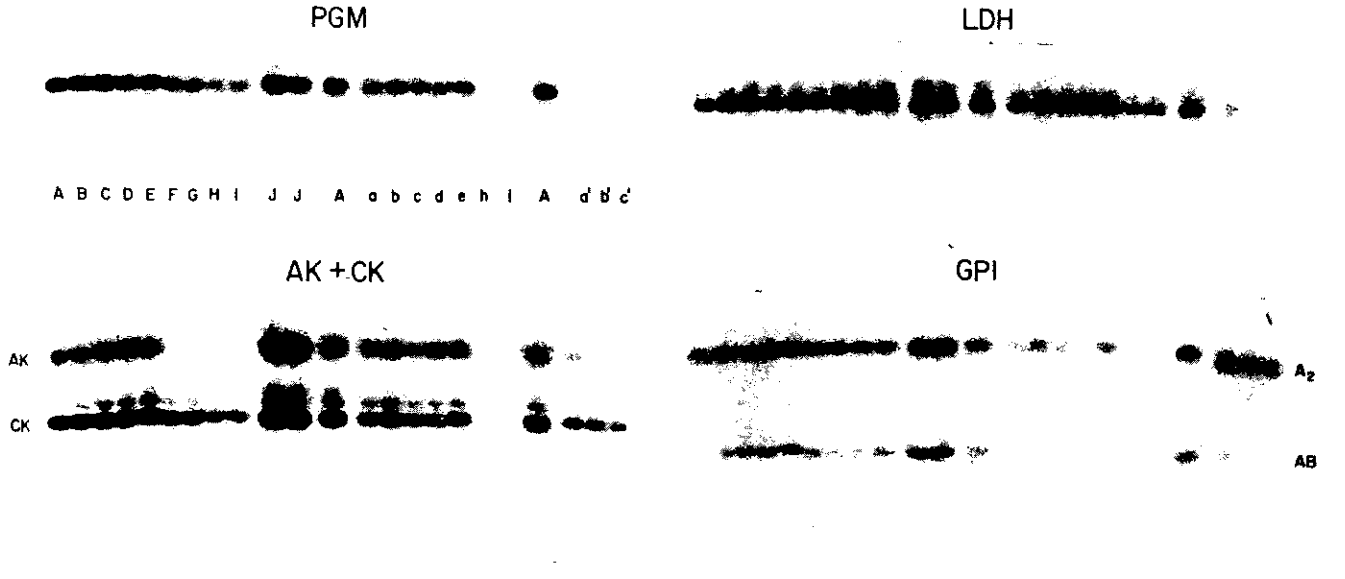


**Figure 4.** The effects of various handling conditions and extraction media on isozyme patterns of barramundi.
**Samples A-I** were freshly prepared from a fish that had been frozen at -20°C for 7 months:
A = standard homogenizing buffer (see Appendix 2);
B = distilled water;
C = tap water;
D = 0.1M Tris-HCl pH 7.0;
E = 0.1M K-$PO_4$ buffer pH 7.0;
F = intact tissue extracted in 2% 2-phenoxyethanol for 24 h at 23°C;
G = extract from tissue homogenized in 2% 2-phenoxyethanol (after sitting for 24 h at 23°C);
H = same as F but extracted for 56 h;
I = same as G but after 56 h;
J = tissue "drip" sample (see text).
**Samples a-i** were extracts stored frozen at -70°C for 18 months prior to electrophoresis. (These extracts had originally been prepared from a fish that had been stored frozen at -20°C for several months.) They were prepared using the same extraction media as samples A-I.
**Samples a' - d'** were freshly prepared extracts from a fish frozen at -20°C for over 21 months, and were prepared using the same extraction media as samples A-D. (d' not shown above, see Figure 14).

included as reference samples to allow the calculation of the relative mobilities of all isozymes (of the other species) on the gels. Such reference samples are essential to provide standardization and permit comparisons among different gels. However, it is not necessary to utilize barramundi as the reference species; indeed in practice it may be advisable to use other species as the reference(s). For example, if one is testing product labelled as species "A", it seems advisable to use extracts known to be from species "A" as the reference.

Note that each of the 45 species represented in Figure 3 exhibit distinctive isozyme patterns at some or all enzyme systems surveyed. Such species-specific patterns form the basis for product identification using electrophoresis. Note also that the species that are represented by multiple individuals on these gels exhibit the same banding pattern in all individuals tested. This constancy of pattern within a species (except in cases of genetic polymorphism; see below and Keenan and Shaklee, 1985) is the

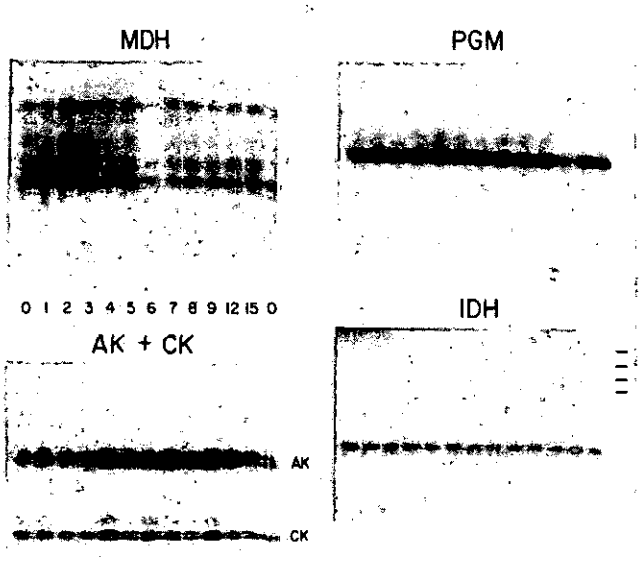


**Figure 5.** Starch gel isozyme patterns of barramundi fillets stored for various periods of time at 5°C. Numbers refer to days at 5°C. The order of samples on all gels is identical to that indicated for MDH. Note the unique, highly anodal IDH bands present in samples stored for 9-15 days.

other major criterion that must be met in order to utilize these characters as reliable markers for species identification.

### *(ii) Sample preparation*

We have used a standard homogenizing buffer throughout our investigations (see Appendix 2) to ensure adequate pH control and long-term stability of the extracts. There is, however, considerable latitude in the choice of extraction media, as indicated in Figure 4. As shown by these examples, comparable electrophoretic results can be obtained with many different extraction solutions. Indeed, the only extraction medium that has not proved suitable for some of the enzyme systems tested is that employing 2-phenoxyethanol, a compound reported to be of use in preparing tissue extracts where refrigerated and/or frozen storage is not available (Nakanishi *et al.*, 1969). Although some enzymes such as LDH and PGM seem unaffected by extraction in 2% 2-phenoxyethanol, several others such as CK, AK, IDH, and MDH are rapidly denatured by this treatment.

Of greatest practical significance is the apparent suitability of tissue "drip" samples as a source of material for electrophoresis. This extremely simple and rapid form of sample preparation (see PROCEDURES A(v) above) seems entirely compatible with the starch gel analyses and obviates the need for actual homogenate preparation and subsequent high-speed centrifugation for routine processing of unknown samples.

### *(iii) Sample storage and handling*

As it is not generally possible to process large numbers of samples on a "same day" basis, it is important to document any effects sample storage may have on subsequent analyses and identification. As shown in Figure 5, refrigerated storage of tissue samples (or intact fillets) at 5°C for up to 72 h has no detectable effect on the resulting enzyme patterns. In fact, most enzymes were not demonstrably affected by 5°C storage of intact tissues for periods up to 15 days! On the other hand, IDH exhibited significant changes in banding pattern after about 8 days with the appearance of one or more additional, highly anodal isozymes.

Because such qualitative changes in isozyme pattern could easily lead to confusion and/or misidentification, prolonged refrigerated storage of samples at 5°C should be avoided. Frozen storage of tissue samples or extracts at either -20°C or -70°C for extended periods resulted in slight decreases in staining activity for some enzymes. However, there was little or no detectable qualitative effect on enzyme banding patterns, even after periods of frozen storage of up to 18 months (cf Figure 5). These results confirm and extend those reported by Cowie (1968).

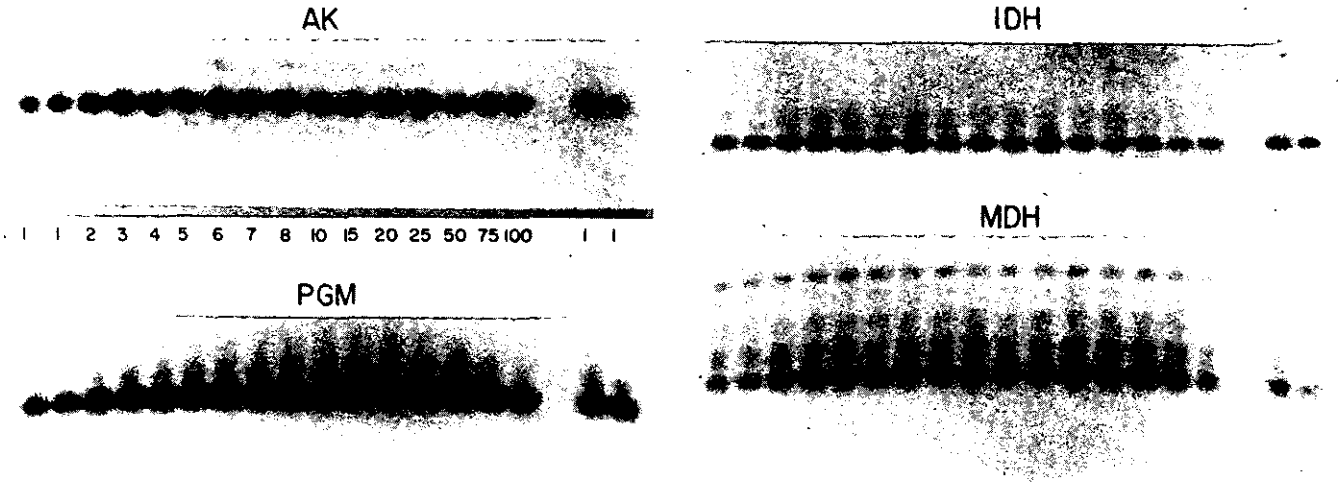


**Figure 6.** The effects of repeated freezing and thawing of muscle extracts on isozyme patterns of barramundi. Numbers refer to the total number of times the extracts have been thawed (and refrozen). The order of samples on all gels is identical to that indicated for AK.

Tests were also conducted to assess the effects of repeated freezing and thawing of extracts on the resulting isozyme patterns. The results of these experiments are shown in Figure 6 for PGM, MDH, IDH, and AK.

For most enzymes tested (and all enzymes shown in Figure 6) repeated cycles of freezing and thawing (up to 100 times over a three-week period) had no detectable effect on the actual isozyme banding patterns. Little effect on quantitative aspects of the isozyme patterns (as judged by visual estimation of staining intensity on the gels) was noticed as well. The results of both the frozen storage and the repeated freezing and thawing experiments indicate that reference samples can be stored and reused for many months without appreciable loss of staining activity or deterioration in electrophoretic resolution.

### *(iv) Red vs. white muscle*

The major body musculature of most species of fishes consists largely of so-called white muscle fibers and is reasonably uniform in its composition. For this reason, it is possible to take a piece of muscle tissue from almost any region of the body and obtain an electrophoretic pattern characteristic of the species. This is shown for barramundi in Figures 7 and 8.

However, other types of muscles (e.g. cardiac, smooth and red skeletal muscle) often exhibit very different distributions of enzymes, and patterns of isozymes, as shown for several species in Figure 8. For barramundi, the enzymes AK and CK exhibit relatively constant patterns in white and red skeletal muscles but are only present at greatly reduced levels in both cardiac and smooth muscle. In the other species tested, similar expression of both of these enzymes in white and red muscle is observed. The major isozyme of PGM in fish

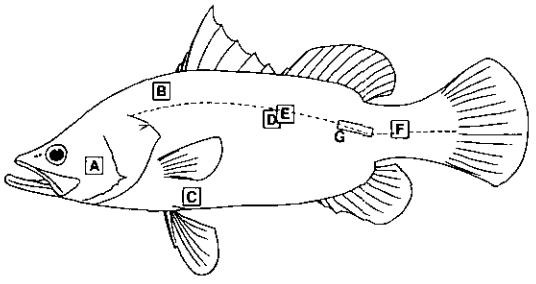


**Figure 7.** Locations of barramundi tissue samples. Note that E and F were superficial samples and contained a mixture of both red and white muscle tissue. D was a "deep" sample consisting of white muscle only. G, a sample from underneath the lateral line, consisted primarily of red muscle.

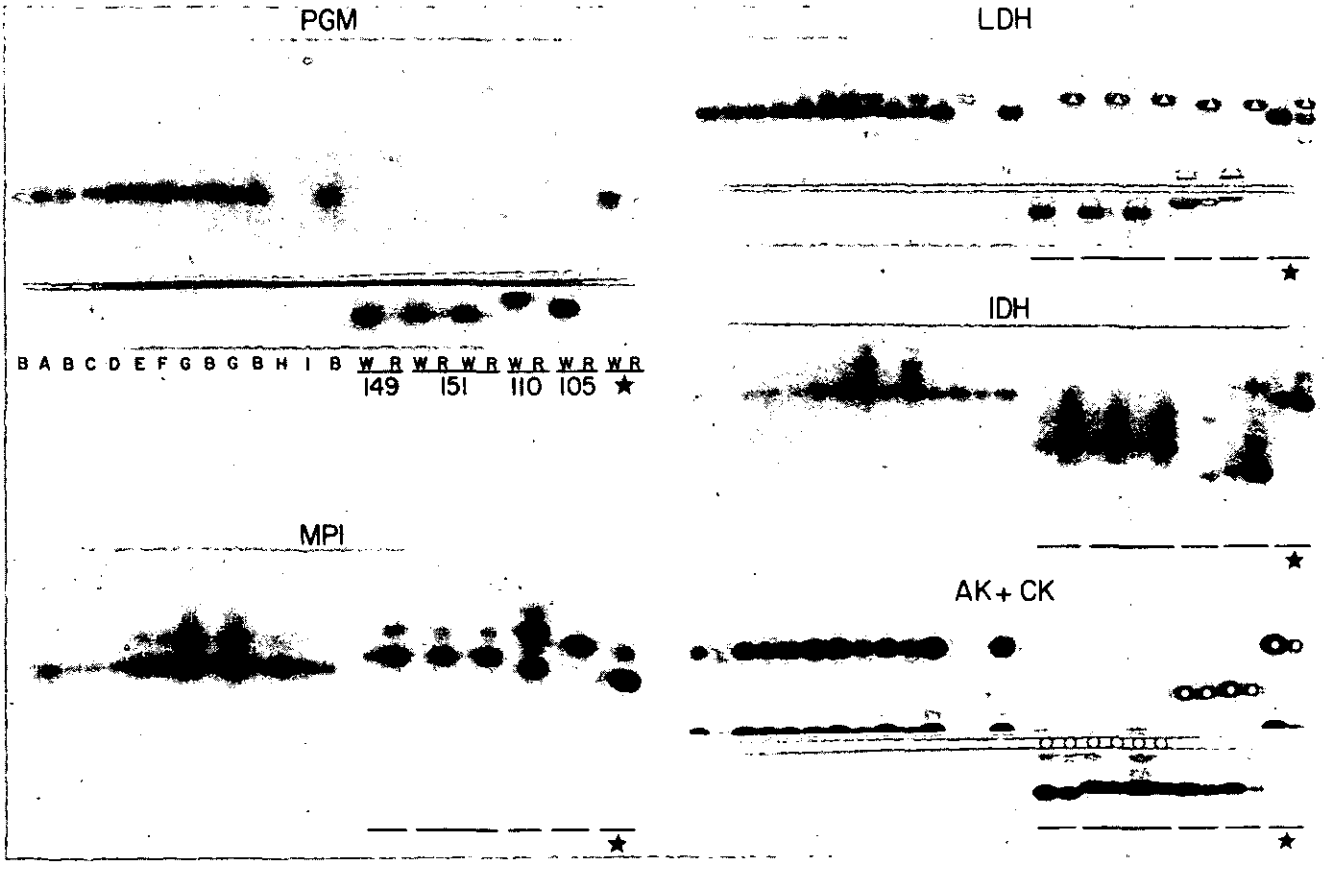


**Figure 8.** Starch gel isozyme patterns of different muscle types of barramundi (A-I), on left of each gel, and of white and red skeletal muscles of five different species numbered as in Appendix 6, on right of each gel. See Figure 7 for locations of fillet samples A-G from barramundi (H = heart, I = stomach [smooth muscle] ). W = white skeletal muscle, R = red skeletal muscle. The $B_4$ isozyme of LDH (which is usually only present at very low levels in white skeletal muscle but predominant in red skeletal muscle) is identified by a solid white triangle to distinguish it from the $A_4$ isozyme. The AK isozyme is similarly identified with a solid white circle to distinguish it from the CK-$A_2$ isozyme. Note: 1) the faint, highly anodal "PGM-1" isozyme present in red muscle extracts of both mackerel species, 2) the minor anodal MPI sub-band prominent in red muscle extracts of most species, 3) the Australian salmon (110) is heterozygous for MPI (exhibiting two bands of equal staining intensity), 4) the minor anodal IDH band(s) present in the red skeletal muscle extracts of all species, and 5) the AK isozyme of both mackerel species (149 and 151) remains at the sample origin under the electrophoretic conditions used in this experiment.

**Table 2**. Approximate amounts of isozymes in fish muscles[1].

| Species and Tissue sample | Isozyme[2] | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LDH | | AK | CK | GPI | | PGM | | IDH | MPI | PEP | m-AAT | G-3-P DH | ADA |
| | A | B | | A | A | B | A | 1 | m | | B | | m | |
| *Lates calcarifer* (barramundi) | | | | | | | | | | | | | | |
| white muscle | 5 | 1 | 5 | 4 | 3 | 5 | 4 | 1 | 2 | 1 | 3 | 1 | 6 | 1 |
| red muscle | 4 | 2 | 5 | 3 | 4 | 5 | 2 | 2 | 4 | 5 | 1 | 4 | 2 | 3 |
| "A" | 5 | 1 | 4 | 2 | 4 | 5 | 4 | 1 | 1 | 2 | 2 | 1 | 2 | 1 |
| "B" | 5 | 1 | 5 | 5 | 3 | 5 | 3 | 1 | 2 | 1 | 3 | 1 | 3 | 1 |
| "C" | 5 | 1 | 5 | 4 | 3 | 5 | 3 | - | 1 | 1 | 2 | 1 | 2 | 1 |
| "D" | 5 | 1 | 5 | 4 | 3 | 5 | 4 | - | 1 | 1 | 2 | 1 | 2 | 1 |
| "E" | 5 | 1 | 5 | 4 | 4 | 5 | 4 | 1 | 2 | 4 | 2 | 3 | 3 | 1 |
| "F" | 5 | 1 | 5 | 4 | 4 | 5 | 5 | 1 | 2 | 4 | 2 | 3 | 3 | 1 |
| "G" | 4 | 3 | 5 | 3 | 4 | 5 | 2 | 2 | 4 | 5 | 1 | 4 | 2 | 3 |
| "H" | 1 | 3 | 1 | - | 5 | 1 | - | 1 | 2 | 3 | 1 | 1 | - | 3 |
| "I" | 1 | 2 | 1 | - | 5 | - | - | 2 | 1 | 2 | 1 | 1 | - | 3 |
| *Scomberomorus queenslandicus* | | | | | | | | | | | | | | |
| white muscle | 5 | 1 | 3 | 4 | 2 | 5 | 5 | 1 | 2 | 1 | 3 | 2 | 5 | 1 |
| red muscle | 1 | 5 | 3 | 4 | 5 | 2 | - | 2 | 5 | 5 | 3 | 4 | 1 | 5 |
| *Scomberomorus commerson* | | | | | | | | | | | | | | |
| white muscle | 5 | 1 | 3 | 4 | 1 | 5 | 5 | - | 2 | 1 | 2 | 1 | 4 | 1 |
| red muscle | 1 | 5 | 3 | 4 | 4 | 1 | 1 | 2 | 4 | 5 | 3 | 4 | 1 | 5 |
| *Arripis trutta* | | | | | | | | | | | | | | |
| white muscle | 5 | 1 | 4 | 4 | 1 | 5 | 5 | - | - | - | 1 | 1 | 5 | 2 |
| red muscle | 2 | 5 | 4 | 4 | 4 | 5 | - | - | 2 | 5 | 3 | 5 | 2 | 5 |
| *Pomatomus saltatrix* | | | | | | | | | | | | | | |
| white muscle | 5 | 1 | 4 | 4 | 1 | 4 | 5 | 1 | 1 | 1 | 3 | 1 | 3 | 2 |
| red muscle | 1 | 5 | 4 | 3 | 4 | 3 | 1 | 2 | 5 | 5 | 2 | 4 | 1 | 5 |

[1] estimated from relative staining intensity on starch gels (see Figure 8)
5 = maximal staining, 1 = minimal staining, - = no staining detected.

[2] isozymes identified according to accepted conventions
m = isozyme predominant in white skeletal muscle
1 = highly anodal isozyme.

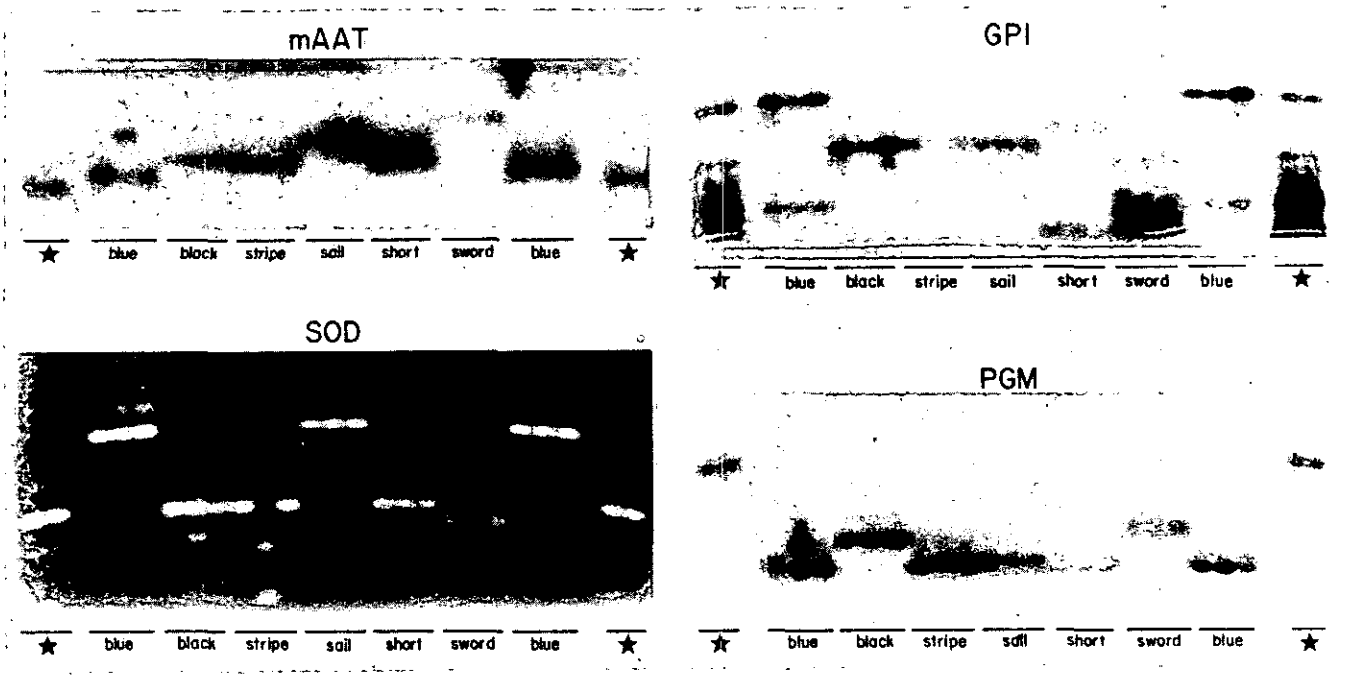


**Figure 9.** Starch gel isozyme patterns of six billfish species: blue = Pacific blue marlin (157), black = black marlin (156), stripe = striped marlin (159), sail = sailfish (155), short = shortnose spearfish (158), and sword = broadbill swordfish (154).
*Note that the sample origin* of the mAAT gel is at the top because this protein migrates cathodally. Also note that the second blue marlin (from the left) is heterozygous for both mAAT and PGM while the second striped marlin is heterozygous for SOD (the mSOD isozyme exhibits light staining on this gel, is less anodal than the cytoplasmic SOD, and has an identical mobility in all species on this gel).

white muscle is almost totally lacking in red, cardiac and smooth muscle, while a second, highly-anodal isozyme ("PGM-1") is present at low levels in the red muscle of several species (even though it is undetectable in white muscle of these same species). The isozyme patterns of LDH are radically different in the two muscle types of most fishes. The LDH-$A_4$ isozyme predominates in white muscle, while the LDH-$B_4$ isozyme predominates in red muscle of most species. Approximate amounts of enzyme activity (based on visual estimates of staining intensity on starch gels) for these and several other enzymes are presented in Table 2.

Because of the often major differences in enzyme composition between white and red muscles, it is important to use fillet samples that consist of white muscle when attempting to identify an unknown species by comparing its isozyme pattern with that of a known reference derived from a piece of white muscle. Similarly, if only red muscle is available for testing of an unknown, reference samples of red muscle from known species should be used as controls.

### *(v) Species discrimination*

The electrophoretic characteristics of ten isozyme systems in over 150 different species of fishes are presented in Appendix 6. Repetitive analyses in this laboratory (involving different gels and different specimens) indicate that the mobilities recorded for each enzyme are generally reproducible to ± 5 Rm units, and almost always within 10 Rm units of the values in Appendix 6. Thus, the data in Appendix 6 are truly characteristic of these species. This or

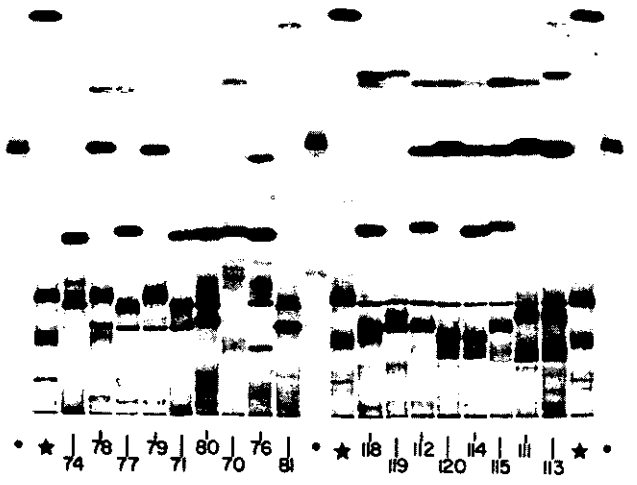


**Figure 10.** Polyacrylamide gel patterns of Coomassie blue stained general proteins of closely related species. On the left are nine species from the subfamily Platycephalinae and on the right, eight species of the genus *Lutjanus*. At either end and in the center are controls of BSA ( ● ) and barramundi ( ★ ).

similar data bases can be used as points of reference for identifying unknown species on the basis of their electrophoretic characteristics.

The most important point to be made from the data in Appendix 6 is that nearly all species tested exhibit distinctive patterns for one or more of the ten isozymes using the two buffers tested. Thus, this approach of using a small number of enzyme stains and only two different electrophoresis buffer systems should be adequate to differentiate most species and thereby identify the vast majority of fresh and frozen fish products.

In some cases this simple methodology does not provide adequate discrimination between closely related species (e.g. billfishes). The billfishes (families Istiophoridae and Xiphiidae) are notoriously difficult to distinguish on both morphological (Nakamura *et al.*, 1968) and biochemical grounds (Yamada and Suzuki, 1982). In such cases, increased resolution can be obtained by: a) staining for additional enzyme systems, b) using additional electrophoresis buffer systems, and/or c) examining patterns of general muscle proteins on polyacrylamide gels using Coomassie blue or silver staining (see below). The increased discriminating power provided by using additional enzyme systems and other electrophoresis buffers is shown for several species of billfishes in Figure 9. The results of these analyses clearly indicate that even closely related species can usually be distinguished using relatively straightforward electrophoretic methods.

## B. Polyacrylamide gel electrophoresis

### *(i) Differences between species*

Figure 10 shows a typical polyacrylamide gel after electrophoresis and staining with Coomassie blue general protein stain. To standardize polyacrylamide gels between runs, both bovine serum albumin (=BSA; at a concentration of 1 mg/ml) and barramundi muscle extract have been included on each gel. These reference samples allow the calculation of relative mobility values for each of the protein bands in the extracts being tested (and they may also be used as standards to allow the determination of the relative protein concentration of bands in these extracts if used in conjunction with a densitometer).

This particular gel (Figure 10) contains closely related species from the subfamily Platycephalinae and from the genus *Lutjanus*. Each species is characterized by the presence of 5-10 different protein bands. Note that although similarities between some species exist for some of the proteins, no two species have identical patterns. Also note the reproducibility of the protein profile of barramundi as evidenced by the three replicated samples on the gel. The species-specific nature of muscle protein profiles visualized by Coomassie blue staining following polyacrylamide gel electrophoresis is well illustrated by the data in Appendix 7, which

summarizes protein mobilities for over 150 species as determined in this laboratory (also see Cowey, 1968; Mitchell and Scott, 1979).

Another test of the power of the technique to identify closely related species involved the electrophoresis of muscle extracts from different species of billfishes (see section A(v) above. Gels containing two individuals of each of six different billfishes are shown in Figure 11. Swordfish (the only species in the family *Xiphiidae*) were easily distinguished from the other billfish species by Coomassie blue staining; the swordfish shows a protein band of low anodal mobility approximately 10 mm from the origin of the gel (Figure 11a). However, three of the other species (black marlin, striped marlin and sailfish) could not be clearly differentiated on the basis of general muscle protein patterns after staining with only Coomassie blue. Subsequent silver staining of this gel revealed subtle but significant differences among these three species in quantitatively minor proteins (Figure 11b).

In contrast to these results for polyacrylamide gel electrophoresis of general muscle proteins, the analysis of specific enzymes after starch gel electrophoresis provided a simpler and more convincing identification of these billfish species because of the clear differences in mobility of several enzymes revealed by this procedure (cf. Figure 9).

### *(ii) Parvalbumins*

Figure 12 displays protein patterns of heated extracts of the same species as in Figure 10. The heat-stable proteins present after heating for approximately 5 min at 100°C are parvalbumins (Keenan and Shaklee, 1985) and possess several unique features.

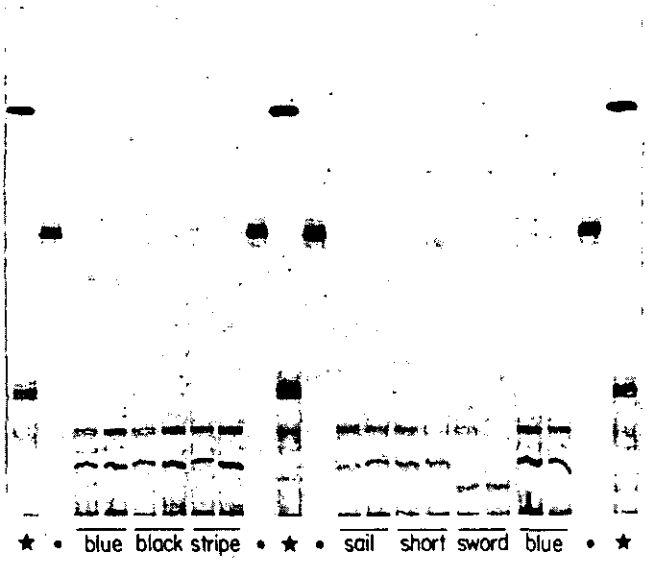


**Figure 11a.** Polyacrylamide gel patterns of Coomassie blue stained general proteins of six billfish species (identified as in Figure 9); ★ = barramundi, ● = BSA.

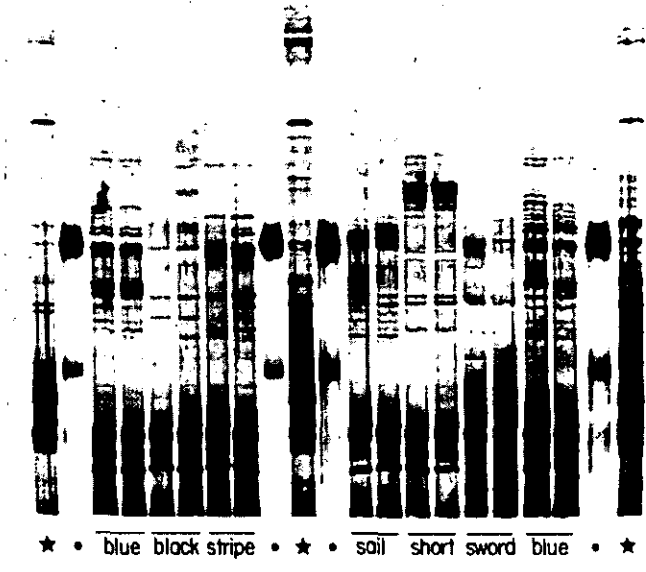


**Figure 11b.** Silver stain of gel in Figure 11a. Note increased sensitivity of stain and minor differences between species. ★ = barramundi, ● = BSA.

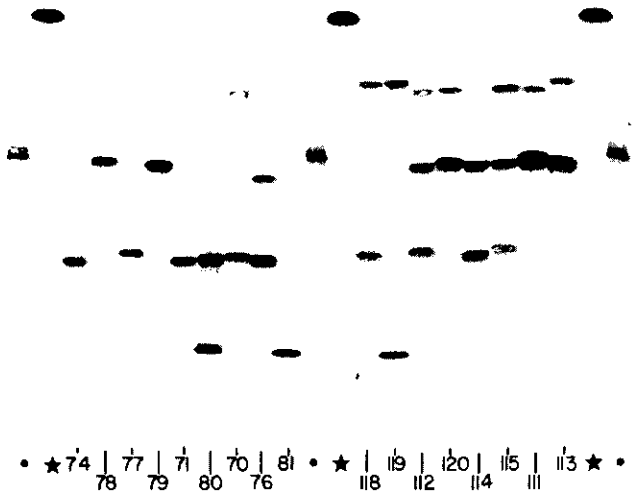


**Figure 12.** Coomassie blue stained parvalbumins of closely related species. The species are in the same order as in Figure 10. ★ = barramundi, ● = BSA.

In addition to their marked heat stability, parvalbumins have an unusual amino acid composition in that tryptophan and tyrosine are typically absent or present in unusually small amounts. The absence of these aromatic amino acids, coupled with a high proportion of phenylalanine residues, results in another unusual characteristic of parvalbumins, namely UV absorbance spectra that exhibit local maxima at wavelengths of 253, 259, 265, and 269 nm; substantially less than the maxima of nearly all other proteins (i.e. 280 nm).

Such characteristic parvalbumin UV spectra are shown in Figure 13 for dusky flathead (lane 4 in Figure 12) and black tip shark. The parvalbumins of the black tip shark display a slightly different spectrum from that exhibited by the flathead parvalbumins (and those of most teleosts) with shoulders at 277 and 283 nm, which are probably due to the presence of one or more tyrosine residues in a parvalbumin of the shark (but see Gerday *et al.*, 1979).

Parvalbumins are ideal protein markers for identifying the species of origin of cooked fish products because of their heat stability. Although only 1-4 parvalbumins are usually present in any species, we have found that their electrophoretic pattern is generally quite species specific as demonstrated in Figure 12 (also see Monaco *et al.*, 1982).

A problem in species identification based solely on parvalbumins may arise, however, following extended frozen storage ( > 6 months) of muscle tissue, because the parvalbumins of some species seem to be present in substantially reduced amounts in extracts made from such samples. This disapearance may be due to denaturation and/or "lock-up" of some muscle proteins when formaldehyde is liberated by fish muscles under these conditions (Bremner and Vail, 1983). Whatever the cause, the reduction in staining intensity after electrophoresis seems most pronounced for highly acidic proteins such as the parvalbumins. There seems to be no such pronounced reduction in parvalbumin staining intensity in tissue extracts stored at -70°C for similar periods of time.

### *(iii) Sample preparation, storage and handling*

As for starch gels, different sample extraction solutions are suitable for electrophoresis on polyacrylamide gels, as illustrated by Figure 14. Nearly all of the different extraction media we have tested produce extracts that give virtually identical protein patterns after polyacrylamide gel electrophoresis and staining with either Coomassie blue or silver. However, the storage conditions of the fish and/or extract do have a substantial effect on the strength of staining. The strongest staining resulted from tissue "drip" samples (see above) followed by fresh extracts made from fresh fish. Weaker staining was produced from old extracts (kept frozen at -70°C for 24 months or longer). Extracts prepared using 2-phenoxyethanol also exhibited a considerable loss of some proteins (also see above). The weakest staining resulted from fish that had remained frozen for over two years (at -20°C) before being extracted and analyzed. The effects of storage at refrigerated and room temperatures on general protein patterns are detailed in Keenan and Shaklee (1985).

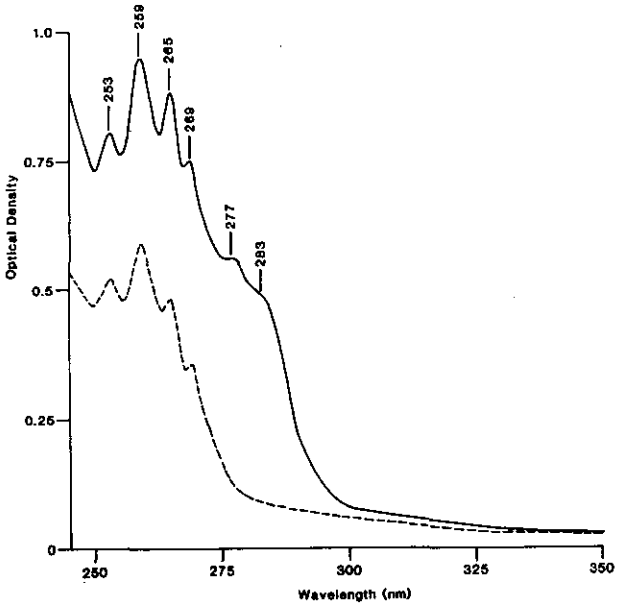


**Figure 13.** UV absorbance spectra for the parvalbumins of black tip shark (solid line) and dusky flathead (broken line). The electrophoretic pattern of dusky flathead parvalbumins can be seen in Figure 12, lane 4.

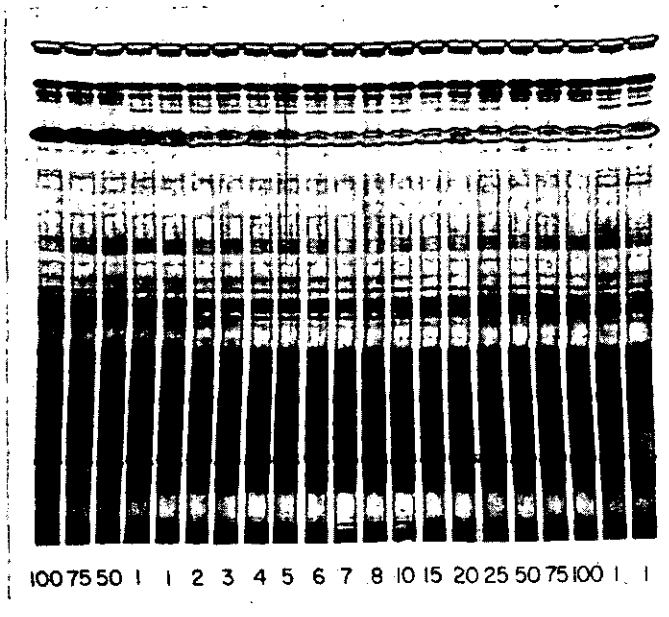


**Figure 15.** *The effects of repeated freezing and thawing* on the general protein pattern (silver stain) of barramundi. Numbers indicate the total number of times each sample was thawed (and refrozen).

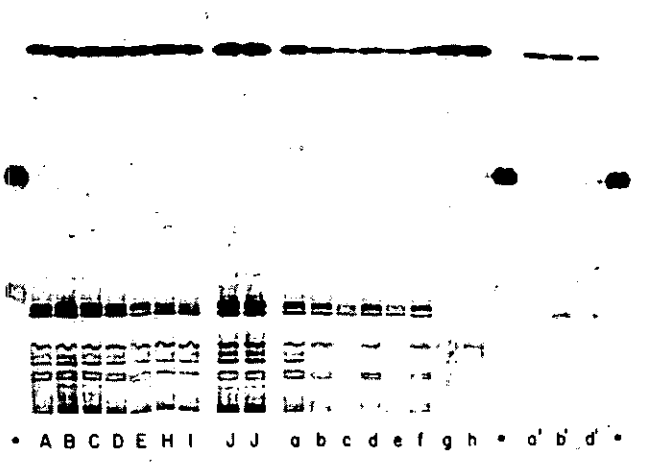


**Figure 14.** The effects of various handling treatments and extraction media on the general protein pattern (Coomassie blue) of barramundi. Details as specified in Figure 4 legend. ● = BSA.

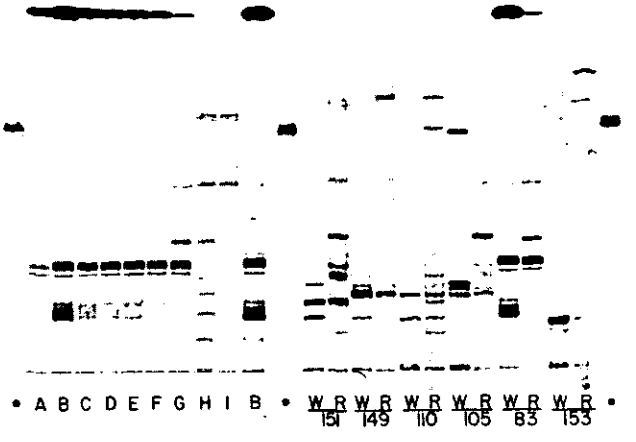


**Figure 16.** Coomassie blue stained general protein patterns of different muscle types of barramundi (A-I), Queensland school mackerel (151), narrow-barred Spanish mackerel (149), Australian salmon (110), tailor (105), barramundi (83) and longtail tuna (153). See Figure 7 for locations of fillet samples from barramundi. H = heart, I = stomach (smooth muscle), R = red skeletal muscle, W = white skeletal muscle. ● = BSA.


**Figure 17.** Silver stained polyacrylamide gel showing the effect of serial dilutions (of equal volume) on staining intensity and protein pattern of an extract of barramundi muscle.

Tests were also conducted to assess the effects of repeated freezing and thawing on general protein profiles. Figure 15 illustrates the slight decrease in staining intensity (presumably due to the denaturation of small amounts of protein) produced by this treatment.

### *(iv) Red vs. white muscle*

General protein staining of extracts from white and red skeletal muscle, heart, and stomach of barramundi reveals many differences in protein composition among these different types of muscle (Figure 16).

Of particular relevance to the problem of identification of cooked fillets is the tissue-specific distribution of the parvalbumins. From Figure 16 it can be seen that, in general, red skeletal muscle contains a lower concentration of parvalbumins than does white muscle. The same parvalbumins are present, however, in both the red (cardiac and skeletal) and white muscles. Gerday *et al.*, (1979) have reported that the concentration of parvalbumins exhibits an inverse relationship with myoglobin. Because of the large differences in the protein profiles of red and white muscle it is important to use proper controls for identification purposes (also see Hamoir *et al.*, 1972).

### *(v) Dilution effects*

Dilution of an extract has little or no effect on the discriminating power of these electrophoretic tests, provided that extreme dilutions are not employed. There are two reasons for this. Firstly, the protein profile (i.e. banding pattern) does not change with dilution, although the strength of staining gradually decreases with increasing dilution, as illustrated in Figure 17. Secondly, the high sensitivity of silver staining can be employed to detect the minutest quantities of protein. For instance, the protein concentration of the sample applied to the control lane of Figure 17 was approximately 18 mg/ml (as determined by the Biuret test). Successive serial dilutions (1:1) of this control extract were made and the entire series run on the gel. The major parvalbumin is detectable at a dilution of 1:64 following staining with Coomassie blue and at dilutions of up to 1:512 after silver staining.

## C. Future computerization of the fillet ID procedure

Polyacrylamide gel electrophoresis of general muscle proteins offers a simple, inexpensive and efficient method for the identification of fish fillets and other seafood products. There are, however, two major interrelated problems that limit its immediate application as a generally accepted and reliable method of seafood identification.

The first major problem is that each species is characterized by an often complex pattern of proteins. The fundamental parameters of use in distinguishing species are differences in: 1) electrophoretic position and 2) relative staining intensity (= concentration) of each protein band contributing to the overall biochemical phenotype of each species. The problem of complicated, species-specific protein patterns is exacerbated by the large numbers of species of both fishes and invertebrates which are contained in various seafoods.

The second major problem is that reliable and correct interpretation of such complex and diverse protein patterns generally requires considerable experience. Fortunately, both these problems lend themselves to computerized analyses. Each species-specific protein pattern can be permanently recorded and recalled as often as necessary using a microcomputer. Although it is possible to measure band positions on a gel manually and then input each measurement individually, this procedure is time-consuming and does not capture any information about the relative quantitative contribution of each individual protein to the overall pattern characterizing each species. A much more efficient method is to employ a scanning densitometer to quantify both position and relative staining intensity of each protein band making up the phenotype, and then to interface the output of the densitometer (through an analog to digital converter) to a microcomputer, which can then store this information in memory or on disk. The microcomputer can be used not only to store and later recall such raw data but also to detect component protein peaks and even "standardize" individual records (in terms of both electrophoretic migration and staining intensity if one or more standards are incorporated on each gel) so that the results of different gels can be compared (for example, see Feltham and Sneath 1979). Examples of two such densitometric scans and their resulting computer analysis are shown in Figure 18 and Table 3.

With appropriate software (for example, see Jackman *et al.*, 1983, Jackman, 1983), the computer can even be programmed to compare the protein profile of an "unknown" sample with that of a designated reference species stored in memory or on disk. If the two profiles match at a specified criterion level, the unknown is assumed to be the same species as the reference it was tested against. If the protein profile of the unknown does not match that of the designated reference specimen, it can then be automatically compared with all other protein profiles stored in the computer to allow tentative identification of the unknown.

If the process of fillet identification were standardized so that all laboratories used the same apparatus and procedures, a common system of computerized data storage and interpretation (for example, see Sapirstein, 1984) could be developed and shared among all laboratories involved in this type of work. This type of computerized system of fillet identification should overcome the problems of complicated and diverse electrophoretic patterns and their interpretation by different individuals with varying expertise in different laboratories. Furthermore, such a standardized system would save substantial duplication of effort since the entire library of reference species would only have to be developed once by one laboratory. Once the library of reference patterns was generated and the programs for analysis developed, these could be shared by all laboratories. This would make the entire procedure of fish fillet identification more efficient and reliable.

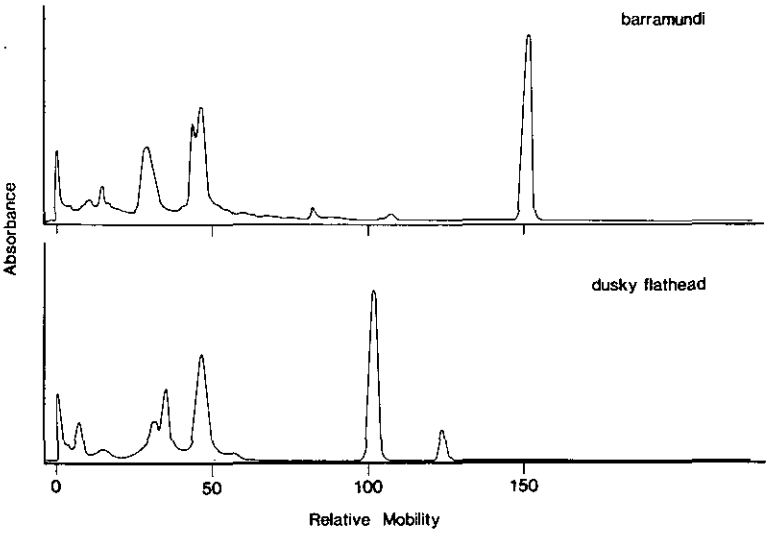


**Figure 18.** Densitometric scans of Coomassie blue stained general muscle proteins after polyacrylamide gel electrophoresis. Lane 13 (barramundi) and 4 (dusky flathead) of the gel in Figure 10 were scanned using a BioRad (model 1650) densitometer in transmittance mode. The output from the densitometer was then analyzed using Chromcard® II (Anadata Inc., 516 North Main Street, Glen Ellyn, Illinois 60137, USA) running on an Apple® IIe microcomputer to provide the graphic output represented in this figure and the data in Table 3.

**Table 3.** Quantitative characterization of protein profiles of barramundi and dusky flathead following densitometry.

| | Peak Number | Mobility (Relative to BSA) | Peak Height | Relative Peak Area (%) |
|---|---|---|---|---|
| **BARRAMUNDI** | 1 | 1 | 62.2 | 6.5 |
| | 2 | 5 | 10.6 | 1.1 |
| | 3 | 9 | 15.6 | 1.6 |
| | 4 | 13 | 25.6 | 2.8 |
| | 5 | 14 | 9.3 | 1.4 |
| | 6 | 29 | 52.5 | 19.1 |
| | 7 | 42 | 73.5 | 10.3 |
| | 8 | 45 | 87.5 | 22.4 |
| | 9 | 52 | 7.4 | 1.3 |
| | 10 | 81 | 9.1 | 1.0 |
| | 11 | 108 | 4.6 | 0.8 |
| | 12 | 152 | 145.1 | 31.8 |
| **DUSKY FLATHEAD** | 1 | 1 | 48.2 | 7.3 |
| | 2 | 6 | 27.3 | 5.3 |
| | 3 | 13 | 6.8 | 3.0 |
| | 4 | 30 | 28.3 | 10.1 |
| | 5 | 33 | 53.1 | 11.7 |
| | 6 | 44 | 80.9 | 28.3 |
| | 7 | 54 | 5.4 | 1.6 |
| | 8 | 101 | 132.0 | 28.8 |
| | 9 | 122 | 22.2 | 4.0 |

## ACKNOWLEDGMENTS

The authors are extremely grateful for the contributions and assistance of many people. JBS is particularly grateful to Drs Clement L. Markert, Gregory S. Whitt, Fred W. Allendorf, and especially Paul B. Samollow, for many of the details of starch gel electrophoresis of enzymes presented in this report. CPK wishes to acknowledge the contributions of Drs Kevin Bell and Chris C. Pollitt to the polyacrylamide gel electrophoresis procedure reported herein. We thank Drs David Woodland, Colin Wrigley, and especially Bill Dall, for helpful discussions and suggestions.

We also wish to thank the many people who contributed specimens used in this study, including S. Bell, S. Blaber, P. Channels, P. Crocos, T. Davis, R. Garrett, N. Gill, R. Griffin, T. Kenchington, P. Last, J. Lyle, L. Nash, M. Potter, I. Rassmussen, K. Sainsbury, J. Stevens, G. White, T. Wassenberg, D. Williams, and M. Wilson. Dr J. de Jersey assisted with the spectrophotometric analysis of parvalbumins.

We are grateful to Dr John Paxton, Curator of Fishes at the Australian Museum, for encouragement and for allowing us to deposit voucher specimens of many fish species in the museum's collection. Finally, we acknowledge the assistance and skill of Ms Narelle Hall in preparing the typescript.

## REFERENCES


1. Aksnes, A. and L.R. Njaa. 1981. Catalase, glutathione peroxidase, and superoxide dismutase in different fish species. *Comparative Biochemistry and Physiology* **69B**: 893-896
2. Avise, J.C. and G.B. Kitto. 1973. Phosphoglucose isomerase gene duplication in the bony fishes: an evolutionary history. *Biochemical Genetics* **8**: 113-132
3. Ayala, F.J. (ed). 1976. Molecular Evolution. Sunderland, Mass. Sinauer Assoc. Inc. 277 pp.
4. Bailey, G.S., A.C. Wilson, J.E. Halver, and C.L. Johnson. 1970. Multiple forms of supernatant malate dehydrogenase in salmonid fishes. Biochemical, immunological, and genetic studies. *Journal of Biological Chemistry* **245**: 5927-5940
5. Baldwin, J. and W.R. England. 1982. Multiple forms of octopine dehydrogenase in *Strombus luhuanus* (Mollusca, Gastropoda, Strombidae): genetic basis of polymorphism, properties of the enzymes, and relationship between the octopine dehydrogenase phenotype and the accumulation of anaerobic end products during exercise. *Biochemical Genetics* **20**: 1015-1025
6. Beaumont, A.R. and C.M. Beveridge. 1983. Resolution of phosphoglucomutase isozymes in *Mytilus edulis* L. *Marine Biology Letters* **4**: 97-103
7. Beaumont, A.R., T.R. Day, and G. Gäde. 1980. Genetic variation at the octopine dehydrogenase locus in the adductor muscle of *Cerastoderma edule* (L.) and six other bivalve species. *Marine Biology Letters* **1**: 137-148
8. Biegniewska, A. and E.F. Skorkowski. 1983. Isolation and some properties of malic enzyme from the shrimp abdomen muscle. *Comparative Biochemistry and Physiology* **74B**: 627-632
9. Bone, Q. 1966. On the function of the two types of mytomal muscle fibre in elasmobranch fish. *Journal of the Marine Biological Association of the United Kingdom* **46**: 321-349
10. Boyer, S.H., D.C. Fainer, and E.J. Watson-Williams. 1963. Lactate dehydrogenase variant from human blood: evidence for molecular subunits. *Science* **141**: 642-643
11. Bremner, H.A. and A.M.A. Vail. 1983. Electrophoretic identification of fish species, or salmon on Friday but barra(on)mundi. *Food Technology in Australia* **35**: 322-326
12. Buroker, N.E., W.K.Hershberger and K.K. Chew. 1975. Genetic variation in the Pacific oyster, *Crassostrea gigas*. *Journal of the Fisheries Research Board of Canada* **32**: 2471-2477
13. Clayton, J.W., W.G. Franzin and D.N. Tretiak. 1973. Genetics of glycerol-3-phosphate dehydrogenase isozymes in white muscle of lake whitefish (*Coregonus clupeaformis*). *Journal of the Fisheries Research Board of Canada* **30**: 187-193
14. Clayton, J.W. and Tretiak, D.N. 1972. Amine-citrate buffers for *p*H control in starch gel electrophoresis. *Journal of the Fisheries Research Board of Canada* ***29***: 1169-1172
15. Clayton, J.W., D.N. Tretiak and A.H. Kooyman. 1971. Genetics of multiple malate dehydrogenase isozymes in skeletal muscle of walleye (*Stizostedion vitreum vitreum*). *Journal of the Fisheries Research Board of Canada* **28**: 1005-1008
16. Cowie, W.P. 1968. Identification of fish species by thin-slab polyacrylamide gel electrophoresis of the muscle myogens. *Journal of Science Food and Agriculture* **19**: 226-229
17. Crabtree, B. and E.A. Newsholme. 1972. The activities of phosphorylase, hexokinase, phosphofructokinase, lactate dehydrogenase and the glycerol 3-phosphate dehydrogenases in muscles from vertebrates and invertebrates. *Biochemical Journal* **126**: 49-58

18. Dando, P.R., K.B. Storey, P.W. Hochachka, and J.M. Storey. 1981. Multiple dehydrogenases in marine molluscs: electrophoretic analysis of alanopine dehydrogenase, strombine dehydrogenase, octopine dehydrogenase and lactate dehydrogenase. *Marine Biology Letters* **2**: 249-257

19. Darnall, D.W. and I.M. Klotz. 1975. Subunit constitution of proteins: a table. *Archives of Biochemistry and Biophysics* **166**: 651-682

20. de Ligny, W. 1968. A routine method for the preservation of starch gels. *Immunogenetics Letters* **5**: 128-129

21. Engel, W., J. Schmidtke and U. Wolf. 1971. Genetic variation of α-glycerophosphate dehydrogenase isoenzymes in clupeoid and salmonoid fish. *Experientia* **27**: 1489-1491

22. Feltham, R.K.A. and P.H.A. Sneath. 1979. Quantitative comparison of electrophoretic traces of bacterial proteins. *Computers and Biomedical Research* **12**: 247-263

23. Ferris, S.D. and G.S. Whitt. 1978. Genetic and molecular analysis of nonrandom dimer assembly of the creatine kinase isozymes of fishes. *Biochemical Genetics* **16**: 811-829

24. Frick, L. 1983. An electrophoretic investigation of the cytosolic di- and tripeptidases of fish: molecular weights, substrate specificities, and tissue and phylogenetic distributions. *Biochemical Genetics* **21**: 309-322

25. Frick, L. 1984. Isolation and characterization of five cytosolic di- and tripeptidases from the skipjack tuna (*Katsuwonus pelamis*). *Comparative Biochemistry and Physiology* **77B**: 533-540

26. Fridovich, I. 1974. Superoxide dismutases. *Advances in Enzymology* **41**: 35-97

27. Gahne, B., R.K. Juneja and J. Grolmus. 1977. Horizontal polyacrylamide gradient gel electrophoresis for the simultaneous phenotyping of transferrin, post-transferrin, albumin and post-albumin in the blood plasma of cattle. *Animal Blood Groups and Biochemical Genetics* **8**: 127-137

28. Geer, B.W., D. Krochko, M.J. Oliver, V.K. Walker and J.H. Williamson. 1980. A comparative study of the NADP-malic enzymes from *Drosophila* and chick liver. *Comparative Biochemistry and Physiology* **65B**: 25-34

29. Gerday, C., B. Joris, N. Gerardin-Otthiers, S. Collin and G. Hamoir. 1979. Parvalbumins from the lungfish (*Protopterus dolloi*). *Biochimie* **61**: 589-599

30. Grant, W.S., R. Bakkala, F.M. Utter, D.J. Teel and T. Kobayashi. 1983. Biochemical genetic population structure of yellowfin sole, *Limanda aspera*, of the North Pacific Ocean and Bering Sea. *Fishery Bulletin* **81**: 667-677

31. Grant, W.S, G.B. Milner, P. Krasnowski, and F.M. Utter. 1980. Use of biochemical genetic variants for identification of sockeye salmon (*Oncorhynchus nerka*) stocks in Cook Inlet, Alaska. *Canadian Journal of Fisheries and Aquatic Sciences* **37**: 1236-1247

32. Hamoir, G., B. Focant and M. Distèche. 1972. Proteinic criteria of differentiation of white, cardiac and various red muscles in carp. *Comparative Biochemistry and Physiology* **41B**: 665-674

33. Harris, H. and D.A. Hopkinson. 1976. Handbook of Enzyme Electrophoresis in Human Genetics. New York, Amer. Elsevier Publ. Co. (looseleaf)

34. Healy, J.A. and M.F. Mulcahy. 1979. Polymorphic tetrameric superoxide dismutase in the pike *Esox lucius* L. (Pisces: Esocidae). *Comparative Biochemistry and Physiology* **62B**: 563-565

35. Hjorth, J.P. 1971. Genetics of *Zoarces* populations. I. Three loci determining the phosphoglucomutase isoenzymes in brain tissue. *Hereditas* **69**: 233-242

36. Hodnett, J.L., J.E. Evans, H.B. Gray, Jr. and A.H. Bartel. 1976. Isolation and partial characterization of supernatant and mitochondrial shrimp muscle malate dehydrogenases. *Comparative Biochemistry and Physiology* **54B**: 271-277

37. Holmes, R.S. and C.J. Masters. 1970. Epigenetic interconversions of the multiple forms of mouse liver catalase. *FEBS Letters* **11**: 45-48

38. Iniesta, M.G., M.J. Cano and A. Garrido-Pertierra. 1985. Properties and function of malate enzyme from *Dicentrarchus labrax* L. liver. *Comparative Biochemistry and Physiology* **80B**: 35-39

39. Jackman, P.J.H. 1983. A program in BASIC for numerical taxonomy of micro-organisms based on electrophoretic band positions. *Microbios Letters* **23**: 119-124

40. Jackman, P.J.H., R.K.A. Feltham and P.H.A. Sneath. 1983. A program in BASIC for numerical taxonomy of micro-organisms based on electrophoretic protein patterns. *Microbios Letters* **23**: 87-98

41. Johnston, I.A. and G. Goldspink. 1973a. A study of the swimming performance of the Crucian carp *Carassius carassius* (L.) in relation to the effects of exercise and recovery on biochemical changes in the myotomal muscles and liver. *Journal of Fish Biology* **5**: 249-260

42. Johnston, I.A. and G. Goldspink. 1973b. The effect of sustained swimming on the concentrations of glycogen and lactate occurring in the myotomal muscle and liver of the coalfish (*Gadus virens* L.). *Journal of the Marine Biological Association of the United Kingdom* **53**: 17-26

43. Kaloustian, H.D., F.E. Stolzenback, J. Everse and N.O. Kaplan. 1969. Lactate dehydrogenase of lobster (*Homarus americanus*) tail muscle. I. Physical and chemical properties. *Journal of Biological Chemistry* **244**: 2891-2901

44. Keenan, C.P. and J.B. Shaklee. 1985. Electrophoretic identification of fresh and cooked fish fillets and other marine products. *Food Technology in Australia* **37**: 117-128

45. Kitto, G.B. and R.G. Lewis. 1967. Purification and properties of tuna supernatant and mitochondrial malate dehydrogenases. *Biochemica Biophysica Acta* **139**: 1-15

46. Kobayashi, T., G.B. Milner, D.J. Teel and F.M. Utter. 1984. Genetic basis for electrophoretic variation of adenosine deaminase in chinook salmon. *Transactions of the American Fisheries Society* **113**: 86-89

47. Learson, R.J. 1969. Collaborative study of a rapid electrophoretic method for fish species identification. *Journal of the Association of Official Analytical Chemists* **52**: 703-707

48. Learson, R.J. 1970. Collaborative study of a rapid electrophoretic method for fish species identification. II. Authentic flesh standards. *Journal of the Association of Official Analytical Chemists* **53**: 7-9

49. Lee, C.Y., C.H. Langley and J. Burkhart. 1978. Purification and molecular weight determination of glucose-6-phosphate dehydrogenase and malic enzyme from mouse and *Drosophila*. *Analytical Biochemistry* **86**: 697-706

50. Leslie, J.F. and R.C. Vrijenhoek. 1977. Genetic analysis of natural populations of *Poeciliopsis monacha*. Allozyme inheritance and pattern of mating. *Journal of Heredity* **68**: 301-306

51. Long, G.L. 1976. The stereospecific distribution and evolutinary significance of invertebrate lactate dehydrogenases. *Comparative Biochemistry and Physiology* **55B**: 77-83

52. Love, R.M. 1980. The Chemical Biology of Fishes. Vol. 2: Advances 1968-1977. With a Supplementary Key to the Chemical Literature. New York: Academic Press 943 pp.

53. Lundstrom, R.C. 1981. Fish species identification by isoelectric focusing: sarcoplasmic protein polymorphism in monkfish (*Lophius americanus*). *Journal of the Association of Official Analytical Chemists* **64**: 32-37

54. Lundstrom, R.C. 1983. Fish species identification by agarose gel isoelectric focusing: collaborative study. *Journal of the Association of Official Analytical Chemists* **66**: 123-127

55. Markert, C.L., J.B. Shaklee and G.S. Whitt. 1975. Evolution of a gene. *Science* **189**: 102-114

56. Menzies, R.A. and J.M. Kerrigan. 1979. Implications of spiny lobster recruitment patterns of the Caribbean - A biochemical genetic approach. *Proceedings of the Gulf and Caribbean Fisheries Institute* **31**: 164-178

57. Merril, C.R., G. Goldman and M.L. Van Keuren. 1982. Simplified silver protein detection and image enhancement methods in polyacrylamide gels. *Electrophoresis* **3**: 17-23

58. Mitchell, C.P. and D. Scott. 1979. Muscle myogens in the New Zealand Galaxiidae. *New Zealand Journal of Marine and Freshwater Research* **13**: 285-294

59. Mo, Y., C.D. Young, R.W. Gracy, N.D. Carter and P.R. Dando. 1975. Isolation and characterization of tissue-specific isozymes of glucosephosphate isomerase from catfish and conger. *Journal of Biological Chemistry* **250**: 6747-6755

60. Monaco, P.J., E.M. Rasch, J.S. Balsano and B.J. Turner. 1982. Muscle protein phenotypes and the probable evolutionary origin of a unisexual fish, *Poecilia formosa*, and its triploid derivatives. *Journal of Experimental Zoology* **221**: 265-274

61. Mulley, J.C. and B.D.H. Latter. 1981a. Geographic differentiation of eastern Australian penaeid prawn populations. *Australian Journal of Marine and Freshwater Research* **32**: 889-895

62. Mulley, J.C. and B.D.H. Latter. 1981b. Geographic differentiation of tropical Australian penaeid prawn populations. *Australian Journal of Marine and Freshwater Research* **32**: 897-906

63. Nakamura, I., T. Iwai and K. Matsubara. 1968. A review of the sailfish, spearfish, marlin and swordfish of the world. *Misaki Marine Biological Institute Special Report* **4**: 1-95 (In Japanese)

64. Nakanishi, M., A.C. Wilson, R.A. Nolan, G.C. Gorman and G.S. Bailey. 1969. Phenoxyethanol: protein preservative for taxonomists. *Science* **163**: 681-683

65. Noda, L., G.E. Schulz and I. Von Zabern. 1975. Crystalline adenylate kinase from carp muscle. *European Journal of Biochemistry* **51**: 229-235

66. Numachi, K. 1971. Genetic polymorphism of α-glycerophosphate dehydrogenase in saury, *Cololabis saira* - I. Seven variant forms and genetic control. *Bulletin of the Japanese Society of Scientific Fisheries* **37**: 755-760

67. Numachi, K. 1981. A simple method for preservation and scanning of starch gels. *Biochemical Genetics* **19**: 233-236

68. Pantelouris, E.M. 1976. Aspartate aminotransferase variation in the Atlantic eel. *Journal of Experimental Marine Biology and Ecology* **22**: 123-130

69. Pechère, J.-F., J.-P. Capony and J. Demaille. 1974. Evolutionary aspects of the structure of muscular parvalbumins. *Systematic Zoology* **22**: 533-548

70. Philipp, D.P., W.F. Childers and G.S. Whitt. 1983. A biochemical genetic evaluation of the northern and Florida subspecies of largemouth bass. *Transactions of the American Fisheries Society* **112**: 1-20

71. Pietkiewicz, J., I. Kustrzeba-Wójcicka and E. Wolna. 1983. Purification and properties of enolase from carp (*Cyprinus carpio*). Comparison with enolases from mammals' muscles and yeast. *Comparative Biochemistry and Physiology* **75B**: 693-698

72. Pollitt, C.C. and K. Bell. 1980. Protease inhibitor system in horses: classification and detection of a new allele. *Animal Blood Groups and Biochemical Genetics* **11**: 235-244

73. Richardson, B.J. 1983. Distribution of protein variation in skipjack tuna (*Katsuwonus pelamis*) from the central and south-western Pacific. *Australian Journal of Marine and Freshwater Research* **34**: 231-251

74. Rodbard, D. and A. Chrambach. 1971. Estimation of molecular radius, free mobility, and valence using polyacrylamide gel electrophoresis. *Analytical Biochemistry* **40**: 95-134

75. Ruth, R.C., D.M. Soja and F. Wold. 1970. Purification and properties of enolases from coho (*Oncorhynchus kisutch*) and chum (*Oncorhynchus keta*) salmon. *Archives of Biochemistry and Biophysics* **140**: 1-10

76. Sapirstein, H,D. 1984. Computer-based wheat cultivar identification and comparative analysis of gliadin electrophoregrams. Unpubl. Ph.D. dissertation, University of Manitoba, Winnipeg

77. Schaal, B.A. and W.W. Anderson. 1974. An outline of techniques for starch gel electrophoresis of enzymes from the American oyster *Crassostrea virginica* Gmelin. *Technical Report of the Georgia Marine Science Center*, **74-3**. 18 pp

78. Schmidtke, J. and W. Engel. 1972. Duplication of the gene loci coding for the supernatant aspartate aminotransferase by polyploidization in the fish family Cyprinidae. *Experientia* **28**: 976-978

79. Scopes, R.K. 1968. Methods for starch-gel electrophoresis of sarcoplasmic proteins. An investigation of the relative mobilities of the glycolytic enzymes from the muscles of a variety of species. *Biochemical Journal* **107**: 139-150

80. Selander, R.K., M.H. Smith, S.Y. Yang, W.E. Johnson and J.B. Gentry. 1971. Biochemical polymorphism and systematics in the genus *Peromyscus*. I. Variation in the old field mouse (*Peromyscus polionotus*). *Studies in Genetics VI. University of Texas Publication* **7103**: 49-90

81. Shaklee, J.B. 1983. Mannosephosphate isomerase in the Hawaiian spiny lobster *Panulirus marginatus*: a polymorphic, sex-linked locus useful in investigating embryonic and larval sex ratios. *Marine Biology* **73**: 193-201

82. Shaklee, J.B. 1984. Genetic variation and population structure in the damselfish, *Stegastes fasciolatus*, throughout the Hawaiian Archipelago. *Copeia* **1984**: 629-640

83. Shaklee, J.B., K.L. Kepes and G.S. Whitt. 1973. Specialized lactate dehydrogenase isozymes: the molecular and genetic basis for the unique eye and liver LDHs of teleost fishes. *Journal of Experimental Zoology* **185**: 217-240

84. Shaklee, J.B. and J.P. Salini. 1985. Genetic variation and population subdivision in Australian barramundi, *Lates calcarifer* (Bloch). *Australian Journal of Marine and Freshwater Research* **36**: 203-218

85. Shaklee, J.B. and P.B. Samollow. 1984a. Genetic variation and population structure in a spiny lobster, *Panulirus marginatus*, in the Hawaiian Archipelago. *Fishery Bulletin* **82**: 693-702

86. Shaklee, J.B. and P.B. Samollow. 1984b. Genetic variation and population structure in a deepwater snapper (*Pristipomoides filamentosus*) in the Hawaiian Archipelago. *Fishery Bulletin* **82**: 703-713

87. Shaklee, J.B. and C.S. Tamaru. 1981. Biochemical and morphological evolution of Hawaiian bonefishes (*Albula*). *Systematic Zoology* **30**: 125-146

88. Sharp, G.D. and S. Pirages. 1978. The distribution of red and white swimming muscles, their biochemistry, and the biochemical phylogeny of selected scombrid fishes, pp. 41-78 in G.D. Sharp and A.E. Dizon (eds) The Physiological Ecology of Tunas. New York: Academic Press 485 pp.

89. Shaw, C.R. and R. Prasad. 1970. Starch gel electrophoresis of enzymes - a compilation of recipes. *Biochemical Genetics* **4**: 297-320

90. Siciliano, M.J. and C.R. Shaw. 1976. Separation and visualization of enzymes on gels, pp. 185-209 in I. Smith (ed.) Chromatographic and Electrophoretic Techniques. Vol. 2. Zone Electrophoresis. 4th edition. London: Heinemann

91. Stoneking, M., B. May and J.E. Wright. 1979. Genetic variation, inheritance, and quaternary structure of malic enzyme in brook trout (*Salvelinus fontinalis*). *Biochemical Genetics* **17**: 599-619

92. Sullivan, B., J. Bonaventura, C. Bonaventura, L. Pennell, J. Elliott, R. Boyum and W. Lambie. 1975. The structure and evolution of parvalbumins. I. Amino acid compositional studies of parvalbumins from four perciform species. *Journal of Molecular Evolution* **5**: 103-116

93. Taggart, J., A. Ferguson and F.M. Mason. 1981. Genetic variation in Irish populations of brown trout (*Salmo trutta* L.): electrophoretic analysis of allozymes. *Comparative Biochemistry and Physiology* **69B**: 393-412

94. Totland, G.K., H. Kryvi and E. Slinde. 1978. LDH isoenzymes in the axial muscle of the sharks *Galeus melastromus* and *Etmopterus spinax*. *Journal of Fish Biology* **12**: 45-50

95. Tsuyuki, H. 1974. Muscle proteins of fishes, pp. 287-305 in M. Florkin (ed.) Chemical Zoology. Vol. VIII. Deuterostomians, Cyclostomes, and Fishes. New York: Academic Press

96. Utter, F.M., H.O. Hodgins, F.W. Allendorf, A.G. Johnson and J.L. Mighell. 1973. Biochemical variants in Pacific salmon and rainbow trout: their inheritance and application in population studies, pp. 329-339 in J.H. Schröder (ed.) Genetics and Mutagenesis of Fish. Berlin: Springer-Verlag, 356 pp.

97. Vourinen, J. 1984. Duplicate loci for supernatant and mitochondrial malic enzymes in vendace, *Coregonus albula* (L.). *Comparative Biochemistry and Physiology* **78B**: 63-66

98. Wallevik, K. and J.C. Jensenius. 1982. A simple and reliable method for the drying of polyacrylamide slab gels. *Journal of Biochemical and Biophysical Methods* **6**: 17-21

99. Walsh, P.J. 1981. Purification and characterization of two allozymic forms of octopine dehydrogenase from California populations of *Metridium senile*. *Journal of Comparative Physiology* **143**: 213-222

100. Winans, G.A. 1980. Geographic variation in the milkfish *Chanos chanos*. I. Biochemical evidence. *Evolution* **34**: 558-574

101. Yamada, J. and A. Suzuki. 1982. Identification of fish species by thin layer isoelectric focusing of sarcoplasmic proteins. *Bulletin of the Japanese Society of Scientific Fisheries* **48**: 73-77 (In Japanese)

102. Zammit, V.A. and E.A. Newsholme. 1976. The maximum activities of hexokinase, phosphorylase, phosphofructokinase, glycerol phosphate dehydrogenases, lactate dehydrogenase, octopine dehydrogenase, phosphoenolpyruvate carboxykinase, nucleoside diphosphate kinase, glutamate-oxaloacetate transaminase and arginine kinase in relation to carbohydrate utilization in muscles from marine invertebrates. *Biochemical Journal* **160**: 447-462

# APPENDIX 1

## ABBREVIATIONS AND SOURCES

All chemicals used should be "reagent grade" unless otherwise indicated.

## A. ABBREVIATIONS

| | |
|---|---|
| ADP | adenosine 5′-diphosphate |
| BIS | N,N′-methylene-bisacrylamide |
| BSA | bovine serum albumin |
| EDTA | ethylenediamine tetraacetic acid |
| MTT | methyl thiazolyl tetrazolium |
| $NAD^+$ | $\beta$-nicotinamide adenine dinucleotide |
| NADH | $\beta$-nicotinamide adenine dinucleotide, reduced form |
| $NADP^+$ | $\beta$-nicotinamide adenine dinucleotide phosphate |
| NBT | nitroblue tetrazolium |
| PMS | phenazine methosulfate |
| TEMED | N,N,N′,N′-tetramethyl-ethylenediamine |
| Tris | Tris(hydroxymethyl)aminomethane |

## B. SOURCES

**Aldrich**

Aldrich Chemical Company
PO Box 355
Milwaukee, Wisconsin 53201
USA

**Connaught**

Connaught Laboratories Limited
Willowdale, Ontario
Canada

**Electrostarch**

Electrostarch Company
PO Box 1294
Madison, Wisconsin 53701
USA

**Flambeau**

Flambeau Products Corporation
PO Box 97
Middlefield, Ohio 44067
USA

**Fluka**

Fluka AG
Chemische Fabrik
CH-9470 Buchs
Switzerland

**Kodak**

Eastman Kodak Company
Rochester, New York 14650
USA

**Schleicher and Schuell**

Schleicher and Schuell, Inc.
Keene, New Hampshire 03431
USA

**Serva**

Serva Fine Biochemicals
Heidelberg
West Germany

**Sigma**

Sigma Chemical Company
PO Box 14508
St. Louis, Missouri 63078
USA

**Whatman**

Whatman Ltd
Springfield Mill, Kent
England

# APPENDIX 2

## REAGENT SOLUTIONS FOR STARCH GEL ELECTROPHORESIS

All chemicals used in this study were obtained from the Sigma Chemical Company and catalogue numbers refer to Sigma (unless otherwise noted).

Quantities in all recipes throughout this manual are for a **final volume** of 1.0 liter unless otherwise stated.

## A. HOMOGENIZING BUFFER

(Selander *et al.*, 1971)

| | |
|---|---|
| Tris | 12.1 g |
| EDTA ($Na_2$) | 336 mg |
| $NADP^+$ | 38 mg |

adjusted to pH 7.0 with HCl
(Store at 5°C)

## B. ELECTROPHORESIS BUFFERS

### TC-1

(buffer #1 of Shaw and Prasad, 1970)

*STOCK SOLUTION*

| | |
|---|---|
| Tris | 16.35 g |
| citric acid monohydrate | 9.04 g |

pH 7.0

***Gel buffer*** - 1:14 dilution of stock
***Electrode buffer*** - undiluted stock

***recommended conditions:*** 175 V, 6 h

### TC-2

(buffer #5 of Selander *et al.*, 1971)

*STOCK SOLUTION*

| | |
|---|---|
| Tris | 83.2 g |
| citric acid monohydrate | 33 g |

pH 8.0

***Gel buffer*** - 1:29 dilution of stock
***Electrode buffer*** - undiluted stock

***recommended conditions:*** 140 V, 6 h

## TC-3

(buffer #4 of Selander *et al.*, 1971)

***Gel buffer -***

| | |
|---|---|
| Tris | 970 mg |
| citric acid monohydrate | 630 mg |

pH adjusted to 6.7 with 1 N NaOH

***Electrode buffer -***

| | |
|---|---|
| Tris | 27.0 g |
| citric acid monohydrate | 18.1 g |

pH adjusted to 6.3 with 1 N NaOH

***recommended conditions:*** 150 V, 4 h

## TC-4

(buffer "a" of Schaal and Anderson, 1974)

*STOCK SOLUTION*

| | |
|---|---|
| Tris | 27 g |
| citric acid monohydrate | 18.1 g |

pH 5.8

***Gel buffer -*** 1:27.5 dilution of stock
***Electrode buffer -*** undiluted stock

***recommended conditions:*** 150 V, 4 h

## CAAPM

(modified buffer of Clayton and Tretiak, 1972)

*STOCK SOLUTION*

| | |
|---|---|
| citric acid monohydrate | 8.4 g |
| N-(3-aminopropyl)-morpholine (# A9028 or Aldrich # 12,309-9) | |

added to pH 6.0

***Gel buffer -*** 1:19 dilution of stock
***Electrode buffer -*** undiluted stock

***recommended conditions:*** 225 V, 6 h

## CAEA

(modified buffer of Clayton and Tretiak, 1972)

*GEL STOCK SOLUTION*

(pH 6.5, 7.0, or 7.5)

| | |
|---|---|
| citric acid monohydrate | 8.4 g |
| N-(3-aminopropyl)-diethanolamine (Fluka # 14870 or Aldrich # 10,944-4) | |

added to desired pH

*ELECTRODE STOCK SOLUTION*

| | |
|---|---|
| citric acid monohydrate | 42 g |
| N-(3-aminopropyl)-diethanolamine | |

added to pH 7.2

***Gel buffer -*** 1:24 dilution of gel stock
***Electrode buffer -*** 1:4 dilution of electrode stock

***recommended conditions:*** 220 V, 5 h

## TRIC

(modified buffer of Clayton and Tretiak, 1972)

*STOCK SOLUTION*

| | |
|---|---|
| citric acid monohydrate | 8.4 g |
| triethanolamine (# T 1377, Fluka # 90282, or Aldrich # T 5,830-0) | |

added to pH 7.2

***Gel buffer -*** 1:19 dilution of stock
***Electrode buffer -*** undiluted stock

***recommended conditions:*** 200 V, 6 h

## EBT

(modified buffer of Boyer *et al.*, 1963)

*STOCK SOLUTION*

| | |
|---|---|
| Tris | 109 g |
| EDTA ($Na_4$) | 7.4 g |
| boric acid | 30.9 g |

pH 8.6

***Gel buffer -*** 1:19 dilution of stock
***Electrode buffer -*** 1:6 dilution of stock

***recommended conditions:*** 300 V, 6 h

## TECB

(buffer of Shaklee and Tamaru, 1981)

*STOCK SOLUTION*

| | |
|---|---|
| Tris | 109 g |
| EDTA ($Na_4$) | 7.4 g |
| citric acid monohydrate | 20 g |
| boric acid | 10 g |
| pH 8.75 | |

***Gel buffer*** - 1:19 dilution of stock
***Electrode buffer*** - 1:6 dilution of stock

***recommended conditions:*** 200 V, 6 h

## TRIS-GLYCINE

(modified buffer of Holmes and Masters, 1970)

*STOCK SOLUTION*

| | |
|---|---|
| Tris | 3.0 g |
| glycine | 14.4g |
| pH 8.5 | |

***Gel buffer*** - undiluted stock
***Electrode buffer*** - undiluted stock

***recommended conditions:*** 425 V, 6 h

## LIOH

(modified buffer #2 of Selander *et al.*, 1971)

*STOCK A*

| | |
|---|---|
| LiOH.($H_2O$) | 1.26 g |
| boric acid | 11.9 g |
| pH 8.15 | |

*STOCK B*

| | |
|---|---|
| Tris | 6.2 g |
| citric acid monohydrate | 1.6 g |
| pH 8.3 | |

***Gel buffer*** - 1 part stock A: 9 parts stock B
***Electrode buffer*** - undiluted Stock A

***recommended conditions:*** 45mA, 6 h

## POULIK

(buffer #3 of Selander *et al.*, 1971)

***Gel buffer*** -

| | |
|---|---|
| Tris | 9.21 g |
| citric acid monohydrate | 1.05 g |
| pH 8.8 | |

***Electrode buffer*** -

| | |
|---|---|
| boric acid | 18.55 g |
| NaOH | 2.4 g |
| pH 8.15 | |

***recommended conditions:*** 40 mA, 6 h

# APPENDIX 3

## REAGENT SOLUTIONS FOR ENZYME STAINING

## A. STAINING BUFFERS

### 0.1M TRIS-HCL

(various pHs from 7.0 to 9.0)

| | |
|---|---|
| Tris | 12.1 g |
| titrated to desired pH with HCl | |

### 0.1M PHOSPHATE pH 7.5

| | |
|---|---|
| $KH_2PO_4$ | 13.6 g |
| $K_2HPO_4$ added to bring the pH to 7.5 | |

### 0.2M PHOSPHATE pH $\approx$ 4.5

| | |
|---|---|
| $NaH_2PO_4.2H_2O$ | 31.2 g |

## B. STOCK ENZYME STAINING SOLUTIONS

All biochemicals (substrates, coenzymes, linking enzymes, and dyes) used for enzyme staining were obtained from Sigma unless otherwise noted. Catalogue numbers refer to Sigma unless otherwise noted. All stock enzyme staining solutions should be stored at 5°C.

### $NAD^+$

| | |
|---|---|
| $\beta$-$NAD^+$ | 500 mg |
| distilled water | 100 ml |

### $NADP^+$

| | |
|---|---|
| $\beta$-$NADP^+$ | 400 mg |
| distilled water | 100 ml |

### MTT

| | |
|---|---|
| MTT | 1.75 g |
| distilled water | 500 ml |
| (must be stored in the dark) | |

### NBT

| | |
|---|---|
| nitroblue tetrazolium | 1.75 g |
| distilled water | 500 ml |
| (must be stored in the dark) | |

### PMS

| | |
|---|---|
| phenazine methosulfate | 1.75 g |
| distilled water | 500 ml |
| (must be stored in the dark) | |

### $MgCl_2$

| | |
|---|---|
| $MgCl_2$ | 2.5 g |
| distilled water | 100 ml |

### G-6-PDH

| | |
|---|---|
| glucose-6-phosphate dehydrogenase (# G 7878) | 500 units |
| distilled water | 50 ml |

### AAT SUBSTRATE SOLUTION

| | |
|---|---|
| $\alpha$-ketoglutarate (# K 1750) | 146 mg |
| L-aspartic acid (# A 9256) | 532 mg |
| EDTA($Na_2$) | 200 mg |
| polyvinylpyrolidone (PVP-10) | 2.0 g |
| $Na_2HPO_4$ | 3.0 g |
| distilled water to a final volume of | 200 ml |

### $\alpha$-N-ACETATE

| | |
|---|---|
| $\alpha$-naphthyl acetate | 750 mg |
| acetone | 25 ml |
| distilled water | 25 ml |

## DL-LACTATE SOLUTION

| | |
|---|---|
| 85% DL-lactic acid | 10.6 ml |
| LiOH added to bring the pH to 7.0 | |
| distilled water to a final volume of | 100 ml |

## DL-MALATE SOLUTION

| | |
|---|---|
| DL-malic acid | 13.4 g |
| NaOH added to bring the pH to 7.0 | |
| distilled water to a final volume of | 100 ml |

# APPENDIX 4
## ENZYME STAINING RECIPES

Staining recipes have now been published for well over 100 different enzymes. However, many of these enzymes are not detectable in fish muscle tissue or in other fishery products. For this reason, we restrict the following list of recipes to those enzymes of general utility in the identification of fishery products (such as fillets). The interested reader is referred to the following sources for more comprehensive listings of enzyme staining recipes: Scopes, 1968; Shaw and Prasad, 1970; Harris and Hopkinson, 1976; Siciliano and Shaw, 1976; and Richardson, 1983. Enzyme abbreviations are derived from the **recommended** enzyme names and Enzyme Commission numbers are according to the Recommendations of the Nomenclature Committee of the IUB on the Nomenclature and Classification of Enzymes as published in "Enzyme Nomenclature 1978" (Academic Press, Inc., New York, 1979).

### AAT

(aspartate aminotransferase EC 2.6.1.1)

*solution stain:*

| | |
|---|---|
| pyridoxal-5'-phosphate | 5 mg |
| fast blue BB salt | 125 mg |
| Tris-HCl pH 8.0 staining buffer | 25 ml |
| AAT substrate solution | 25 ml |

### AK*

(adenylate kinase EC 2.7.4.3)

*agar overlay:*

| | |
|---|---|
| α-D-glucose | 40 mg |
| ADP | 20 mg |
| hexokinase | 75 units |
| Tris-HCl buffer | 12 ml |
| $MgCl_2$ solution | 1 ml |
| G-6-PDH solution | 1 ml |
| MTT solution | 1 ml |
| PMS solution | 1 ml |
| $NADP^+$ solution | 1 ml |

* see ARGK and CK recipes

### ADA

(adenosine deaminase EC 3.5.4.4)

*agar overlay:*

| | |
|---|---|
| phosphate buffer pH 7.5 | 7 ml |
| Tris-HCl buffer | 3 ml |
| adenosine | 30 mg |
| MTT solution | 1 ml |
| xanthine oxidase | 0.3 units |
| purine nucleoside phosphorylase | 0.4 units |
| PMS solution | 1 ml |

### ALPDH

(alanopine dehydrogenase)

*agar overlay:*

| | |
|---|---|
| pyruvate | 4 mg |
| L-alanine | 134 mg |
| NADH | 10 mg |

view dark, defluorescent bands under UV light (note: this enzyme is only present in some molluscs)

## ARGK*

(arginine kinase EC 2.7.3.3)

*agar overlay:*

| | |
|---|---|
| α-D-glucose | 40 mg |
| phosphoarginine | 15 mg |
| ADP | 15 mg |
| hexokinase | 75 units |
| Tris-HCl buffer | 12 ml |
| $MgCl_2$ solution | 1 ml |
| G-6-PDH solution | 1 ml |
| MTT solution | 1 ml |
| PMS solution | 1 ml |
| $NADP^+$ solution | 1 ml |

(note: this enzyme is only present in invertebrates)

* Because AK also stains using this recipe, it is necessary to stain a "control" slice of the same gel for AK; bands appearing with both stains are AK, those unique to the ARGK stain are ARGK.

## CK*

(creatine kinase EC 2.7.4.3)

*agar overlay:*

| | |
|---|---|
| α-D-glucose | 40 mg |
| phosphocreatine | 25 mg |
| ADP | 20 mg |
| hexokinase | 75 units |
| Tris-HCl buffer | 12 ml |
| $MgCl_2$ solution | 1 ml |
| G-6-PDH solution | 1 ml |
| MTT solution | 1 ml |
| PMS solution | 1 ml |
| $NADP^+$ solution | 1 ml |

(note: this enzyme is only present in vertebrates)

* Because AK also stains using this recipe, it is necessary to stain a "control" slice of the same gel for AK; bands appearing with both stains are AK, those unique to the CK stain are CK.

## ENO

(enolase EC 4.2.1.11)

*agar overlay:*

| | |
|---|---|
| 2-phosphoglycerate | 20 mg |
| ADP | 20 mg |
| α-D-glucose | 40 mg |
| KCl | 75 mg |
| NADH | 10 mg |
| hexokinase | 75 units |
| pyruvate kinase | 20 units |
| lactate dehydrogenase | 20 units |

view dark, defluorescent bands under UV light

## EST-D

(esterase-D EC 3.1.1.?)

*agar overlay:*

| | |
|---|---|
| 4-methylumbelliferyl acetate (dissolve in a few drops of acetone) | 1 mg |
| Tris-HCl pH 7.0 buffer | 12 ml |

view bright, fluorescent bands under UV light

## GPI

(glucosephosphate isomerase EC 5.3.1.9)

*agar overlay:*

| | |
|---|---|
| fructose-6-phosphate | 20 mg |
| Tris-HCl buffer | 12 ml |
| G-6-PDH solution | 1 ml |
| MTT solution | 1 ml |
| PMS solution | 1 ml |
| $NADP^+$ solution | 1 ml |

## G-3-PDH

(glycerol-3-phosphate dehydrogenase EC 1.1.1.8)

*agar overlay:*

| | |
|---|---|
| α-glycerophosphate | 500 mg |
| Tris-HCl buffer | 12 ml |
| $NAD^+$ solution | 1 ml |
| MTT solution | 1 ml |
| PMS solution | 1 ml |

## IDH

(isocitrate dehydrogenase EC 1.1.1.42)

*agar overlay:*

| | |
|---|---|
| sodium isocitrate | 30 mg |
| Tris-HCl buffer | 12 ml |
| $MgCl_2$ solution | 1 ml |
| MTT solution | 1 ml |
| PMS solution | 1 ml |
| $NADP^+$ solution | 1 ml |

## LDH

(lactate dehydrogenase EC 1.1.1.27)

*agar overlay:*

| | |
|---|---|
| DL-lactate solution* | 4 ml |
| Tris-HCl buffer | 8 ml |
| $NAD^+$ solution | 1 ml |
| MTT solution | 1 ml |
| PMS solution | 1 ml |

* (note: certain invertebrates can be distinguished from fishes by comparing a gel slice stained using D-lactate with a slice stained using L-lactate as indicated in Table 1.)

## MDH

(malate dehydrogenase EC 1.1.1.37)

*agar overlay:*

| | |
|---|---|
| DL-malate solution | 5 ml |
| Tris-HCl buffer | 7 ml |
| $NAD^+$ solution | 1 ml |
| MTT solution | 1 ml |
| PMS solution | 1 ml |

## MDH($NADP^+$)

(malate dehydrogenase ($NADP^+$) EC 1.1.1.40)

*agar overlay:*

| | |
|---|---|
| DL-malate solution | 5 ml |
| Tris-HCl buffer | 7 ml |
| $MgCl_2$ solution | 1 ml |
| MTT solution | 1 ml |
| PMS solution | 1 ml |
| $NADP^+$ solution | 1 ml |

## MPI

(mannose phosphate isomerase EC 5.3.1.8)

*agar overlay:*

| | |
|---|---|
| mannose-6-phosphate | 40 mg |
| Tris-HCl buffer | 12 ml |
| $MgCl_2$ solution | 1 ml |
| glucosephosphate isomerase | 10 units |
| G-6-PDH solution | 1 ml |
| MTT solution | 1 ml |
| PMS solution | 1 ml |
| $NADP^+$ solution | 1 l |

## ODH

(octanol dehydrogenase EC 1.1.1.73)

*solution stain:*

| | |
|---|---|
| 1-octanol | 3 ml |
| ethanol | 1 ml |
| Tris-HCl buffer | 50 ml |
| $NAD^+$ solution | 1 ml |
| MTT solution | 1 ml |
| PMS solution | 1 ml |

## OPDH

(octopine dehydrogenase EC 1.5.1.15)

*agar overlay:*

| | |
|---|---|
| octopine | 10 mg |
| $MgCl_2$ solution | 1 ml |
| $NAD^+$ | 1 ml |
| MTT solution | 1 ml |
| PMS solution | 1 ml |

(note: this enzyme is only present in some molluscs)

## PEP

(peptidase)

*agar overlay:*

| | |
|---|---|
| peptide substrate* | 10 mg |
| o-dianisidine (# D 3252) | 10 mg |
| amino acid oxidase (# A 5147) | 2 mg |
| peroxidase (# P 8250) | 1 mg |

* to stain PEP-A (EC 3.4.13.11) use glycyl-L-leucine as substrate.
* to stain PEP-B (EC 3.4.11.4) use L-leucyl-glycyl-glycine.
* to stain PEP-D (EC 3.4.13.9) use L-phenylalanyl-L-proline.

## PGM

(phosphoglucomutase EC 2.7.5.1)

*agar overlay:*

| | |
|---|---|
| glucose-1-phosphate (# G 7000) | 50 mg |
| Tris-HCl buffer | 12 ml |
| $MgCl_2$ solution | 1 ml |
| G-6-PDH solution | 1 ml |
| MTT solution | 1 ml |
| PMS solution | 1 ml |
| $NADP^+$ solution | 1 ml |

## SOD

(superoxide dismutase EC 1.15.1.1)

*agar overlay:*

| | |
|---|---|
| Tris-HCl buffer* | 12 ml |
| MTT solution | 1 ml |
| NBT solution | 1 ml |
| PMS solution | 1 ml |

* use pH 8.0 buffer for gels of pH > 8.
* use pH 9.0 buffer for gels of pH < 8.

## STRDH

(strombine dehydrogenase)

*agar overlay:*

| | |
|---|---|
| pyruvate | 4 mg |
| glycine | 113 mg |
| NADH | 10 mg |

view dark, defluorescent bands under UV light (note: this enzyme is only present in some molluscs)

# APPENDIX 5

## REAGENT SOLUTIONS FOR POLYACRYLAMIDE GEL ELECTROPHORESIS

## A. HOMOGENIZING BUFFER

Samples are prepared as for starch gel electrophoresis.

(Selander *et al.*, 1971)

| | |
|---|---|
| Tris | 12.1 g |
| EDTA ($Na_2$) | 336 mg |
| $NADP^+$ | 38 mg |

adjusted to pH 7.0 with HCl
(Store at 5°C)

## B. ELECTROPHORESIS BUFFERS

(Store at 5°C)

### *ACRYLAMIDE STOCK*

| | |
|---|---|
| acrylamide (Kodak) | 160 g |
| BIS (Kodak) | 4 g |

dissolve in distilled water to approximately 475 ml

(treat for 60 minutes with 5 g Amberlite MB-1 resin and filter solution to remove Amberlite, see Pollitt and Bell, 1980)

distilled water to a final volume of 500 ml

### *AMMONIUM PERSULPHATE*

| | |
|---|---|
| ammonium persulphate (Kodak) | 150 mg |
| distilled water to a final volume of | 100 ml |

(prepare fresh stock weekly)

### *GEL BUFFER STOCK*

| | |
|---|---|
| LiOH ($H_2O$) | 252 mg |
| boric acid | 2.38 g |
| Tris | 11.16 g |
| citric acid monohydrate | 2.88 g |
| TEMED (Sigma) | 1.5 ml |
| $\beta$-mercaptoethanol | 0.75 ml |
| distilled water to a final volume of | 500 ml |

### *ELECTRODE BUFFER*

LIOH Stock A (undiluted) as for starch gels.

| | |
|---|---|
| LiOH.($H_2O$) | 1.26 g |
| boric acid | 11.9 g |

pH 8.15

## C. STAINING SOLUTIONS

### *COOMASSIE BLUE STAINING SOLUTION*

| | |
|---|---|
| Coomassie blue R-250* (Sigma) stock (1% in $H_2O$) | 4 ml |
| glacial acetic acid | 15 ml |
| methanol | 60 ml |
| distilled water | 75 ml |

* Serva violett #49 (Serva) can be used in place of Coomassie blue

### *SILVER STAIN SOLUTIONS*

**Fixing Solution**

| | |
|---|---|
| methanol | 500 ml |
| acetic acid | 120 ml |
| distilled water to a final volume of | 1000 ml |

**Silver Nitrate Solution**

| | |
|---|---|
| silver nitrate | 2.04 g |
| distilled water to final volume | 1000 ml |

**Developer**

| | |
|---|---|
| sodium carbonate | 29.68 g |
| formalin | 0.5 ml |
| distilled water to a final volume of | 1000 ml |

# APPENDIX 6

## ELECTROPHORETIC MOBILITIES OF TEN ENZYMES ON STARCH GELS[1]

| No. | Scientific Name | Enzyme | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | AK | CK-A | GPI-A | GPI-B | G-3-PDH | PGM | IDH | LDH-A | LDH-B | PEP-B |
| 1 | *Orectolobus maculatus* | 85 | 165 | NA | 160 | 195 | 105 | | -50 | 195 | |
| 2 | *Galeus boardmani* | 5 | 100 | NA | 20 | 30 | 40 | | -105 | 265 | 215 |
| 3 | *Carcharhinus amblyrhynchoides* | 0 | 30 | NA | -80 | -40 | 60 | 15 | -80 | | |
| 4 | *C. amboinensis* | 0 | 30 | NA | 5 | -40 | 60 (40) | 15 | -80 | 285 | |
| 5 | *C. brevipinna* | 0 | -20 | NA | -90 | -40 | 60 | 15 | -100 | 285 | |
| 6 | *C. cautus* | 0 | 30 | NA | -40 | -40 | 60 | 10 | -105 | 300 | |
| 7 | *C. dussumieri* | 0 | -15 | NA | -115 | -40 | 60 | | -65 | | |
| 8 | *C. fitzroyensis* | 0 | 30 | NA | -65 | -40 | 60 | 15 | -95 | 285 | |
| 9 | *C. limbatus*[2] | 0 | 30 | NA | -80 | -40 | 60 (40) | 15 | -80 | 220 | |
| 10 | *C. "limbatus"* [2] | 0 | 30 | NA | -80 | -40 | 60 (40) | 15 | -80 | 220 | |
| 11 | *C. macloti* | 0 | | NA | -80 | -20 | 60 | 15 | -85 | 280 | |
| 12 | *C. melanopterus* | 0 | 35 | NA | -35 | -40 | 60 | 15 | -110 | 285 | |
| 13 | *C. sorrah* | -25 | -20 | NA | -60 | | 60 | 15 | -80 | 280 | |
| 14 | *Galeorhinus australis* | 35 | -25 | NA | -50 | -65 | 30 | | -90 | 195 | 185 |
| 15 | *Rhizoprionodon taylori* | 0 | -20 | NA | -95 | -55 | 60 (40) | 15 | -75 | 280 | |
| 16 | *Centroscymnus crepidator* | 5 | 220 | NA | 80/35 | -10 | 80 | 15 | -5 | 185 | 95 |
| 17 | *C. owstoni* | 30 | 170 | NA | 120 | -10 | 80 | | -5 | | 175 |
| 18 | *Deania calcea* | 35 | 220 | NA | 30 | 120/50 | 60 | 65 | 75 | | 80 |
| 19 | *Etmopterus baxteri* | 0 | 130 | NA | 105 | 30 | 100 | 70 | -15 | | 135 |
| 20 | *E. lucifer* | 5 | 100 | NA | 135 | 125 | 85 | 70 | -5 | 190 | |
| 21 | *Squalus blainvillei* | -20 | 20 | NA | 55 | -55 | 85 | | -55 | | 130 |
| 22 | *S. megalops* | -20 | 15 | NA | 10 | -60 | 60 | 45 | -50 | | 130 |
| 23 | *Squatina tergocellata* | 70 | 5 | NA | -60 | -55 | 65 | | -55 | | |
| 24 | *Aptychotrema rostrata* | 10 | 70 | NA | -45 | -65 | 70 | | -50 | 195 | 45 |
| 25 | *Bathyraja sp.* | 40 | 190 | NA | 195 | 245 | 85 | -5 | 0 | | 325 |
| 26 | *Raja cerva* | 20 | 55 | NA | 60 | 45 | 80 | 15 | 10 | | |
| 27 | *Rajidae sp.* | 15 | 105 | NA | 110/75 | 15 | 85 | | 15 | | |
| 28 | *Urolophus testaceus* | -5 | 90 | NA | -75 | -40 | 40 | | -60 | 135 | 155 |
| 29 | *U. viridus* | 20 | 20 | NA | | -35 | 55 | | -65 | 70 | 75 |
| 30 | *Hydrolagus ogilbyi* | -55 | 140 | NA | 90 | 80 | 265 | | 120 | 150 | 160 |
| 31 | *Decapterus russelli* | 10 | -35 | 125 | -135 | -105 | -50 | 40 | -5 | 120 | 250 |
| 32 | *Elops hawaiiensis* | 5 | 30 | 85 | -125 | -175 | 55 | 40 | -10 | 225 | 170 |
| 33 | *Megalops cyprinoides* | 40 | 65 | 75 | 60 | -135 | 85 | 75/30 | 15 | 225 | 175 |
| 34 | *Albula glossodonta* | 10 | | 125 | -105 | -140 | 10 | | 5 | 180 | 180 |
| 35 | *A. neoguinaica* | 15 | | | | -160 | 0 | 45 | -10 | 180 | 130 |
| 36 | *Sardinella gibbosa* | | | | | | -25 | | -15 | 285 | 90 |
| 37 | *Saurida tumbil* | 40 | 20 | 130 | -15/10 | -80 | 50 | 100 | 105 | | 125 |
| 38 | *Chlorophthalmus nigripinnis* | 5 | 25 | 140/120 | 15 | -70 | 70 | 110 | 90 | | 240 |

Cont'd..

| No. | Scientific Name | Enzyme | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | AK | CK-A | GPI-A | GPI-B | G-3-PDH | PGM | IDH | LDH-A | LDH-B | PEP-B |
| 39 | *Arius sp.* | 55 | 50 | 95 | -100 | 155 | -10 | 55 | 60 | | 60 |
| 40 | *Euclichthys polynemus* | | 85 | 145 | 65 | 220 | 125 | 105 | 45 | 210 | 160 |
| 41 | *Halagyreus johnsoni* | 35 | 210 | 105 | (140) 105 | 220 155 | 180 | 90 | 125 | 180 | 240 |
| 42 | *Mora moro* | 35 | 175 | 105 | 70 | | 165 | 90 | 60 | 190 | 215 |
| 43 | *Tripterophycis gilchristi* | 35 | 60 | 100 | 100 | | 100 | | 40 | 210/ 130 | 230 |
| 44 | *Macruronus novazelandiae* | 35 | 75 | 140 | -15 | 200 | 130 | 95 | -10 | 300 | 210 |
| 45 | *Genypterus blacodes* | 95 | 70 | 115 | 60(25) | 245 | 160 | 70 | 130 | 245 | 145 (120) |
| 46 | *Cetonurus globiceps* | 50 | 65 | 85 | 20 | 225 | 240 | 75 | -30 | 210 | |
| 47 | *Coelorhynchus australis* | 50 | 85 | 85 | -30 | 220 | 85(80) | 150 | -25 | 190 | 190 |
| 48 | *C. fasciatus* | 50 | 85 | 110/95 | -35 | 190 | 120 | 130 | -25 | 210 | 175 |
| 49 | *Coryphaenoides serrulatus* | 50 | 175 | | 60 | | 160 | | 125 | | 230 |
| 50 | *Gadomus sp. A* | 75 | 110 | 85 | 15 | | 200 | | 30 | 210 | |
| 51 | *Lepidorhynchus denticulatus* | 50 | 70 | 115 | 30 | 215 | 240 (220) | 115 | 10 | 195 | 185 |
| 52 | *Ventrifossa nigromaculata* | 45 | 10 | 135 | | | | 95 | 0 | 210 | 245 |
| 53 | *Macrourus carinatus* | 50 | 75 | 105 | -20 | 215 | 255 240 185 155 | 135 | -30 | 260 | 150 |
| 54 | *Hyporhamphus ardelio* | 60 | 45 | 120 | -55 | -5 | 30 | 100 | 25 | 125 | 130 |
| 55 | *H. quoyi* | 20 | 15 | 155 | -90 | 30 | 5 | 100 | 20 | 125 | 165 |
| 56 | *Hoplostethus atlanticus* | 45 | 105 | 140 (80) | 5(40) | | (155) 120 | | 140 | | 310 |
| 57 | *Beryx splendens* | 50 | 35 | 90 | -40 | 55 | 95 | 105 | 155 | | 200 |
| 58 | *Centroberyx affinis* | 60 | 110 | 120 | -85 | 70 | 105 | 95 | 55 | | 245 |
| 59 | *Cyttus australis* | | | 75 | -80 | | 55 | | -30 | | 205 |
| 60 | *C. traversi* | 45 | | 80 | -65 | 95 | 110 | 75 | 65 | | 260 |
| 61 | *Zenopsis nebulosus* | 10 | | 65 | -100 | -20 | 75 | | 15 | | 230 |
| 62 | *Allocyttus verrucosus* | 45 | 155 (130) | | (255) 215 | | (185) 165 | 90 | 105 | 200 | 285 |
| 63 | *Neocyttus rhomboidalis* | 40 | 120 | | (170) 135 (115) | 80 | 155 | | 100 | 200 | 275 |
| 64 | *Pseudocyttus maculatus* | 45 | 105 | | 235 | 155 | 155 (140, 125) | 90 | 100 | 195 | 280 |
| 65 | *Centriscops humerosus* | 55 | 40 | 145 | -35 | 70,40 | 80 | 45 | 90 | | 265 |
| 66 | *Helicolenus percoides* | 60 | 55 | | | 45 | (45)30 | 135 | 65 | | 190 |
| 67 | *Pterygotrigla polyomatta* | 20 | -10 | 115 | -125 | -35 | -15 | 90 | 25 | | |
| 68 | *Cymbacephalus nematophthalmus* | 65 | 110 | | -85 | 10 | 55 | 115 | 80,25 | | 195 |
| 69 | *Elates ransonneti* | 65 | 90 | 130 | -55 | 80 | 80 | 115 | 95 | | |
| 70 | *Leviprora inops* | 65 | 155 | | -80/-55 | | 120 | 115 | 40 | | 270 |
| 71 | *Platycephalus arenarius* | 65 | 80 | 115 | -100 | -20 | 25 | 115 | 25 | 190 | 195 |
| 72 | *P. sp. nov. A* | 20 | 40 | | -105 | -25 | -15 | 245 | -20 | 250 | |
| 73 | *P. sp. nov. B* | 65 | 75 | 85 | -100 | -20 | -10 | 115 | 25 | | 240 |
| 74 | *P. caeruleopunctatus* | 65 | 75 | | | -15 | -15 | 115 | 20 | 195 | 240 |
| 75 | *P. castelnaui* | 65 | 80 | | | -25 | -20 | 120 | 20 | 190 | 220 |
| 76 | *P. conatus* | 60 | 125 | 115 | -60 | 40 | 85 | 115 | 85 | 190 | 175 |

Cont'd...

| No. | Scientific Name | Enzyme | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | AK | CK-A | GPI-A | GPI-B | G-3-PDH | PGM | IDH | LDH-A | LDH-B | PEP-B |
| 77 | *P. endrachtensis* | 20 | 75 | 85 | -130 | -20 | 80 | 115 | 25 | | 140 |
| 78 | *P. fuscus* | 65 | 110 | 85 | -115 | -40 | 80 | 115 | 25 | | 135 |
| 79 | *P. indicus* | 65 | 110 | 85 | -115 | 20 | 80 | 115 | 25 | | 140 |
| 80 | *P. leavigatus* | 60 | 75 | 110 | -80 | -20 | 50 | 115 | 95 | 195 | 220 |
| 81 | *P. longispinis* | 20 | 75 | | 0 | -70 | 15 | 115 | (85)30 | 195 | 190 |
| 82 | *P. richardsoni* | 60 | 65 | | -80 | | 80 | | 85 | | |
| 83 | *Lates calcarifer* | 100 | 100 | 100 | -100 | -100 | 100 | 100 | 100 | | 100 |
| 84 | *Psammoperca waigiensis* | 100 | 110 | 175, 170 | -100 | -15 | 125 | 75 | 80 | 130 | 105 |
| 85 | *Macquaria australasica*[3] | 60 | 110 | 125 | -50 | 100 | 135 | 80 | 125 | | 215 |
| 86 | *M. australasica*[3] | 60 | 110 | 125 | -50 | 100 | 180 | 55 | 125 | | 225 |
| 87 | *M. novemaculeata* | 60 | 45 | 130 | -55,-25 | 95 | 95 | 80 | 75 | 195 | 200 |
| 88 | *Cephalopholis miniatus* | 65 | 95 | 85/75 | -70 | -30 | 105 | 70 | 150 | | 210 |
| 89 | *Epinephalus fasciatus* | 65 | 80 | 95/85 | -110 | -160 | 90 | 85 | 95 | | 190 |
| 90 | *E. malabaricus* | 60 | 100 | 85 | -55 | -25 | 100 | 95 | 95 | | 200 |
| 91 | *E. megachir* | 65 | 90 | 115 | -60 | -45 | 105 | 100 | 100 | | 180 |
| 92 | *Plectropomus laevis* | 60 | 105 | 100 | -60 | -15 | 100 | 95 | 80 | | 230 |
| 93 | *P. sp.* | 60 | 105 | 100 | -60 | -15 | 100 | 95 | 80 | | 230 |
| 94 | *P. sp.* | 60 | 105 | 100 | -60 | -15 | 100 | 95 | 80 | | 230 |
| 95 | *P. leopardus*[4] | 60 | 105 | 90 | -60 | | 100 | 95 | 80 | | |
| 96 | *Promicrops lanceolatus* | 60 | 95 | 85 | -50 | -100 | 100 | 95 | 95 | | 220/ 200 |
| 97 | *Glaucosoma scapulere* | 65 | 80 | 85 | -65 | -30 | 115 | 95 | 75 | | 175 |
| 98 | *Pelates quadrilineatus* | 60 | 40 | 115 | -100 | | 85 | 60 | 80 | | 135 |
| 99 | *Epigonus denticulatus* | 60 | 35 | 75 | 0 | 0 | 135/ 105 | 120 | 90 | 210 | 180 |
| 100 | *E. robustus* | 60 | 35 | 65 | 40(0) | 55 | 170 | 90 | 85 | | 200 |
| 101 | *E. telescopus* | 60 | 35 | | -30 | 55 | 110 | 90 | 70 | | 150 |
| 102 | *Sillago analis* | 30 | 45 | 105 | -90 | -50 | 75 | 100 | 80 | | |
| 103 | *S. ciliata* | 25 | 55 | 115 | -115 | -105 | 25 | 100 | 20 | 125 | |
| 104 | *S. maculata* | 25 | 50 | 135/ 110 | -115 | -50 | (100) 80 | 100 | 80 | | 130 |
| 105 | *Pomatomus saltatrix* | 60 | 10 | 90 | -110 | -115 | -25 | 45 | 5 | 125 | 110 |
| 106 | *Caranx bucculentus* | 25 | 35 | 135/ 115 | -105 | -10 | 10 | 85 | 25 | 195 | 190 |
| 107 | *Scomberoides lysan* | 60 | 45 | 105 | -130 | -10 | 15 | 45 | -15 | 125 | 190 |
| 108 | *Seriola dumerilii* | 5 | -25 | | | -30 | 35 | 85 | 20 | 130 | 175 |
| 109 | *Trachurus declivis* | 5 | 25 | 95 | -120 | -70 | -35 | (100), 75 | 25 | 120 | 215 |
| 110 | *Arripis trutta* | 60 | 5 | 75 | -125 | -45 | -15 | 30 | 5 | 125 | 100 |
| 111 | *Lutjanus argentimaculatus* | 65 | 75 | 120/ 105 | -85 | 165 | 35 | 80 | 100 | | 180 |
| 112 | *L. erythropterus* | 65 | 50 | 150/ 140 | -85 | 40 | 0 | 85 | 105/ 45 | | 175 |
| 113 | *L. johni* | 40 | 75 | 105 | -85 | 20 | 45 | 80 | 90 | | 175 |
| 114 | *L. lutjanus* | 65 | 20 | 115 | -60 | -15 | 35 | 80 | 95 | | 175 |
| 115 | *L. malabaricus* | 65 | 50 | 145 | -55 | 40 | 35 | 85 | 105 | | 205 |
| 116 | *L. quinquelineatus* | 65 | 45 | 110 | -60 | | 40/15 | 80 | 100 | | 155 |
| 117 | *L. rivulatus* | 65 | 105 | 140 | -60 | -20 | 35 | 80 | 95 | | 130 |
| 118 | *L. russelli* | 65 | 45 | 125 | -60 | -15 | 35 | 80 | 100/35 | | 140 |
| 119 | *L. sebae* | 65 | 85 | 140 | -55 | 60 | 35 | 85 | 150 | | 175 |

Cont'd..

| No. | Scientific Name | Enzyme | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | AK | CK-A | GPI-A | GPI-B | G-3-PDH | PGM | IDH | LDH-A | LDH-B | PEP-B |
| 120 | *L. vittus* | 65 | 20 | 140/ 125 | -60 | -15 | 35 | 80 | 95 | | 185 |
| 121 | *Pterocaesio chrysozona* | -10 | 15 | 145 | -100 | 40 | -30 | 85 | 100 | | 155 |
| 122 | *Plectorhinchus gibbosus* | 60 | 80 | 125 | -65 | -125 | 110 | 95 | 80 | 195 | 235 |
| 123 | *Pomadasys argenteus* | 60 | 25 | 160 | -105 | 25 | 80 | 120 | 75 | | 195 |
| 124 | *Lethrinus choerynchus* | 25 | 30 | | -110 | -95 | 15 | 100 | 30 | | 200 |
| 125 | *Acanthopagrus australis* | 60 | 70 | 120 | (-125), -95 | (50) -20 | 15 (-15) | 85 | 85 | | 120 |
| 126 | *A. berda* | 60 | 95 | 100 | -95 | 110 | 55 | 80 | 75 | | 145/ 120 |
| 127 | *Rhabdosargus sarba* | 20 | 80 | 135/ 115 | -40/0 | -50 | 25 | 105 | 25 | 205 | 120 |
| 128 | *Johnius diacanthus* | 60 | 105 | 130 | -105 | 30 | 80 | 90 | 95 | | 145 |
| 129 | *Argyrosomus hololepidotus* | 70 | 155 | 130 | -80 | -50 | 85 | 95 | 90 | 125 | 200 |
| 130 | *Nibea solado* | 65 | 110 | 125 | -85 | -80 | 85 | 90 | 105 | 195 | 215 |
| 131 | *Upeneus tragula* | 60 | 75 | 125 (105) | -95 | -105 | 30 | 55 | 100 | | |
| 132 | *Monodactylus argenteus* | 65 | 70 | 95 | -115 | 30 | 70 | 65 | 25 | | 160 |
| 133 | *Girella tricuspidata* | 60 | 70 | 125 | -105 | 65 | 20 | 85 | 25 | 125 | 180 |
| 134 | *Selenotoca multifasciata* | 60 | 45 | 110 | -130 | -65 | 20 | 55 | 55 | | 160 |
| 135 | *Nemadactylus macropterus* | 20 | 10 | | -105 | -95 | -45(-20) | 50 | -20 | 125 | 210 |
| 136 | *Liza dussumiera* | 60 | 25 | 145 | | 30 | 80 | 60 | 80 | | 170 |
| 137 | *Mugil cephalus* | 60 | 30 | 150 | -90 | -45 | 5 | 25 | 20 | 125 | 210/ 190 |
| 138 | *M. georgii* | 65 | 85 | 140 | | -20 | 5 | 40 | 70 | | 200 |
| 139 | *Sphyraena bleekeri* | 15 | 10 | 95 | -120 | -80 | 45 | 65 | 45 | 125 | 160 |
| 140 | *S. flavicauda* | 60 | 85 | 125 | -95 | -70 | 60 | | 35 | 130 | 185 |
| 141 | *Eleutheronema tetradactylum* | 65 | 70 | 145 | | -20 | 55 | 65 | 40 | 190 | 230 |
| 142 | *Polydactylus sheridani* | 65 | 120 | 150 | -50 | -80 | 135 | 65 | 140 | | 215 |
| 143 | *Ichthyscopus lebeck sannio* | 65 | 90 | 115 | -70 | 110 | 80 | 80 | 75 | | 280 |
| 144 | *Rexea solandri* | 35 | 15 | 100(80) | -110 | 0 | 5 | 100,60 | 5 | 115 | 205 |
| 145 | *Lepidopus caudatus* | 20 | 75 | 85 | -65 | -15 | -25 | | 25 | | 185 |
| 146 | *Acanthocybium solandri* | 5 | -20 | | | -140 | -45/ -25 | 40 | -25 | 195 | 210/ 180 |
| 147 | *Cybiosarda elegans* | 20 | -25 | 85 | -150 | -175 | -70 | | -30 | 130 | 185 |
| 148 | *Scomberoides commersonianus* | 60 | 45 | 105 | -130 | -10 | 15 | 45 | -15 | 125 | 190 |
| 149 | *Scomberomorus commerson* | 15 | 10(-20) | 100 (95) | -135 | -145 | -45 (-25) | 60 | -30 | 125 | 240 |
| 150 | *S. munroi* | 20 | -10 | 120 | -135 | -135 | -85/ -70 | 60 | -30 | 125 | 240 |
| 151 | *S. queenslandicus* | 15 | 0 | 115 | -135 | -135 | -35 | 60 | -30 | 125 | 240 |
| 152 | *S. semifasciatum* | 15 | 65 | 115/ 105 | -135 | -135 | -30 | 60 | 20 | 125 | 240 |
| 153 | *Thunnus tonggol* | -25 | 40 | 75 | -145 | -170 | -60 | 30 | -35 | 125 | 180 |
| 154 | *Xiphias gladius* | -15 | -5 | 85 | 20 | -70 | -35 | 45 | -60 | 125 | |
| 155 | *Istiophorus platypterus* | -20 | -15,-10 | 55 | -140 | -180 | -70 | 25 | -60 | 190 | |
| 156 | *Makaira indica* | -25 | -15 | 55 | -140 | -180 | -55 | 25 | -60 | 190 | |
| 157 | *M. nigricans* | -25 | -15 | 85 | -140 | -205, -180 | -70 | 25 | -60 | 190 (120) | |
| 158 | *Tetrapterus angustirostris* | -20 | -15 | 60 | -140 | -180 | -70 | 25 | -60 | 190 | |
| 159 | *T. audax* | (-45) | -15 | 55 | -140 | -205, | -70 | 25 | -60 | 260 | |

Cont'd...

| No. | Scientific Name | Enzyme | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | AK | CK-A | GPI-A | GPI-B | G-3-PDH | PGM | IDH | LDH-A | LDH-B | PEP-B |
| | | -25 | | | | -180 | (-55) | | | (190) | |
| 160 | *Hyperglyphe antarctica* | 60 | 10 | 65 | -125 | -40 | -40, -25 | 45 | 10 | 120 | 210 |
| 161 | *Seriolella punctata* | 15 | 35 | | -125 | -40(0) | -30 (-20) | 35 | -30 | 185 | 230 (205) |
| 162 | *Pseudorhombus arsius* | 25 | 90 | 90 | -75, -100 | -90 | 65 | 115 | 70 | | 215 |
| 163 | *P. jenynsii* | 65 | 55 | | -125 | -20 | (80) 65 | 100 | 65 | | 280 |
| 164 | *Azygopus pinnifasciatus* | 65 | 85 | | | 80 | 95 | 130 | 15 | | |

1 Enzyme abbreviations as in Appendix 4.
Enzymes AK, CK-A, GPI-A, GPI-B and G-3-PDH were resolved on the CAEA pH 7.0 buffer.
Enzymes PGM, IDH, LDH-A, LH-B, and PEP-B were resolved on the TC-1 buffer.

All mobilities are relative to the mobility of the common form of the homologous isozyme of barramundi (*Lates calcarifer*) except for LDH-B which is relative to the LDH-A of barramundi. Negative values indicate cathodal mobility. Multiple entries indicate heterozygous fish (e.g. 80/35) or rare alleles -- indicated by parentheses. Blanks indicate no data. NA indicates not applicable as elasmobranchs lack GPI-A. All relative mobilities have been rounded to the nearest 5%.

2 *Carcharhinus limbatus* is a relatively rare species in northern Australian waters and is probably the true *C. limbatus* (type locality = the Bahamas). *C. "limbatus"* is an abundant species in northern Australian waters; similar to, but distinct from, *C. limbatus*.

3 *"Macquaria australasica"* appears to consist of two distinct species, one occurring east of the Great Dividing Range (represented by Species No. 86) and one west of the Great Dividing Range (represented by Species No. 85).

4 *Plectropomus leopardus* appears genetically distinct from specimens of coral trout examined. All other specimens (Species numbers 92, 93 and 94) have identical enzyme mobilities and general protein patterns (Appendix 7). Because very closely related species can theoretically be difficult or impossible to distinguish on electrophoretic grounds, these data do not prove that all of these specimens are actually conspecific. However, the lack of electrophoretic differences among them is consistent with the possibility that they are simply different "forms" of the same species.

# APPENDIX 7

## RELATIVE MOBILITIES OF GENERAL PROTEINS ON POLYACRYLAMIDE GELS

| No. | Scientific Name | Museum No.[1] | Protein Relative Mobilities (Rms) |
|---|---|---|---|
| 1 | *Orectolobus maculatus* | I.25553-001 | 3 29 36 52 **98 169** |
| 2 | *Galeus boardmani* | I.25701-003 | 2 31 47 **92 128 137 148** |
| 3 | *Carcharhinus amblyrhynchoides* | | 1 22 27 32 **64 84 97** |
| 4 | *C. amboinensis* | | 1 24 **68 151** |
| 5 | *C. brevipinna* | | 1 13 35 41 47 **62 95** |
| 6 | *C. cautus* | I.25597-001 | 1 24 33 42 53 **67 99 152** |
| 7 | *C. dussumieri* | | 1 3 23 **67** |
| 8 | *C. fitzroyensis* | | 1 24 34 **98 124** |
| 9 | *C. limbatus*[2] | | 1 26 30 36 **68 99** |
| 10 | *C. "limbatus"*[2] | | 2 26 36 **68 99 130** |
| 11 | *C. macloti* | | 1 21 24 42 **99 125** |
| 12 | *C. melanopterus* | | 1 23 31 38 **63 97** |
| 13 | *C. sorrah* | | 1 8 32 38 |
| 14 | *Galeorhinus australis* | | 2 32 **62 94 141 150** |
| 15 | *Rhizoprionodon taylori* | | 1 9 19 30 39 44 **122 147** |
| 16 | *Centroscymnus crepidator* | | 3 36 59 **128 151 171 211** |
| 17 | *C. owstoni* | | 3 16 29 51 **97 125 151 211** |
| 18 | *Deania calcea* | I.25652-001 | 1 20 26 58 **99 125 151 211** |
| 19 | *Etmopterus baxteri* | | 3 16 21 50 **125 150 211** |
| 20 | *E. lucifer* | I.25688-001 | 1 16 47 49 **121** |
| 21 | *Squalus blainvillei* | I.25592-001 | 1 34 41 **59 94** |
| 22 | *S. megalops* | I.25591-003 | 1 28 45 **59 119 127** |
| 23 | *Squatina tergocellata* | I.25701-001 | 1 10 20 28 30 59 **94 129 154 172** |
| 24 | *Aptychotrema rostrata* | I.25711-001 | 1 36 **119 128** |
| 25 | *Bathyraja sp.* | | 1 37 49 **103 153** |
| 26 | *Raja cerva* | I.25591-005 | 1 17 32 **130 152 169** |
| 27 | *Rajidae sp.* | I.25712-001 | 1 27 33 42 **130 147 150** |
| 28 | *Urolophus testaceus* | I.25675-001 | 1 3 42 **111 120** |
| 29 | *U. viridus* | I.25591-001 | 1 **116 136** |
| 30 | *Hydrolagus ogilbyi* | I.25680-001 | 2 3 5 8 9 41 44 **97** |
| 31 | *Decapterus russelli* | I.25455-001 | 1 3 21 27 34 **70 124** |
| 32 | *Elops hawaiiensis* | I.25634-001 | 1 17 32 39 49 102 **108 129 162** |
| 33 | *Megalops cyprinoides* | I.25607-001 | 3 22 23 24 36 49 59 **104** |
| 34 | *Albula glossodonta* | | 1 2 3 24 **71 103 131** |
| 35 | *A. neoguinaica* | | 1 2 3 24 **71 103** |
| 36 | *Sardinella gibbosa* | I.25658-001 | 2 4 9 13 15 38 **67** |
| 37 | *Saurida tumbil* | I.25678-001 | 4 5 6 25 35 38 **71** 89 **146** 157 |
| 38 | *Chlorophthalmus nigripinnis* | I.25465-001 | 2 16 37 46 98 **108 126 196** |

Cont'd...

| No. | Scientific Name | Museum No.[1] | Protein Relative Mobilities (Rms) |
|---|---|---|---|
| 39 | *Arius sp.* | I.25662-001 | 1 2 6 30 **71 104 148** |
| 40 | *Euclichthys polynemus* | I.25555-001 | 3 36 **129 151 173** |
| 41 | *Halagyreus johnsoni* | | 1 10 27 37 55 57 **101 128 146 151** |
| 42 | *Mora moro* | | 1 3 58 **66 98 145 150** |
| 43 | *Tripterophycis gilchristi* | I.25550-001 | 3 30 37 **142 149 153** |
| 44 | *Macruronus novazelandiae* | I.25629-001 | 1 4 18 22 39 62 97 **131** |
| 45 | *Genypterus blacodes* | | 1 20 21 26 38 39 **96 164** |
| 46 | *Cetonurus globiceps* | I.25554-001 | 3 33 41 60 116 **149** 158 |
| 47 | *Coelorhynchus australis* | | 1 3 23 38 41 **126 156 165** |
| 48 | *C. fasciatus* | I.25696-001 | 3 22 39 56 **148 156 163 166 168** |
| 49 | *Coryphaenoides serrulatus* | I.25477-001 | 4 27 51 **149 164 175** |
| 50 | *Gadomus sp. A* | I.25680-001 | 4 39 **98 148 176 183** |
| 51 | *Lepidorhynchus denticulatus* | I.25546-001 | 3 21 23 25 37 45 **155 162 164** |
| 52 | *Ventrifossa nigromaculata* | I.25459-001 | 3 21 25 155 **162 166 207** |
| 53 | *Macrourus carinatus* | | 3 19 22 23 29 45 55 **124 155** |
| 54 | *Hyporhamphus ardelio* | I.25677-001 | 1 29 33 **67 102 127** |
| 55 | *H. quoyi* | I.25605-001 | 1 3 29 66 **100 127** |
| 56 | *Hoplostethus atlantius* | I.25466-001 | 1 28 48 **63 118 144** |
| 57 | *Beryx splendens* | I.25558-001 | 1 14 16 28 40 **69 99 123** |
| 58 | *Centroberyx affinis* | I.25591-001 | 1 13 21 30 44 **100** |
| 59 | *Cyttus australis* | | 1 23 28 68 **126 151** |
| 60 | *C. traversi* | I.25464-001<br>I.25646-001 | 1 5 14 31 40 69 **101 125 169** |
| 61 | *Zenopsis nebulosus* | | 1 22 26 33 **128 145 149** |
| 62 | *Allocyttus verrucosus* | I.25590-001 | 1 26 49 54 **103 147 173** |
| 63 | *Neocyttus rhomboidalis* | I.25593-001 | 1 7 19 27 49 56 69 **131 149** |
| 64 | *Pseudocyttus maculatus* | I.25463-001<br>I.25651-002 | 1 23 45 57 **120 148 213** |
| 65 | *Centriscops humerosus* | I.25648-001 | 1 3 15 18 36 43 51 **71** |
| 66 | *Helicolenus percoides* | | 1 16 23 37 49 **101 126** |
| 67 | *Pterygotrigla polyomatta* | I.25599-001<br>I.25591-002 | 1 3 29 33 35 **38** |
| 68 | *Cymbacephalus nematophthalmus* | | 2 41 48 51 **67** |
| 69 | *Elates ransonneti* | | 2 26 40 48 51 **65** |
| 70 | *Leviprora inops* | | 1 25 27 51 55 57 **68 94 123** |
| 71 | *Platycephalus arenarius* | | 2 11 32 41 45 **66 122** |
| 72 | *P. sp. nov. A* | | 1 14 34 37 40 44 46 **120** |
| 73 | *P. sp. nov. B* | I.25708-001 | 1 8 11 38 41 46 **67 124** |
| 74 | *P. caerueopunctatus* | | 1 9 40 45 49 **65 96 120** |
| 75 | *P. castelnaui* | | 1 32 38 41 45 76 **95 110 120** |
| 76 | *P. conatus* | I.25551-001 | 1 4 7 25 42 49 56 **67 94** |
| 77 | *P. endrachtensis* | | 1 5 32 **40 69 121** |
| 78 | *P. fuscus* | I.25549-001 | 1 6 29 33 43 54 **99 121** |

Cont d.

| No. | Scientific Name | Museum No.[1] | Protein Relative Mobilities (Rms) |
|---|---|---|---|
| 79 | *P. indicus* | I.25676-002 | 1 5 32 36 43 **98 120 123** |
| 80 | *P. leavigatus* | | 1 4 15 **35** 41 44 **67 93 145** |
| 81 | *P. longispinis* | | 1 3 15 **32** 40 43 **142** |
| 82 | *P. richardsoni* | | 7 15 32 38 (42) 54 **67 96 119** |
| 83 | *Lates calcarifer* | I.25594-001 | 1 4 10 14 16 27 42 44 80 106 **150** |
| | | I.25609-001 | |
| 84 | *Psammoperca waigiensis* | | 1 4 9 13 30 33 41 44 **103 127 150** |
| 85 | *Macquaria australasica*[3] | I.25543-001 | 1 20 21 28 31 34 45 69 91 **102 123** |
| | | I.25673-001 | |
| 86 | *M. australasica*[3] | I.25460-001 | 1 20 21 28 31 34 45 69 87 **102 123** |
| | | I.25460-002 | |
| | | I.25685-001 | |
| 87 | *M. novemaculeata* | I.25460-003 | 1 2 7 19 29 35 41 87 91 **100 105 126** |
| | | I.25461-001 | |
| 88 | *Cephalopholis miniatus* | | 1 20 29 34 43 48 **129** |
| 89 | *Epinephalus fasciatus* | | 1 2 28 41 **129** |
| 90 | *E. malabaricus* | I.25705-001 | 1 3 19 21 39 44 **102 112 147** 155 **163** |
| 91 | *E. megachir* | | 1 29 32 40 43 **129** |
| 92 | *Plectropomus laevis* | I.25606-001 | 1 2 13 15 18 38 42 56 71 **103 133** |
| | | I.25654-001 | |
| | | I.25703-001 | |
| 93 | *P. sp. Nov. A* | I.25587-001 | 1 2 13 15 18 38 42 56 71 **(103) 133** |
| | | I.25600-001 | |
| | | I.25601-001 | |
| | | I.25657-001 | |
| 94 | *P. leopardus* | I.25613-001 | 1 2 13 15 18 38 42 56 71 **103 133** |
| | | I.25633-001 | |
| 95 | *P. leopardus* [4] | I.25647-001 | 1 13 16 18 38 42 77 **104 130** |
| 96 | *Promicrops lanceolatus* | | 1 3 18 25 38 41 **127 138 154** |
| 97 | *Glaucosoma scapulere* | | 1 2 7 14 19 36 41 **103 126** |
| 98 | *Pelates quadrilineatus* | | 1 2 5 7 16 30 32 36 **101 104 126** |
| 99 | *Epigonus denticulatus* | I.25598-001 | 1 20 32 48 62 **118 131 150** |
| 100 | *E. robustus* | I.25458-001 | 1 6 14 18 21 30 31 46 **125 128** |
| 101 | *E. telescopus* | | 1 12 22 33 35 40 **100 131** |
| 102 | *Sillago analis* | I.25676-001 | 1 4 14 15 22 32 34 **70 105 127** |
| 103 | *S. ciliata* | I.25687-002 | 1 4 16 20 32 34 **70 105 126** |
| 104 | *S. maculata* | I.25589-001 | 1 4 14 18 22 32 34 **72 105 127** |
| 105 | *Pomatomus saltatrix* | I.25679-001 | 1 2 15 21 33 34 **38 103** |
| 106 | *Caranx bucculentus* | I.25661-001 | 1 21 28 32 35 37 **67 99 123** |
| 107 | *Scomberoides lysan* | | 1 10 15 21 32 35 **66 100** |
| 108 | *Seriola dumerilii* | | 1 10 18 21 24 **32 100** |
| 109 | *Trachurus declivis* | I.25547-001 | 1 9 13 20 31 **53 66** 96 |
| 110 | *Arripis trutta* | I.25653-001 | 1 8 13 28 |

Cont d...

| No. | Scientific Name | Museum No.[1] | Protein Relative Mobilities (Rms) |
|---|---|---|---|
| 111 | *Lutjanus argentimaculatus* | I.25478-001 | 1 5 11 18 21 38 42 **100 123** |
| 112 | *L. erythropterus* | I.25693-002 | 1 5 24 33 42 **70 97 122** |
| 113 | *L. johni* | I.25704-001 | 1 4 9 16 21 38 42 **99 126 145** |
| 114 | *L. lutjanus* | I.25697-001 | 1 5 9 22 29 42 **69 98 123** |
| 115 | *L. malabaricus* | I.25693-001 | 1 5 10 21 33 42 **70 98 124** |
| 116 | *L. quinquelineatus* | I.25544-001 | 3 4 6 14 18 30 37 **98 125** |
| 117 | *L. rivulatus* | I.25596-001 | 2 11 18 21 24 39 42 44 **99 127** |
| 118 | *L. russelli* | I.25686-001 | 1 3 19 28 33 42 **68 122 125** |
| 119 | *L. sebae* | I.25681-001 | 1 3 5 18 19 32 38 42 **121 125** |
| 120 | *L. vittus* | I.25692-001 | 1 5 8 22 29 42 **98 123** |
| 121 | *Pterocaesio chrysozona* | I.25692-002 | 1 3 18 22 27 **64 97** |
| 122 | *Plectorhinchus gibbosus* | I.25612-001 | 1 3 18 22 37 39 41 44 **91 112 125** |
| 123 | *Pomadasys argenteus* | I.25642-001 | 2 12 17 23 29 33 **94 114** |
| 124 | *Lethrinus choerynchus* | | 2 4 20 29 33 **99 126** |
| 125 | *Acanthopagrus australis* | I.25659-001 | 2 8 13 17 28 34 **93 97 122** |
| 126 | *A. berda* | I.25656-001 | 1 4 12 17 21 28 32 38 111 **123 125** |
| 127 | *Rhabdosargus sarba* | I.25660-001 | 1 13 18 28 34 **57 90 96** |
| 128 | *Johnius diacanthus* | | 2 4 14 40 42 44 47 **111 123 168** |
| 129 | *Argyrosomus hololepidotus* | I.25605-002 | 3 15 19 22 31 46 **98 147** |
| 130 | *Nibea solado* | I.25586-001 | 1 15 24 39 41 44 47 50 **130 146 150** |
| 131 | *Upeneus tragula* | I.25673-001 | 1 6 17 25 37 40 42 **(69) 100 126** |
| 132 | *Monodactylus argenteus* | I.25557-001 | 3 6 24 32 39 42 **102 124** |
| 133 | *Girella tricuspidata* | I.25676-003 | 3 20 22 **35** 38 41 43 **101** |
| 134 | *Selenotoca multifasciata* | I.25462-001 | 4 13 15 22 **34 98 125** |
| 135 | *Nemadactylus macropterus* | I.25545-001 | 23 30 33 37 **99 146** |
| | | I.25695-001 | |
| 136 | *Liza dussumiera* | I.25689-001 | 7 13 21 27 28 31 39 **68 128** |
| 137 | *Mugil cephalus* | I.25689-002 | 1 4 21 31 34 **67 102** |
| 138 | *M. georgii* | I.25552-001 | 5 14 21 24 **36** 39 41 **127** |
| 139 | *Sphyraena bleekeri* | I.25595-001 | 2 5 13 23 31 34 50 **67 73 96** |
| | | I.25682-001 | |
| 140 | *S. flavicauda* | I.25588-001 | 5 21 33 40 **99 125** |
| 141 | *Eleutheronema tetradactylum* | I.25694-001 | 2 5 19 24 33 **38** 39 68 **126** |
| 142 | *Polydactylus sheridani* | I.25655-001 | 1 23 26 30 33 39 **127** |
| 143 | *Ichthyscopus lebeck sannio* | I.25687-001 | 6 13 20 39 41 **143 145** |
| 144 | *Rexea solandri* | | 4 23 31 **33 95 99** |
| 145 | *Lepidopus caudatus* | | 1 15 19 37 40 **60 99 125** |
| 146 | *Acanthocybium solandri* | | 1 10 11 18 23 **97** |
| 147 | *Cybiosarda elegans* | I.25677-002 | 1 13 15 21 **69 100** |
| 148 | *Scomberoides commersonianus* | I.25694-002 | 1 10 15 21 32 35 **66 100** |
| 149 | *Scomberomorus commerson* | I.25632-001 | 1 13 15 22 30 **69 99** |
| 150 | *S. munroi* | I.25628-001 | 1 23 26 **36 99** |
| 151 | *S. queenslandicus* | I.25628-001 | 1 15 16 23 28 **36 100** |

Cont'd.

| No. | Scientific Name | Museum No.[1] | Protein Relative Mobilities (Rms) |
|---|---|---|---|
| 152 | *S. semifasciatum* | | 2 16 17 24 29 39 41 **71 100** |
| 153 | *Thunnus tonggol* | I.25540-001 | 1 2 14 17 22 26 29 42 |
| 154 | *Xiphias gladius* | | 10 30 61 144 |
| 155 | *Istiophorus platypterus* | | 18 30 66 105 |
| 156 | *Makaira indica* | | 18 30 66 95 |
| 157 | *M. nigricans* | | 18 23 30 83 86 99 104 |
| 158 | *Tetrapterus angustirostris* | | 18 30 79 96 122 |
| 159 | *T. audax* | | 17 29 65 95 103 |
| 160 | *Hyperglyphe antarctica* | | 2 17 19 29 30 |
| 161 | *Seriolella punctata* | | 1 17 **35** |
| 162 | *Pseudorhombus arsius* | I.25585-001<br>I.25674-001 | 1 3 4 16 28 33 41 **61 99 150** |
| 163 | *P. jenynsii* | I.25706-001 | 1 3 15 18 23 27 37 39 **97 126 150** |
| 164 | *Azygopus pinnifasciatus* | I.25696-003 | 1 2 3 25 29 40 45 **93 127** |

( ): indicates polymorphic protein.
**Bold type**: parvalbumins visible with Coomassie stain
Normal type: heat-labile proteins visible with Coomassie stain

1 Full Museum No.AMS-I._____-____
Tissue sample stored in ultrafreezer, see "Reference samples and specimens" (p.3)

2 *Carcharhinus limbatus* is a relatively rare species in northern Australian waters and is probably the true *C. limbatus* (type locality = the Bahamas). *C. "limbatus"* is an abundant species in northern Australian waters; similar to, but distinct from, *C. limbatus.*

3 *"Macquaria australasica"* appears to consist of two distinct species, one occurring east of the Great Dividing Range (represented by Species No. 86) and one west of the Great Dividing Range (represented by Species No. 85).

4 *Plectropomus leopardus* appears genetically distinct from other specimens of coral trout examined. All other specimens (Species numbers 92, 93 and 94) have identical enzyme mobilities (Appendix 6) and general protein patterns. Because very closely related species can theoretically be difficult or impossible to distinguish on electrophoretic grounds, these data do not prove that all of these specimens are actually conspecific. However, the lack of electrophoretic differences among them is consistent with the possibility that they are simply different "forms" of the same species.

**CSIRO**

**Marine Laboratories**

comprises

Division of Oceanography
Division of Fisheries Research

**HEADQUARTERS**
Castray Esplanade, Hobart, Tas
G.P.O. Box 1538, Hobart, Tas 7001,
Australia

**QUEENSLAND LABORATORY**
233 Middle Street, Cleveland, Qld
P.O. Box 120, Cleveland, Qld 4163

**WESTERN AUSTRALIAN LABORATORY**
Leach Street, Marmion, WA
P.O. Box 20, North Beach, WA 6020

ISSN 0725-4598