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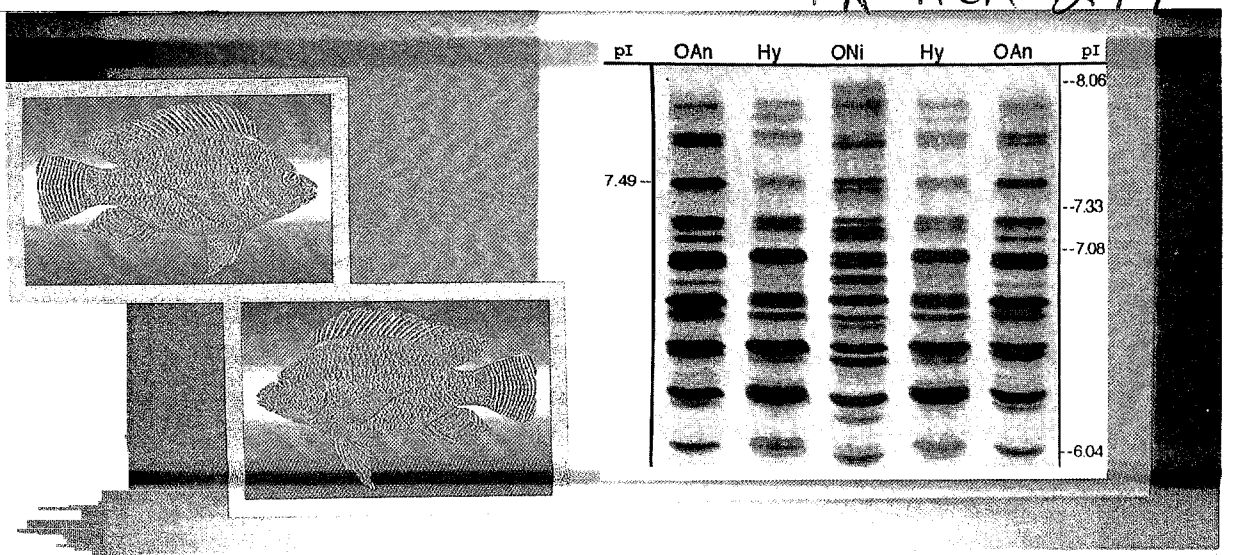

A Biochemical Laboratory Manual for Species Characterization of Some Tilapiine Fishes

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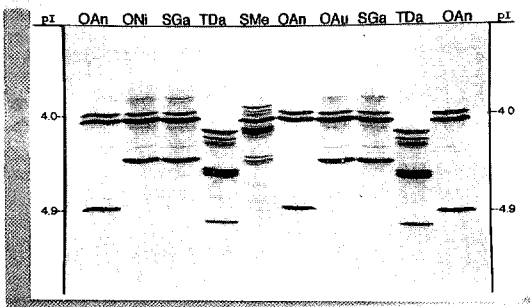

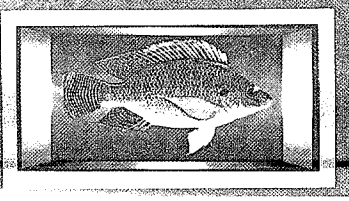

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International Center for Living Aquatic Resources Management

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Zoologisches Institut und Zoologisches Museum, Universität Hamburg

A

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Contents

List of Acronyms

AAE	agglutination assays of erythrocytes
AU PAGE	acidic urea polyacrylamide gel electrophoresis
AUT PAGE	acidic urea triton polyacrylamide gel electrophoresis
BMZ	Bundesministerium für Wirtschaftliche Zusammenarbeit und Entwicklung
BSA	bovine serum albumin
CBB	Coomassie brilliant blue
GTZ	Deutsche Gesellschaft für Technische Zusammenarbeit
IAB	Institute of Aquatic Biology
ICLARM	International Center for Living Aquatic Resources Management
IEF	isoelectric focusing
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
SGE	starch gel or allozyme electrophoresis
ZIM	Zoologisches Institut und Zoologisches Museum, Universität Hamburg

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A

Foreword

The capacity to conserve and to use natural resources wisely requires scientific understanding of the resources themselves. In the case of fish, captured from the wild or cultured, experience has long shown the necessity of identifying taxa, such as species and genera, and variation at the individual or population level. Identification and discrimination demand scientific methods whose application is demonstrated through sound empirical studies.

The present publication is both a manual of such methods for the biochemical analysis of several tissues of tilapias and an empirical demonstration of the methods for several species. Given the great and growing importance of tilapia in aquaculture and the importance of knowing the genetic composition of broodstock, the present work pays particular attention to blood-based tests in an effort to determine whether non-destructive sampling of fish tissue is feasible.

A manual such as the present publication is intended to have several impacts. First, it provides a reference and training manual for those embarking on new studies in fish genetics. Second, by guiding other studies, it is intended to lead to greater uniformity of methods and hence to a much greater ability to compare results across studies. The 1995 meeting of the Steering Committee of the International Network on Genetics in Aquaculture identified lack of comparable methods and tissues sampled as a major impediment to comparisons across studies. Third, the manual publishes results for some species by species analyses and between species comparisons, mainly taken from natural populations in Africa. Future studies may then use these results for comparisons and build on them to enable even more discrimination between tilapia species.

Meryl J. Williams
Director General, ICLARM

Preface

In Africa, tilapias are the mainstay of inland fisheries and of most inland aquaculture. In African countries that prize freshwater fish as human food (for example, Ghana), there is an urgent need to increase fish supply to match the needs of the growing population. In addition to this, African inland fisheries and aquaculture could target some of the rapidly expanding export markets for high-quality freshwater fish, particularly tilapia. Tilapia farming is expanding rapidly in the tropics and subtropics and tilapia products are traded internationally, but Africa is so far missing out on this tilapia farming boom.

One basic prerequisite to the effective management of tilapia fisheries and the expansion of tilapia aquaculture in Africa is the accurate characterization of this fish's biodiversity and genetic resources. Despite a wealth of taxonomic and other biological studies on tilapias (more than on any other group of tropical freshwater fishes), the morphological similarity among different species, their great facility for hybridizing when transferred to waterbodies outside their native habitats, and the mixed nature of many populations because of transfers for aquaculture or fisheries, mean that tilapia characterization is not easy. Identifying tilapias to species, or detecting hybrids, can pose problems, particularly in the field. Identifying population characteristic markers which are stable within each population and which are expressed by each individual of the population studied is difficult especially for the underequipped laboratories of developing countries.

It was for this reason that the Institute of Aquatic Biology (IAB), Ghana, the Zoologisches Institut und Zoologisches Museum (ZIM), Universität Hamburg, Germany, and the International Center for Living

Aquatic Resources Management (ICLARM), Manila, Philippines, began in 1991 a collaborative project to characterize the tilapia genetic resources of Ghana for their conservation and sustainable use in aquaculture and fisheries. This was aimed not only at assisting Ghana, as the project's host country, but also at devising and testing methods for tilapia characterization in general.

The overall idea was to identify taxonomically useful biochemical markers for differentiating tilapia species that are not easily distinguishable by classical morphometric and meristic characters. The majority of species studied may easily be distinguished by morphology with the exception of *Tilapia zillii, T. dageti* and *T. guineensis;* and to some extent, *Oreochromis niloticus* and *O. aureus.*

For a long period, that still continues, the main alternative to morphometric and meristic characterization has been with allozyme electrophoresis, using muscle extracts. However, this method has one main disadvantage: the specimen most often has to be killed before testing. Reflecting on this, one of us (Prof. Dr. Wolfgang Villwock) put forward the idea of using blood components as discriminatory tools for tilapia species that are used in aquaculture or might have potential for it. The advantage of using blood samples is that the donors survive and therefore may be used in further experiments, as well as for breeding purposes according to their evaluation in terms of breeding value and their genetic factors.

This idea was discussed with an experienced physiologist of ZIM, Prof. Dr. Lothar Renwrantz, who made the first concrete suggestions for transforming it into practice by application of different biochemical techniques.

Ghana and its IAB in Accra were a natural choice for partnership with ZIM and ICLARM. Ghana represents one of the few countries in the continent of origin of tilapias where undisturbed populations of fish important for aquaculture still occur.

In this manual, the ZIM group, together with Dr. Eddie K. Abban of IAB, present progress in the step-by-step series of biochemical investigations over the last five years. The results seem promising. They represent not only the successful completion of a research project but also, hopefully, contributions to the training of scientists in differentiating reliably among a number of tilapias, some of which are used for aquaculture. We also hope that these results and methods will contribute to efforts to conserve and to use tilapia genetic resources of Ghana, free from contamination by exotic species. During our studies

towards this manual, the protection of the biodiversity of Ghanaian inland waters has come to much greater prominence. We anticipate that the methods described will be of national and regional use.

Dr. Roger S.V. Pullin
Leader
Biodiversity and Genetic
Resources Program
ICLARM

Prof. Dr. Wolfgang Villwock
Zoologisches Institut
und Zoologisches
Universität Hamburg

1.0 Introduction

1.1 Background

The entry into force of the International Convention on Biological Diversity, in December 1993, has put the assessment of natural resources and the formulation and enactment of policies for their management and sustainable use higher on national and international agendas than ever before in the history of humankind. Almost every nation, including Ghana, has now signed and ratified this convention, thereby asserting their rights over the living resources within their borders and acknowledging their obligations to conserve and to use these sustainably for the benefit of present and future generations. The success of the convention depends upon the sustained support of concerned authorities, professional experts and the general public.

It is vital that all who work for the conservation and sustainable use of living organisms are able to describe them accurately, in terms of their identity, diversity and abundance. Legislation, resource conservation and utilization plans and supportive research, training and information activities can succeed only if all concerned use the same descriptors for living resources and have confidence in the characterization methods used. To this end, the world's experts on all living organisms, from viruses to primates, are joining in a project, "Species 2000", to create a global standard list of the names of all known species (about 1.75 million) by the year 2000 and to update the list thereafter. A global database on fishes, FishBase (Froese and Pauly 1996), is a contributor.

Most scientists regard the first significant system, describing living organisms as species and giving them a standardized nomenclature, to be that devised by Linnaeus (1758) and indeed his system remains

the basis of scientific nomenclature today. Traditional societies have also named and categorized living organisms from the earliest days of human history and the so-called "common names" of species are sometimes as good (and occasionally better) than the delineations recognized by scientists. Moreover, common names are often most of what the general public knows about living organisms. Common names are, however, impossible to standardize among communities and countries and pose severe limitations where reliable and accurate identification of an individual or a population is needed for conservation or utilization purposes or both.

Living resources, whether for present or future use, are in effect genetic resources, within the species communities and ecosystems that house them. Many of their useful or potentially useful attributes are coded for by genes. Therefore, all concerned with their conservation and use need to be able to determine their identity: at least at the species level, through the branch of science known as taxonomy or systematics, and where possible their genetic identity within species; both using standard methods and descriptors. This applies to living resources used or of potential use for agriculture, aquaculture, fisheries and forestry, medicine, the pharmaceutical industry, etc.

The living resources available for aquaculture and fisheries are mostly to be found in wild or near-wild populations. Domestication for aquaculture is centuries behind crop and livestock breeding. There is a wide array of aquatic species to be accurately identified and their populations to be characterized for evaluation of aquaculture potential. Among the finfishes, the tilapias, African fishes of the family Cichlidae, are generating high interest for aquaculture around the world. In Africa, the home of tilapias, and in the rest of the tropics and subtropics where they are widely farmed or under investigation for farming (Pullin 1996), the need to characterize and to name tilapias, consistently and accurately, is extremely important for conservationists, farmers and researchers.

As tilapia farming progresses, and farmed breeds are developed, such characterization will increasingly require descriptors based on molecular genetics. However, given the similarity in appearance of many tilapias (especially in their juvenile forms) and their high propensity to hybridize when transferred for aquaculture or fisheries purposes or when escaped from fish farms, there is an immediate need for the characterization of pure tilapia species and hybrids in

natural waters, on farms and as subjects for research and development studies.

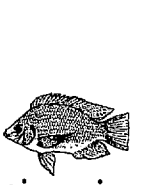
The methods for such characterization must be affordable and usable in the field and in the laboratories of the tropical and subtropical developing countries where tilapias occur in natural waters and on farms. This manual describes the development of some biochemical methods towards the goal of discriminating among some of the main tilapias of interest. Before addressing these methods, it is useful to summarize the recent history of tilapia characterization.

1.2 Biochemical approaches to discrimination and characterization of species and populations

The bulk of systematic information in fish, including tilapias, is based on measurements and counts of comparable body parts and characters (Thys van den Audenaerde 1970; Trewavas 1983; Lévêque and Paugy 1984; Teugels and Thys van den Audenaerde 1992). The theory, practice and results of this is known as *morphological systematics*. It is, however, accepted that characters upon which morphological systematics are based may be influenced by environmental conditions. Thus, measurements of the same morphological characteristics within different populations of a species can produce different results. This often presents a dilemma to systematists when determining acceptable limits of a morphological character variation within a taxonomic unit.

With the view of reducing the overall influence of environment on systematic information, biologists have borrowed approaches from protein chemists. This has mainly involved the analysis of deoxyribonucleic acid (DNA) or of the primary products (proteins) for which it codes in order to provide information on biochemical character states of individuals and populations within and among taxonomic units.

The advantages of using biochemical characters to support or to refine morphological systematics were recognized at the beginning of this century (e.g., Nuttall 1901; Bateson 1913). Since then, the major approaches used have comprised immunological and various electrophoretic techniques, peptide analysis and amino acid sequencing (Ferguson 1980). In tilapias, as in the majority of other fish groups, the most common biochemical approach used for the characterization and identification of species, natural populations and



aquaculture strains has focused on allozyme electrophoresis using starch gels (McAndrew and Majumdar 1983; 1984; Macaranas et al. 1986; Abban 1988; Seyoum 1990; Sodsuk and McAndrew 1991; Eknath et al. 1991; Macaranas et al. 1995; Pouyaud and Agnèse 1995; Rognon et al. 1996; and Agnèse et al., in press).

In addition, polyacrylamide gel electrophoresis (PAGE), isoelectric focusing (IEF) and immunological assays have been successfully applied to identify genetically controlled, nonenzymatic species characteristics, for example, in tilapia plasma samples (Avtalion et al. 1975; 1976; Avtalion 1982; and and Oberst et al. 1992; 1993); tilapia muscle extracts (Abban 1988; Focant and Vandewalle 1991; Oberst et al. 1993; Focant et al. 1994; and Oberst et al. 1996); hemoglobin and globin chain samples of tilapias (Chen and Tsuyuki 1970; Hines et al. 1971; Oberst et al. 1989; Macaranas et al. 1996; and Falk et al., in press); and on erythrocyte membranes (Oberst et al. 1988; 1989; 1996). More recently, molecular genetic techniques have been introduced in this area of tilapia research (Seyoum and Kornfield 1992b; Bardakci and Skibinski 1994; Carvalho and Pitcher 1995; Naish et al. 1995; Dinesh et al. 1996; and Agnèse et al., in press). Especially microsatellites, a class of variable numbers of tandem repeats (VNTR) loci of nDNA, are expected to become a promising tool for population genetic analyses (Wright and Bentzen 1995).

Not all of these techniques are presently applicable everywhere, especially in developing-country laboratories. However, it is in the tropical developing countries where species richness, often accompanied by morphological similarities, is greatest. Hence, in this manual, detailed procedures are described for wide application in biochemical systematics where it is most needed. These include: agglutination assays of erythrocytes (AAE), polyacrylamide gel electrophoresis (PAGE), starch gel or allozyme electrophoresis (SGE) and isoelectric focusing (IEF).

To develop these methods and to demonstrate their application as tools for species discrimination, we used tilapiine fishes, mainly from Ghana. In addition, the diagnostic test results presented in this manual should also serve as reference data on biochemical genetic markers for some tilapiine species. However, it is anticipated that these methods could be successfully applied to other fish groups.

2.0 Tilapias Studied for This Manual, with Their Collection Localities

Most of the tilapias used for these characterization studies were obtained from wild populations in Ghanaian rivers or lakes. Populations of some species not indigenous to Ghana or more widely spread (e.g., *Oreochromis aureus, O. andersonii, O. niloticus* and *Sarotherodon galilaeus*) were included in several tests to broaden comparisons. Table 2.1 lists all the tilapia samples used, the sample locations (Fig. 2.1) and the numbers investigated, with reference to the biochemical techniques applied.

Table 2.1. Tilapias (*Oreochromis, Sarotherodon* and *Tilapia* spp.) studied, location of the source (Ghana, unless otherwise stated), populations used and numbers obtained.

Species	Sample location	Number
For erythrocyte agglutination assays		
O. aureus	Israel	7
O. niloticus	Volta lake at Kpandu	>20
O. niloticus	University of Stirling, Scotland	7
S. galilaeus	Israel	7
S. galilaeus	Southern part of Volta lake	>20
S. melanotheron	Southern part of Volta lake	>20
T. dageti	Volta lake at Kpandu	>20
T. guineensis	Southern part of Volta lake	>20
T. guineensis	Densu river at Weija	>20
T. zillii	Southern part of Volta lake	>20
T. zillii	Densu river at Weija	>20
T. zillii	Black Volta at Bamboi	>20
T. zillii	White Volta at Nawuni	>20
T. zillii	Oti river at Sabari	>20
T. zillii	Pru river at Asabende	>20

continued...

Table 2.1 continued

Species	Sample location	Number
For electrophoretic analysis of blood plasma		
O. niloticus	Southern part of Volta lake	10-15
S. galilaeus	Southern part of Volta lake	10-15
S. melanotheron	Southern part of Volta system	10-15
T. guineensis	Southern part of Volta system	10-15
For electrophoretic analysis of parvalbumins		
O. andersonii	University of Stirling, Scotland	7
O. aureus	University of Stirling, Scotland	2
O. aureus	Lake Manzala, Egypt	5
O. niloticus	Southern part of Volta lake	10-15
O. niloticus	Densu river at Weija	4
S. galilaeus	Southern part of Volta lake	10-15
S. melanotheron	Southern part of Volta lake	10-15
T. busumana	Lake Bosomtwi	16
T. dageti	Volta lake at Kpandu	>20
T. discolor	Lake Bosomtwi	18
T. guineensis	Southern part of Volta lake	10-15
T. zillii	Southern part of Volta lake	>20
T. zillii	Densu river at Weija	>20
T. zillii	Black Volta at Bamboi	>20
T. zillii	White Volta at Nawuni	>20
T. zillii	Oti river at Sabari	>20
T. zillii	Pru at Asabende	>20
For hemoglobin and globin chain analysis:		
O. andersonii	University of Stirling, Scotland	10
O. aureus	Lake Manzala, Egypt	5
O. aureus	University of Stirling, Scotland	10
O. niloticus	Southern part of Volta lake	12
O. niloticus	Densu river at Weija	14
O. niloticus	Lake Manzala, Egypt	3
O. niloticus	University of Stirling, Scotland	23
S. galilaeus	Israel	7
S. galilaeus	Densu river at Weija	8
S. galilaeus	Black Volta at Busunu	4
S. melanotheron	Densu river at Weija	20
S. melanotheron	Lower part of Volta lake	12
T. busumana	Lake Bosomtwi	16
T. dageti	Volta lake at Kpandu	14
T. discolor	Lake Bosomtwi	18
T. guineensis	Volta lake at Battor	10
T. guineensis	Densu river at Weija	8
T. guineensis	Lower Volta at Sogakofe	7
T. guineensis	Layo/Abidjan, Côte d'Ivoire	8
T. zillii	Black Volta at Busunu	4

continued...

Table 2.1 continued

Species	Sample location	Number
T. zillii	Lower Volta at Battor	10
T. zillii	Densu river at Weija	12
T. zillii	Bouaké, Côte d'Ivoire	8
T. zillii	Sassandra, Côte d'Ivoire	10
Hybrids (*O. niloticus* x *O. andersonii* both directions: 10 specimens each)	University of Stirling	20
For allozyme analysis of tilapia muscle extracts		
O. niloticus	Volta lake	>20
O. niloticus	Black Volta	>20
O. niloticus	White Volta	>20
O. niloticus	Oti river at Sabari	>20
O. niloticus	Pru river at Asabende	>20
S. galilaeus	Volta lake	>20
S. galilaeus	Black Volta	>20
S. galilaeus	White Volta	>20
S. galilaeus	Oti river at Sabari	>20
S. galilaeus	Pru river at Asabende	>20
S. melanotheron	Lower part of Volta lake	>20
S. melanotheron	Densu river at Weija	>20
S. melanotheron	Okye river at Nakua	>20
S. melanotheron	Fosu lagoon at Cape Coast	>20
T. busumana	Lake Bosomtwi	>20
T. dageti	Volta lake at Kpandu	>20
T. discolor	Lake Bosomtwi	>20
T. guineensis	Densu river at Weija	>20
T. guineensis	Ankobra	>20
T. guineensis	Volta lake at Battor	>20
T. guineensis	Lower Volta at Sogakofe	>20
T. guineensis	Layo/Abidjan, Côte d'Ivoire	>20
T. zillii	Southern part of Volta lake	>20
T. zillii	Densu river at Weija	>20
T. zillii	Black Volta at Bamboi	>20
T. zillii	White Volta at Nawuni	>20
T. zillii	Oti river at Sabari	>20
T. zillii	Pru river at Asabende	>20

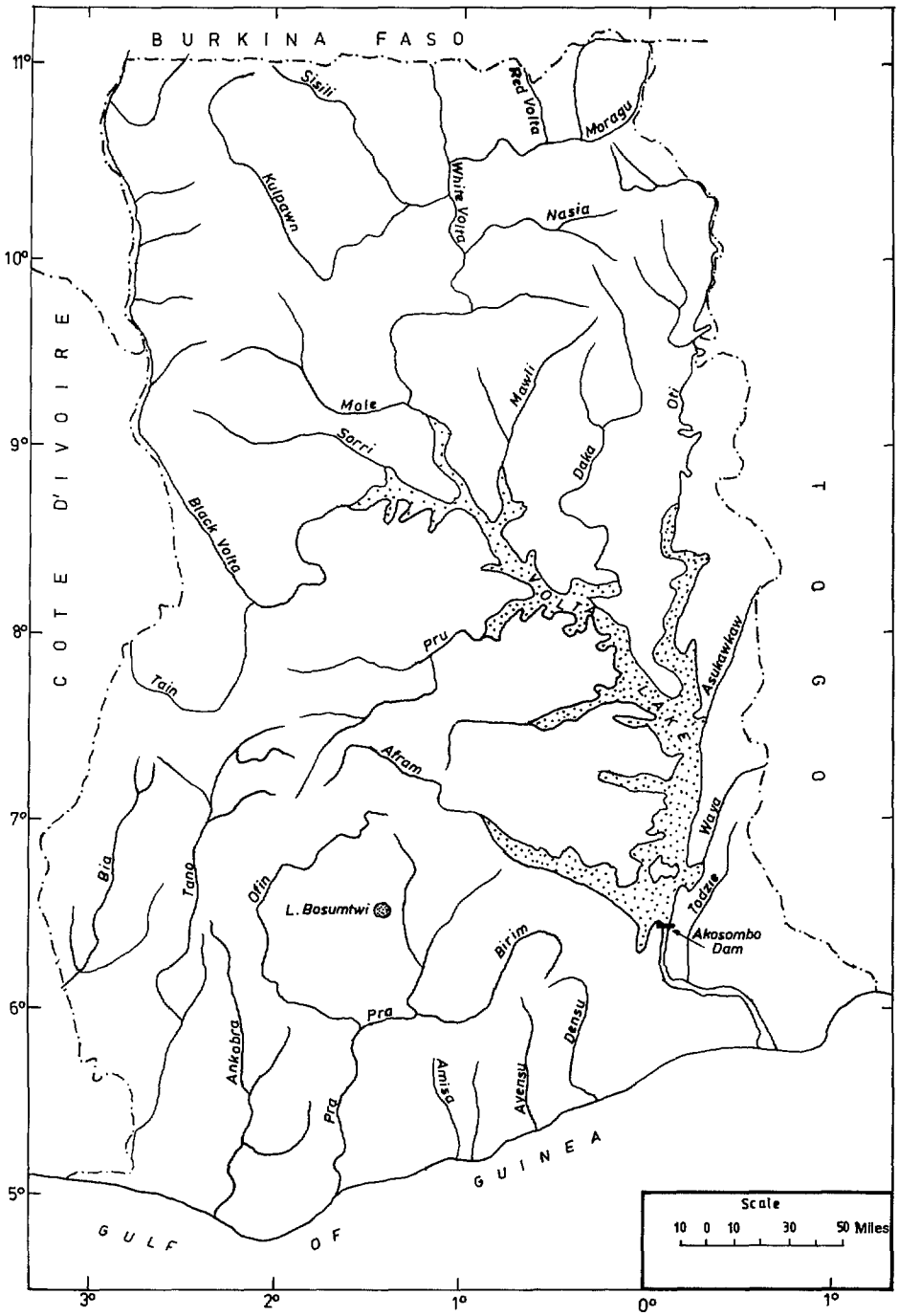


Fig. 2.1. Drainage map of Ghana.

3.0 Choice of Tissue Samples Used and Their Preparation

3.1 Background

Identification of biochemical taxonomic markers for species is based on subjecting tissue or tissue component samples of the taxa under investigation to biochemical tests or assays. The validity of biochemical tests depends greatly upon the quality of samples. Thus, this section describes the preparation of samples of tilapia tissue components used in the various biochemical tests and assays outlined in this manual. These tissue components are: blood plasma, erythrocytes, hemolysate (erythrocyte content), soluble skeletal muscle proteins, including parvalbumins (see Fig. 3.1).

3.2 Preparation of blood plasma, erythrocyte and hemolysate samples

Plasma, erythrocytes and hemolysate are all components of blood. Therefore whole blood must be obtained from the fishes under study.

3.2.1 EQUIPMENT AND REAGENTS

a. Swing-out bench top centrifuge with refrigeration (maximum, 3 000 g)
b. Refrigerated high-speed centrifuge (up to 30 000 g)
c. Eppendorf pipettes: (200-1 000 µl; 50-200 µl)
d. Magnetic stirrer and stir bars
e. Centrifuge tubes (5-10 ml)
f. Eppendorf tubes (1.5 ml)

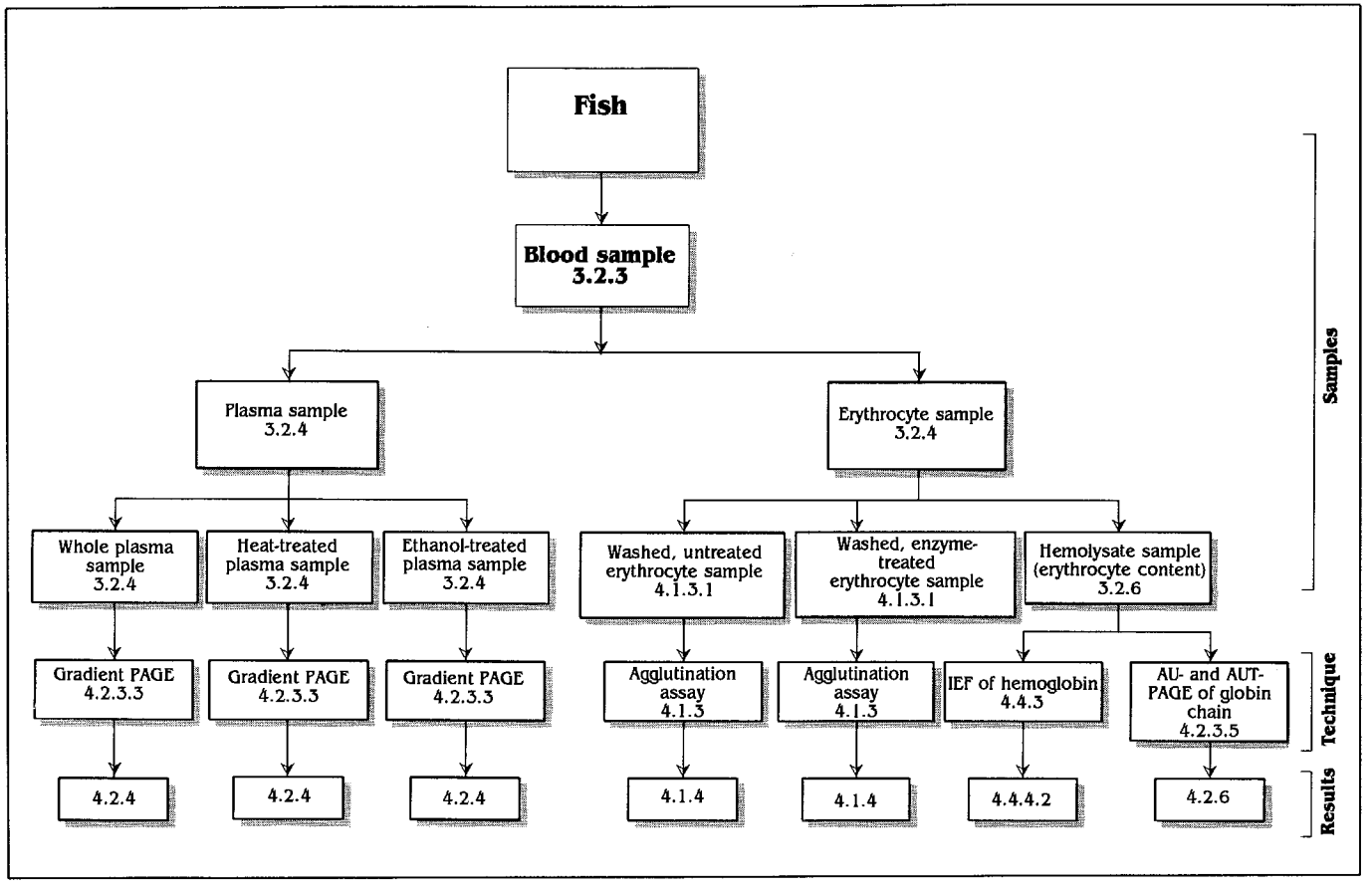


Fig. 3.1. Combined flow diagram of sample source, biochemical techniques applied and their results. This diagram summarizes the samples required for use in the various biochemical techniques described in the manual. Numbers in the diagram indicate the sections where the procedures involved as well as the results obtained have been described.

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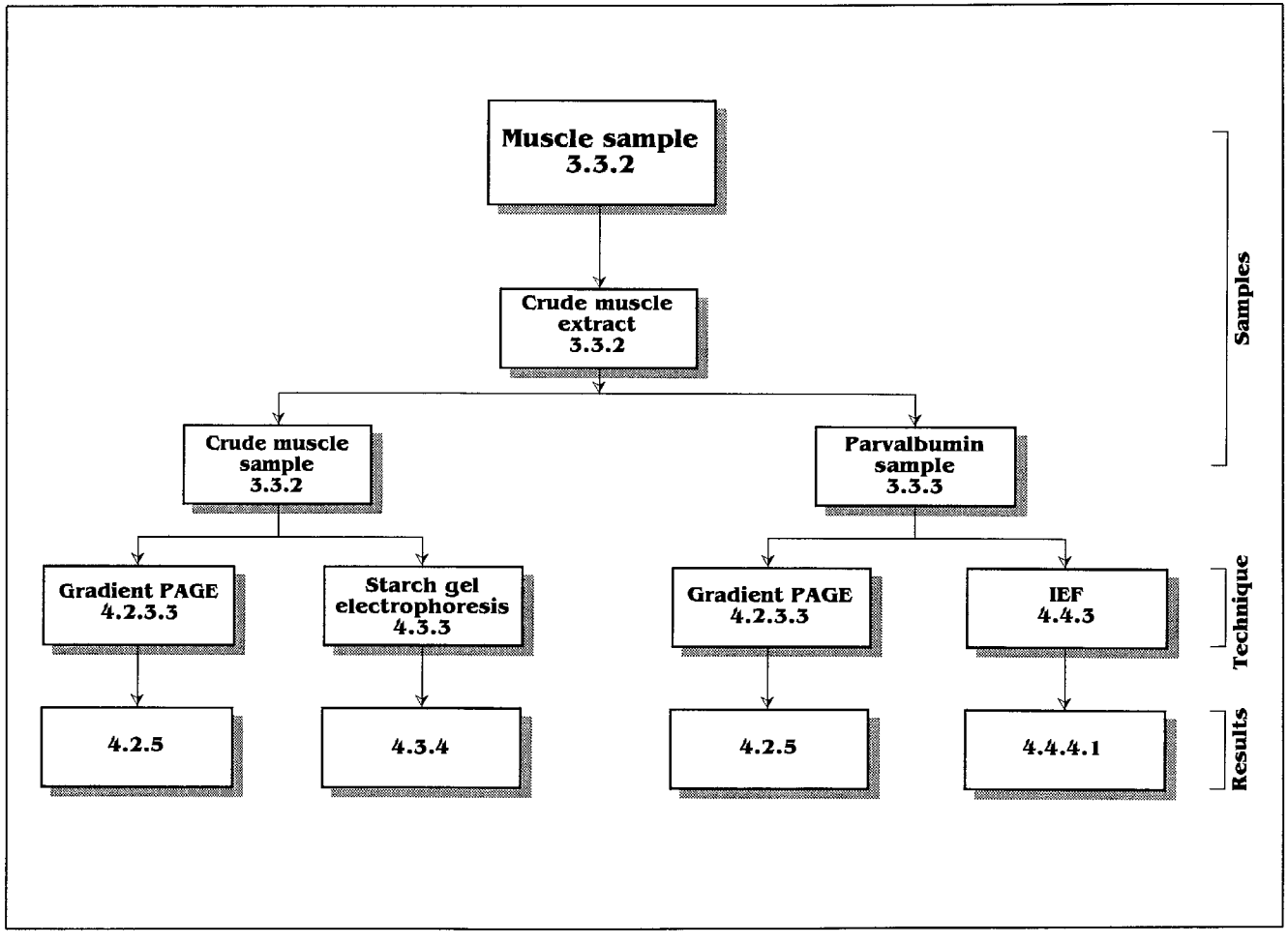
Fig. 3.1 continued



g. Syringes (5-10 ml) with fitting needles - 0.6 x 30 mm for small samples; 0.9 x 40 mm for large samples
h. Pasteur pipettes
i. Dialysis tubes, Visking 27/32, Serva, Heidelberg
j. Benzocaine (ethyl-4-aminobenzoate, Merck, Darmstadt) or other fish anesthetic
k. Acetone, Merck, Darmstadt
l. Ethanol (96%), Merck, Darmstadt
m. Glycerol (100%), Merck, Darmstadt
n. Sodium heparinate (Liquemin 20.000 IU/ml, Hoffmann la Roche)
o. Buffer salts: KH_2PO_4, Na_2HPO_4 (dihydrate), NaCl, KCl, $CaCl_2$ (dihydrate), $MgCl_2$ (hexahydrate), $MnCl_2$ (tetrahydrate), $NaHCO_3$, Merck, Darmstadt
p. Crushed ice
q. Distilled water

3.2.2 STOCK SOLUTIONS

a. Anaesthetizing solution (0.05% Benzocaine):
   i. Dissolve 10 g ethyl-4-aminobenzoate in 80 ml acetone and make up to a final volume of 100 ml with acetone.
   ii. Add 20 ml of ethyl-4-amino-benzoate solution to 3 980 ml aquarium water and mix well.

b. Phosphate buffered saline (PBS) recipe, 300 mOsm/kg, pH 7.4:

KH_2PO_4	1.815 g
Na_2HPO_4	9.496 g
NaCl	5.072 g
$MgCl_2$	0.094 g
$CaCl_2$	0.110 g
$MnCl_2$	0.125 g

Make up to a final volume of 1 000 ml with distilled water.

c. Fish Ringer solution, pH 8.0:

NaCl	0.8%
KCl	0.02%
$CaCl_2$	0.02%
$NaHCO_3$	0.02%

d. PBS buffered glycerol (80%):
   i. To 80 ml of glycerol, add 20 ml of PBS and mix well. The PBS used here does not contain $CaCl_2$, $MgCl_2$ or $MnCl_2$.

3.2.3 BLOOD SAMPLING PROCEDURE

a. Draw a little anticoagulant (for example, 50-100 µl Liquemin) into a syringe (volume: 5-10 ml).
b. Put the live fish into anaesthetizing solution.
c. Wait until the fish becomes almost immobilized (3-5 min).
d. Insert the needle into the caudal vein at the base of the anal fin: the point of insertion is approximately under the third scale behind the anal fin.
e. Draw blood slowly into the syringe until the flow stops; do not fill the syringe entirely - see h. below.
f. Remove the needle, applying tight finger pressure at the insertion point to stop bleeding.
g. Remove the needle from the syringe.
h. Draw a little air into syringe with blood.
i. Mix blood and anticoagulant in syringe gently by shaking.
j. Transfer the blood samples into appropriately sized, labelled tubes for centrifugation (3-10 ml volume).
k. Store the tubes with blood on ice.

3.2.4 SEPARATION OF PLASMA FROM BLOOD CELLS, PLASMA COLLECTION AND TREATMENT OF PLASMA SAMPLES

Although whole blood samples are relatively stable for a few days at 5°C, immediate separation of the major blood components is recommended.

a. Centrifuge blood at 750 g (about 1 800-2 000 rpm) and 4-5°C for 10 min.
b. Pipette the clear supernatant (plasma) into new centrifugation tubes leaving a thin layer above the compacted cells.
c. Centrifuge the plasma at 15 000 g and 4-5°C for 15 min.
d. Freeze the clear and colorless plasma samples and store frozen until required.

Important:

i. Reddish-colored plasma after the first centrifugation indicates some hemolysis of erythrocytes. Such plasma samples are not good to use.
ii. Slightly yellowish or whitish-colored plasma indicates the presence of high amounts of free fatty acids. These samples are acceptable.
iii. Good plasma samples may be stored frozen for a maximum of four to eight weeks.

All tissue samples used for allozyme analysis should be as fresh as possible. In this manual, allozyme electrophoretic studies using plasma are not covered.

Preparation of heat-treated plasma samples

a. Transfer aliquots (0.5 ml) of plasma into Eppendorf tubes, using Eppendorf pipettes.
b. Incubate the tubes in a water bath at 70-75°C for 15 min.
c. Transfer the tubes into iced water for about 10 min.
d. Centrifuge the samples at 8 000 g and 5°C for about 20 min.
e. Withdraw, by micropipette (50-200 µl), the clear supernatants as heat-treated plasma samples.
f. Store at -20°C until required. Samples were kept for five months at this temperature without loss of quality.

Preparation of ethanol-treated plasma samples

a. Transfer aliquots (0.5 ml) of plasma into Eppendorf tubes (volume 2.0 ml).
b. Slowly add 1.17 ml of 96% ethanol to each plasma sample under constant whirling of sample, by use of a small magnetic stirrer and small stirring bar.
c. Leave the samples for about 2 hours at room temperature.
d. Centrifuge the sample at 8 000 g and 5°C for 20 min.
e. Pipette the clear supernatants into dialysis bags. When you order the bags, you will get clear instructions on how to use them.

f. Dialyze the samples against a 10-fold volume of fish Ringer solution for about 24 hours at 5°C.
g. Transfer the dialyzate samples retained in the bags into Eppendorf tubes (1.5 ml) and store at -20°C until required. The possible storage time is probably several months.

3.2.5 ERYTHROCYTE PREPARATION

After removal of the blood plasma from centrifuged whole blood:

a. Add precooled (5°C) PBS to the compacted erythrocyte pellets in each tube: seven volumes of PBS to three volumes of pellet.
b. Mix gently to resuspend cells by shaking the tubes.
c. Centrifuge the homogeneous cell suspension at 750 g and 5°C for 10 min.
d. Carefully remove the supernatant by pipette.
e. Repeat resuspension, centrifugation and removal of the supernatant three times. The whole washing buffer volume (supernatant) as well as the thin, white, cloudy layer of cells (mainly leucocytes and platelets) on top of the red erythrocyte pellet must be carefully removed and discarded after each centrifugation step.

Important:

i. Erythrocyte samples for agglutination tests (see chapter 4.0) and for preparation of hemolysates should be further processed *almost immediately.*
ii. The erythrocyte sample preparation method given above is *not* sufficient to remove all leukocytes, but these cells are in such a minority compared to the erythrocytes that their presence can usually be neglected for purposes of the tests outlined in this manual.

3.2.6 PREPARATION OF HEMOLYSATE SAMPLES

After the third washing of erythrocytes (3.2.5.e):

a. Take one volume (50 Myl-1 ml) of pelleted erythrocytes from the bottom of tube.
b. Add four volumes of ice-cooled distilled water and mix well.
c. Centrifuge at 2 200 g (about 4 000-5 000 rpm) and 5°C for 30 min.
d. Pipette the clear supernatants (hemolysate solution) leaving the pelleted cellular debris.
e. Centrifuge the hemolysate solutions at 15 000-30 000 g and 5°C for 15 min.
f. Pipette the clear supernatants as final hemolysate samples.
g. To stabilize the samples and to prevent precipitation of hemoglobin molecules, add 1 ml of PBS buffered glycerol (80%) to each milliliter of hemolysate and mix well by use of a whirler or shaking by hand.
h. Store at -20°C until required.

Important:

i. Hemolysate samples prepared as above contain approximately 30-35 mg hemoglobin per milliliter Samples can be stored at -20°C in liquid form for a maximum of 1.5 years without precipitation or color change due to oxidation of hemoglobins.
ii. The high-speed centrifugation step (e) is not essential, but it improves sample quality for electrophoretic studies.

3.3 Preparation of soluble skeletal muscle proteins and parvalbumin samples

3.3.1 EQUIPMENT AND REAGENTS

a. Refrigerated centrifuge (maximum, 3 000 g)
b. Refrigerated high speed centrifuge (maximum, 30 000 g)
c. Dissecting kit
d. Homogenizer or ceramic pestle and mortar
e. Eppendorf pipettes: (50-200 µl) and (200-1 000 µl)
f. Centrifuge test tubes (1-5 ml)
g. Eppendorf tubes (1.5 ml)
h. Hot water bath

i. Crushed ice
j. Distilled water

3.3.2 PREPARATION OF SOLUBLE MUSCLE PROTEIN SAMPLES

a. Take a piece of white muscle (1-3 g) from fish.
b. Add an equal volume of ice-cold distilled water.
c. Homogenize mechanically or manually into a paste over ice.
d. Put the homogenized muscle samples into centrifugation tubes.
e. Centrifuge the samples at about 8 000 g and 5°C for 30 min.
f. Pipette the supernatants (water-soluble muscle proteins) into Eppendorf tubes and centrifuge at 8 000 to 30 000 g and 5°C for 15 min.
g. Transfer the second supernatants into new Eppendorf tubes.
h. Store at -20°C until required (maximum, about 2-6 months).

3.3.3 PREPARATION OF PARVALBUMIN SAMPLES

a. Transfer aliquots (0.5 ml) of the second supernatants of soluble muscle proteins in Eppendorf tubes (1.5 ml).
b. Incubate the tubes with samples in a water bath at 70-75°C for 15 min.
c. Transfer the tubes into iced water for 10 min.
d. Centrifuge the samples at 3 000 to 30 000 g at 5°C for 15-30 min.
e. Pipette the clear supernatants as parvalbumin sample solutions.
f. Store at -20°C until required (maximum storage of about 1.5 years).

Important:

High speed centrifugation (>3 000 g) (d.) is not essential, but it improves sample quality for subsequent isoelectric focusing (IEF).

4.0 Techniques in the Characterization of Tilapia Species Used in the Project Study

4.1 Erythrocyte agglutination assay

4.1.1 BACKGROUND

Agglutination of erythrocytes may basically be considered as a process of cell clumping brought about by a binding agent. However, an appropriate binding agent for erythrocytes of a species has to have very specific biochemical properties to react with specific molecules on the surface of cell membranes. Binding agents tested here were lectins but antibodies may be used to agglutinate red blood cells.

Lectins are carbohydrate binding proteins or glycoproteins with at least two binding sites for specific carbohydrates. They can be isolated from different organisms (Lis and Sharon 1973; Gold and Balding 1975; Goldstein et al. 1980; Ey and Jenkin 1982; Renwrantz 1986). Erythrocytes of vertebrates are known to have a variety of carbohydrate moieties on their membrane surfaces, the characteristics of which are genetically determined.

Therefore, the tests conducted here were aimed at characterizing species by specific carbohydrate moieties on their erythrocyte surfaces, as indicated by their positive or negative agglutination reaction with lectins. It is, however, possible for a specific carbohydrate to occur on the surface of the erythrocytes of a species and yet not be in a position accessible to an appropriate lectin. Thus, enzyme treatment of erythrocytes with N-acetyl-neuraminidase or pronase prior to agglutination tests (Uhlenbruck 1969a; 1969b; 1971) sometimes

changes the reactions of erythrocytes of a species with specific lectins. These enzyme-mediated effects are generally based on the exposure of new, otherwise hidden, sugar moieties on the surface of the erythrocyte membranes.

4.1.2 EQUIPMENT AND REAGENTS

a. Swing-out bench top centrifuge (3 000 g)
b. Water bath, 37°C
c. Microdilution (microtiter) plates with V-shaped bottoms, 96 wells
d. Eppendorf pipettes: 200-1 000 µl and 50-200 µl
e. Four-way micropipette (25 µl each) and tips, see Fig. 4.1
f. Lectins (see Table 4.1)
g. Lectin inhibiting substances (see Table 4.1)
h. N-acetyl-neuraminidase (Type V from *Clostridium perfringens*, 1.8 U/mg), Sigma, St. Louis, USA
i. Pronase (Type E, 70 000 PUK/g), Merck, Darmstadt
j. PBS (300 mOsm·kg^{-1}, pH 7.0): for recipe, see chapter 3.0, but adjust pH to 7.0 by use of 4M HCl.

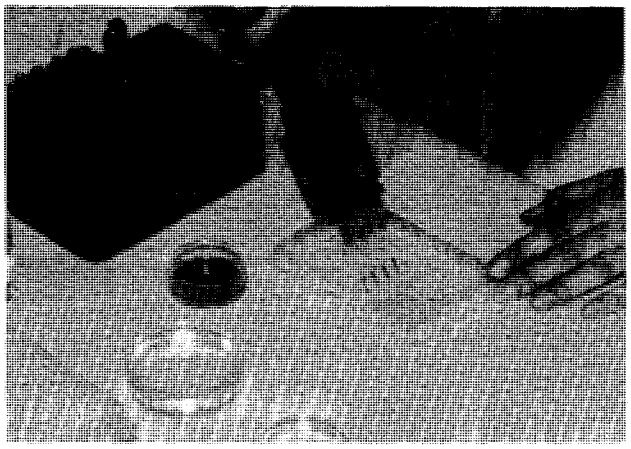
4.1.3 AGGLUTINATION ASSAY PROCEDURE

Tests described here require a Takatsy microtiter system (Kabat 1971) and standard microdilution plates with 12 columns (1-12) and eight rows (A-H). A plate allows for the comparison of the agglutination reactions of erythrocyte samples of eight different species against one lectin, eight individual samples of a species against one lectin or combinations of samples of different species and lectins. All procedures, except enzyme treatment, can be performed at room temperature.

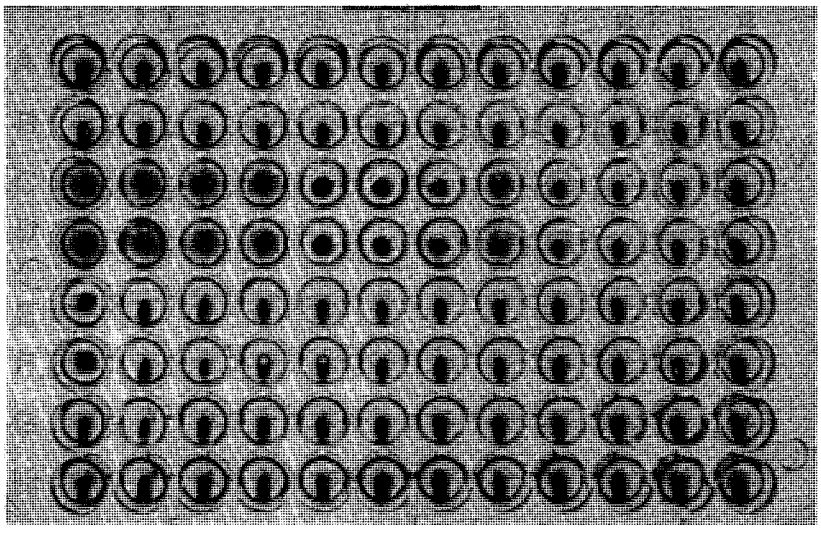
4.1.3.1 Erythrocyte Sample Preparation for Agglutination Tests

For agglutination tests outlined in this manual, three different erythrocyte suspensions are required. Their preparations are described below. Enzyme treatment of erythrocytes (nos. 2 and 3) aims at exposing hidden carbohydrate moieties.

a. Agglutination test (step 1 of section 4.1.3.3).



b. Agglutination test (step 2 of section 4.1.3.3).



c. Evaluation of agglutination reactions. Nonagglutinated cells: rows A, B, G and H. Agglutinated cells: rows C, D (up to titer 8), E and F (up to titer 1).

Fig. 4.1. Agglutination assay.

Table 4.1. Lectins tested and the concentrations of stock solutions used (modified from Oberst et al. 1988 and 1996).

Lectin	Acronym	Concentration used ($mg \cdot ml^{-1}$ PBS)	Inhibiting substance
Abrus precatorius	APA	0.020	D-galactose
Anguilla anguilla	AAA	0.250	L-fucose
Arachis hypogaea	PNA	0.313	Mucin
Canavalia ensiformis	Con A	1.250	D-mannose
Datura stramonium	DSA	0.625	Asialofetuin
Dolichos biflorus	DBA	2.500	N-acetyl-D-galactosamine
Euonymus europaeus	EEA	2.500	Fetuin
Glycine maximus	SBA	1.500	D-galactose
Helix pomatia	HPA	1.250	N-acetyl-D-galactosamine / N-acetyl-D-glucosamine
Lens culinaris	LCA	5.000	D-mannose
Limulus polyphemus	LPA	1.000	N-acetyl-neuraminic acid
Lycopersicon esculentum	LEA	1.000	Chitotriose
Phaseolus vulgaris-E	PHA-E	2.500	Asialofetuin
Phaseolus vulgaris-L	PHA-L	5.000	Asialofetuin
Phytolacca americana	PWM	0.156	Chitotriose
Pisum sativum	PSA	5.000	D-mannose
Ptilota plumosa	PPA	5.000	D-galactose
Ricinus communis-60	RCA-60	1.250	N-acetyl-D-galactosamine
Ricinus communis-120	RCA-120	1.250	D-galactose
Sambucus nigra	SNA	0.125	N-acetyl-D-galactosamine
Solanum tuberosum	STA	2.500	Chitotriose
Tetragonolobus purpurea	TPA	1.000	L-fucose
Triticum vulgaris	WGA	0.100	Chitotriose, N-acetyl-neuraminic acid
Ulex europaeus I	UEA I	0.156	Galactogen
Ulex europaeus II	UEA II	5.000	Galactogen
Vicia villosa	VVA	1.500	N-acetyl-D-galactosamine

Notes:
1. All lectins were obtained from Sigma, except *Lens culinaris* and *Pisum sativum* (both Serva). *Helix pomatia* lectin was isolated in own laboratories.
2. Inhibiting substances (sugars) were obtained from Sigma and/or Serva.

a. Untreated 3% erythrocyte suspension
   i. Take 900 µl fresh-washed erythrocyte pellets (for preparation, see 3.2.5).
   ii. Add 29.1 ml PBS (300 $mOsm \cdot kg^{-1}$, pH 7.0; see chapter 3.2.2b) and resuspend the cells (homogeneous cell suspensions are required for tests).

b. N-acetyl-neuraminidase treated 3% erythrocyte suspension
   i. Resuspend and incubate appropriate volumes of washed pelleted erythrocytes in PBS containing 0.2% neuraminidase (final concentration) for 20 min at 37°C.
   ii. Compact the erythrocytes by centrifugation (750 g, 10 min).
   iii. Remove the supernatant by pipette.

iv. Wash the cells three times with PBS (see 3.2.5.e).
v. Prepare 3% erythrocyte suspensions as above.

c. Pronase-treated 3% erythrocyte suspension
   i. Incubate appropriate volumes (1-4 ml) of freshly washed erythrocyte pellets in PBS containing 0.1% pronase (final concentration) for 10 min at 37°C.
   ii. Compact the erythrocytes by centrifugation (750 g) and remove the supernatant.
   iii. Immediately resuspend and wash the cells three times using PBS (see 3.2.5e).
   iv. Prepare 3% erythrocyte suspensions as above.

4.1.3.2 Preparation of Lectin Stock Solutions

All the lectins tested for tilapia characterization studies and their stock solutions required are listed in Table 4.1. PBS (pH 7.0) is used throughout as the dilution medium.

4.1.3.3 Practical Procedures

a. Label plate(s) according to lectin(s) and samples to be tested.
b. Pipette 25 µl of buffer into all wells of plate except those of the first column.
c. Pipette 50 µl of lectin stock solution(s) (see Table 4.1) into wells of column 1.
d. Transfer 25 µl of the contents of each well of column 1 into its corresponding second column well.
e. Mix the lectin/buffer solutions in 2nd column wells (with about 20 "stokes" of re-entry and expulsion from the pipette, leaving the contents in the wells).
f. Transfer 25 µl of wells of column 2 into the wells of column 3.
g. Repeat steps (e) and (f) along rows up to and including last well of each row. The process results in serial dilutions of lectin(s) along each row; e.g., the wells of the second column contain 1:2 lectin:buffer dilutions; the third column wells 1:4, etc., until all the wells of column 12 contain 1:2 048 dilutions) (see Fig. 4.1a).
h. Remove 25 µl from the wells of column 12 after mixing.
i. Add 25 µl of 3% erythrocyte suspensions to each well (see Fig. 4.1b).

j. Agitate the plates gently by hand shaking and leave covered for about 45 min.
k. Inspect plates for positive and/or negative agglutination by lifting each plate and tilting it slightly: agglutinated cells do not flow on a tilted plate. See Fig. 4.1c.

4.1.3.4 Verification of Specificity of Positively Reacting Lectins

The specificity of a positively reacting lectin can be verified by use of an appropriate inhibitor (see Table 4.1). The verification procedures is as follows:

a. Pipette 50 µl of the stock solution of a positively reacting lectin into a well.
b. Prepare a serial lectin dilution along a row of plate as above (4.1.3.3).
c. Add 25 µl of a 0.2 M solution of an appropriate inhibitor (e.g., sugar) to each well.
d. Allow the plate to stand (covered) for approximately 30 min.
e. Add to each well 50 µl of a 3% suspension of the erythrocytes that react positively (agglutinate) with this lectin.
f. Inspect the plate for agglutination as before.

Important:

i. The carbohydrates used for inhibition tests must be dissolved in 1:3 diluted PBS (one volume PBS/two volumes distilled water) to obtain a final osmolarity of about 300 $mOsm \cdot kg^{-1}$.
ii. Only freshly prepared erythrocyte suspensions should be used in agglutination tests and the whole procedure, including blood collection and erythrocyte sample preparation, should be performed within the same day.
iii. Prolonged pronase treatment of erythrocytes will lead to a considerable loss of red blood cells, caused by hemolysis.

4.1.4 EXAMPLES AND INTERPRETATION OF AGGLUTINATION ASSAYS

Agglutination assays with the lectins specified here (Table 4.1) have shown that many lectins react similarly with erythrocytes from different tilapia species (Tables 4.2 and 4.3). However, the erythrocyte

Table 4.2. Reactions of lectins with untreated erythrocytes of tilapias (modified from Oberst et al. 1988 and 1996): + indicates agglutination; - indicates nonagglutination.

Lectin	Species tested ONi[a]	OAu[b]	SGa[c]	SMe[d]	TZi[e]	TDa[f]	TGu[g]
Con A	-	-	-	-	-	-	-
EEA	-	-	-	-	-	-	-
HPA	-	-	-	-	-	-	-
PSA	-	-	-	-	-	-	-
TPA	-	-	-	-	-	-	-
VVA	-	-	-	-	-	-	-
LEA	+	+	+	+	+	+	+
PHA-E	+	+	+	+	+	+	+
WGA	+	+	+	+	+	+	+
LPA	+	+	+	+	-	-	-
STA	+	+	+	-	-	-	-
RCA120	-	-	-	+	+	+	+
SBA	-	-	-	-	+	-	-

[a] *Oreochromis niloticus*
[b] *O. aureus*
[c] *Sarotherodon galilaeus*
[d] *S. melanotheron*
[e] *Tilapia zillii*
[f] *T. dageti*
[g] *T. guineensis*

Table 4.3. Reactions of lectins with untreated and enzyme-treated erythrocytes: N-acetyl-neuraminidase (NANAse) or pronase (modified from Oberst et al. 1988).

	Oreochromis niloticus			*Oreochromis aureus*			*Sarotherodon galilaeus*		
Lectin	untreated	NANAse	pronase	untreated	NANAse	pronase	untreated	NANAse	pronase
APA	-	+	+	-	+	+	-	+	+
RCA 120	-	+	+	-	+	+	-	+	+
TPA	-	±	+	-	±	+	-	+	+
PNA	-	+	-	-	+	-	-	+	-
EEA	-	+	-	-	+	-	-	+	-
SBA	-	+	-	-	+	-	-	+	-
UEA II	-	+	-	-	+	-	-	+	-
RCA 60	-	+	-	-	+	-	±	+	±
Con A	-	-	±	-	-	±	-	-	±
PSA	-	-	±	-	-	+	-	-	+
PWM	-	+	-	-	+	-	-	-	-
SNA	-	+	-	-	+	-	-	-	-
VVA	-	+	-	-	+	-	-	-	-
PHA-L	-	-	+	-	-	+	-	-	-
HPA	-	-	-	-	+	-	-	-	-

agglutination reactions of some lectins, e.g., LPA and, to some extent, STA and RCA 120, with untreated erythrocytes can be used to separate *Oreochromis* and *Sarotherodon* species from *Tilapia* species. In addition, *Sarotherodon melanotheron* could be identified by a test combination of two different lectins: a positive reaction with LPA in combination with a negative agglutination reaction with STA is unique for *S. melanotheron* erythrocytes. Moreover, the positive reaction of SBA to untreated erythrocytes of *Tilapia zillii* is specific to this species among the species investigated (Table 4.2).

As indicated earlier, enzyme treatment of erythrocytes can alter their agglutination reactions with lectins. Table 4.3 compares the reactions of some lectins tested with untreated and with N-acetyl-neuraminidase (NANAse)- and pronase-treated erythrocytes of *Oreochromis niloticus, O. aureus* and *S. galilaeus.* Most of the lectins used here react negatively with untreated erythrocytes, whereas enzyme-treated cells (especially after NANAse treatment) are often agglutinated by the same lectin.

These reactions may be explained as follows. NANAse treatment removes terminal N-acetyl-neuraminic acid molecules from sugar moieties on the erythrocyte membrane. This exposes carbohydrates which, as the table suggests, could be more species-specific. As demonstrated in Table 4.3, *O. aureus* can be distinguished from *O. niloticus* and *S. galilaeus* by the reaction of their NANAse-treated red blood cells with *Helix* lectin (HPA). Also *O. niloticus* can be distinguished from *O. aureus* and *S. galilaeus* by a test combination with *Sambucus* (SNA) or *Vicia* (VVA) and *Helix* lectins after NANAse treatment of erythrocytes. Similarly, *S. galilaeus* erythrocytes can be distinguished from those of *O. niloticus* and *O. aureus* by comparative agglutination tests, using PHA-L in combination with pronase-treated cells.

Some lectins, however, showed negative agglutination reactions with both untreated and enzyme-treated erythrocytes: e.g., *Anguilla, Dolichos, Lens, Ptilota* and UEA I lectins. Some lectins reacted positively with both untreated and enzyme-treated erythrocytes: e.g., *Datura, Limulus, Lycopersicon,* PHA-E, *Solanum* and WGA.

Notes:

Polyclonal antisera, raised in tilapias by cross-immunization with untreated or enzyme-treated xenoantigenic erythrocytes (erythrocytes

of a different species were used for immunization) are also helpful in discriminating among tilapia species by agglutination assays (Oberst et al. 1989). Strong, species-specific antisera have been successfully raised against the erythrocyte surface antigens of *O. aureus, O. niloticus* and *S. galilaeus,* enabling their easy identification. This biochemical approach of species differentiation by means of erythrocyte surface component analysis was started on common carp *(Cyprinus carpio)* erythrocytes by Groth et al. 1984.

The occurrence of intraspecific blood group properties in both *O. niloticus* and *O. aureus* has been indicated by erythrocyte agglutination assays using polyclonal antisera raised against untreated alloantigenic erythrocytes (erythrocytes of different individuals of the *same* species were used for immunization) by tilapia cross-immunization (Oberst et al. 1993).

Such blood group properties may be widespread in fishes. For example, differences in the distribution of erythrocyte antigens seem to be present in the Atlantic herring (*Clupea harengus)* (Sinderman and Mairs 1959), cod (*Gadus morhua)* (Möller 1967), the common and grass carps (*Cyprinus carpio* and *Ctenopharyngodon idella)* (Balakhnin and Zraszhevskaya 1968) as well as in some elasmobranchs (Sinderman and Mairs 1961; Sinderman and Honey 1964). Further studies on fish blood types include those of Truvepper (1979a, 1979b); Tong et al. (1987); Kaastrup et al. (1989); Stet et al. (1990); and Tong and Wu (1993).

Our findings, especially concerning the antigenic sites on tilapia red blood cells that are species-characteristic, have led to a new direction in our research: the production of monoclonal antibodies raised against tilapia erythrocyte membrane components. Such work is enabling the development of an immunological field kit for identification of tilapia species and abolishing the need for costly laboratory equipment on site. Monoclonal antibody techniques are also expected to give further insights into tilapia population genetics and evolutionary genetics.

4.2 Polyacrylamide gel electrophoresis

4.2.1 BASIC CONCEPTS AND OBJECTIVES OF ELECTROPHORESIS

Electrophoresis can be explained as the movement or migration of proteins in an electric field based on their electric charge. Thus,

the general objective of subjecting proteins to electrophoresis has been to characterize samples as a result of their migration. Generally, the samples to be compared are obtained from different tissues of an organism; the same tissue of an organism at different times during its life history; the same tissue of individuals of a population; the same tissue of related organisms (e.g., species) or any combination of the above.

Proteins under investigation are usually provided a solid medium within which to move. Common media used include cellulose acetate paper, polyacrylamide gels, agarose and hydrolyzed starch gels. The electrophoretic techniques described in this manual include PAGE, SGE or allozyme electrophoresis and IEF. Like most electrophoresis procedures, the specific name for PAGE is derived from the supporting medium used (polyacrylamide). The separation of proteins in acrylamide gels is dependent on several factors, like the net charge, size and shape of the molecules and the field strength, ionic strength and pore size of the sieving medium in which they are moving. Different gel and sample preparation techniques are therefore required with reference to proteins and characteristics to be investigated. The techniques for PAGE separation of tilapia tissue components used in our studies are outlined below.

4.2.1.1 Gradient Gel Electrophoresis

Gradient PAGE, using pore size gradients of the supporting medium, separates proteins mainly on the basis of their molecular size and allows for estimation of molecular weights. For example, a decreasing pore size gradient (from top to bottom of a separation gel), created by a linearly increasing acrylamide concentration causes larger molecules to move more slowly and also to stop their migration earlier than smaller ones (supposedly at points in the medium where the pore size is smaller than the molecular size of protein). Moreover, sharp banding patterns are achieved using discontinuous gel systems which are characterized by sample collection and separation gel layers with different pH values and acrylamide concentrations.

4.2.1.2 Acidic Urea PAGE and Acidic Urea Triton PAGE

Acidic urea PAGE aims both to dissociate tetrameric hemoglobin molecules into their monomeric forms (globin chains) and to separate

dissociated chains simultaneously. These chains usually have very similar molecular weights. Globin chain separation within acidic urea gels is therefore mainly based on net charge differences. The dissociation process is achieved by the inclusion of a dissociating agent in both the sample preparation buffer and the gel solution by the presence of 8M urea, which generates high ionic pressure. Homogeneous 12% polyacrylamide gels are used as supporting media.

A slightly modified procedure, described as AUT-PAGE, is adopted for separating dissociated globin chains on the basis of differences in net charge and additionally in their hydrophobicity, achieved by application of a non-ionic detergent (triton x 100) present in collection and separation gel solutions. Using the AUT-PAGE system, neutral-to-neutral amino acid replacements among globin molecules sometimes become detectable (Alter et al. 1980; Andrews 1981; Di Luccia et al. 1991).

The following two sections outline procedures for PAGE analysis of tilapia plasma proteins, skeletal muscle parvalbumins and globin chains.

4.2.2 EQUIPMENT AND REAGENTS

4.2.2.1 Equipment

a. Vertical slab gel electrophoresis unit SE 600, Hoefer
b. Macrodrive 5 power supply, Pharmacia LKB
c. Thermostatic circulator
d. Gradient maker (2 x 20 ml reservoirs) and magnetic stir bars, Pharmacia LKB
e. Magnetic stirring stand
f. Gel casting stand and cams, Pharmacia LKB
g. Glass plates, dimensions: 160 x 180 x 4 mm, Pharmacia LKB
h. Clamps, spacers (1.5 mm thickness), combs (15 and 20 wells, 1.5 mm thickness) and silicone rubber gaskets, Pharmacia LKB
i. Deaeration flasks
j. Eppendorf pipettes: 5-40 µl and 200-1 000 µl

Important:

Equipment parts a, f, g and h are available as electrophoresis setup kit, "The SE 600 vertical slab gel unit", Hoefer.

4.2.2.2. Chemicals for Gel, Buffer and Sample Preparations

a. Acrylamide, 4x crystallized, Serva, Heidelberg
b. Bis-acrylamide, 2x crystallized (N,N', methylenebisacrylamide), Serva, Heidelberg
c. Ammonium persulfate per analysis, Merck, Darmstadt
d. Temed (Tetramethylethylendiamine) per analysis, Serva, Heidelberg
e. Urea for molecular biology use, Merck, Darmstadt
f. Acetic acid 100% per analysis, Merck, Darmstadt
g. Tris (Trihydroxymethylaminomethane) per analysis, Merck, Darmstadt
h. Glycine per analysis, Merck, Darmstadt
i. HCl Merck, Darmstadt
j. Glycerol 100% per analysis, Serva, Heidelberg
k. Bromophenol blue (Na-salt), Serva, Heidelberg
l. Methylene green (Na-salt), Serva, Heidelberg
m. 2-mercaptoethanol, Serva, Heidelberg
n. Triton X 100, extra pure, Serva, Heidelberg
o. Cello-seal compound, Pharmacia LKB
p. CHAPS (3-cholamidopropyl-dimethyl-amino-1-propanesulfonate), Serva, Heidelberg

Protein Fixation and Staining Reagents

q. Trichloro acetic acid per analysis, Merck, Darmstadt
r. Coomassie brilliant blue G-250, Serva, Heidelberg
s. Methanol per analysis, Merck, Darmstadt

Marker Proteins for Molecular Weight Determination

t. Ferritin 450 kD, Serva, Heidelberg
u. Catalase 240 kD, Serva, Heidelberg
v. Bovine serum albumin (BSA) 67 kD and 134 kD (dimer), Sigma, St. Louis
w. Ovalbumin 45 kD, Serva, Heidelberg

4.2.3 PROCEDURES

4.2.3.1 Stock Solutions for Gradient PAGE (Laemmli 1970):

All chemicals should be of highest purity and double distilled water must be used in preparing the stock solutions.

Warning: Acrylamide and bis-acrylamide are neurotoxins. Avoid inhalation and skin contact. Wear gloves.

a. Acrylamide/bis-acrylamide solution:
29.1 g acrylamide
0.9 g N,N'-methylenebisacrylamide
Dissolve in 15 ml distilled water by stirring until the solution becomes clear, and make up to 50 ml with distilled water. Gentle heating (35°C) will accelerate this step. Store in the dark at 4°C for a maximum of two weeks.

b. Ammonium persulfate, 60%:
600 mg ammonium persulfate
Dissolve and make up to a final volume of 1 ml with distilled water. Prepare fresh solution every day.

c. Separation gel buffer, pH 8.8:
23.64 g Tris
Dissolve in 70 ml distilled water and adjust pH to 8.8, adding appropriate volumes of 4N HCl. Make up to 100 ml with distilled water. Store in the dark at 4°C for a maximum of two weeks.

d. Collection gel buffer, pH 6.8:
7.88 g Tris
Dissolve in 70 ml distilled water and adjust pH to 6.8, adding appropriate volumes of 4N HCl. Make up to 100 ml with distilled water. Store in the dark at 4°C for a maximum of two weeks.

e. Electrode buffer, about pH 8.3:
15.15 g Tris
72 g glycine
Dissolve in 5 000 ml distilled water; do not adjust the pH.

f. Bromophenol blue solution 1%:
200 mg bromophenol blue Na-salt
Make up to 20 ml with distilled water.

g. Sample preparation buffer, pH 6.8:
6.25 ml collection gel buffer (d)
15 ml 66.7% glycerol
2.5 ml 1% bromophenol blue solution (f)
Make up to 50 ml, adding distilled water. Store in the dark at 4°C.

4.2.3.2 Stock Solutions for Acidic Urea and Acidic Urea Triton PAGE (Modified after Alter et al. 1980):

h. Acrylamide/bis-acrylamide solution, 60%/0.6%:
30 g acrylamide
0.3 g N,N'-methylenebisacrylamide
Dissolve in 15 ml distilled water by stirring and careful heating (35°C) within a water bath until the solution is clear. Make up to 50 ml with distilled water. Store in a dark bottle at 4°C for a maximum of two weeks.

i. Ammonium-persulfate, 60%:
600 mg ammonium persulfate
Dissolve within a final volume of 1 ml distilled water. Prepare fresh solution every day.

j. Urea solution, 12 M:
18 g urea
Make up to a final volume of 25 ml, adding distilled water. Stir until the solution is clear. Gentle heating (40°C) is recommended to accelerate this step.

k. Methylene green/glycerol solution:
300 µl 1% methylene green solution (10 mg/ml)
700 µl glycerol (100%)
Mix both volumes well and store in the dark at 4°C for a maximum of one week.

l. Electrode solution:
250 ml acetic acid
Add 4 750 ml distilled water, mix well and precool the 5% acetic acid solution (15°C).

m. Sample preparation buffer:
3.5 ml 12 M urea solution (j)
0.25 ml acetic acid (100%)
0.4 ml 2-mercaptoethanol
Make up to 5 ml by adding 0.85 ml distilled water. Use only freshly prepared sample preparation buffer.

4.2.3.3 Preparation of Samples for Gradient PAGE

All tissue or tissue component sample preparations outlined above may be considered as stock samples. For various electrophoresis procedures, further steps may be necessary to make the samples suitable for analysis. For gradient PAGE, stock samples of plasma proteins, soluble skeletal muscle proteins and parvalbumins are prepared for analysis as follows (25 µl of each sample prepared are required for an electrophoretic run using a 20 well comb):

a. Plasma samples:
   i. Dilute untreated plasma stock samples 1:10 with sample preparation buffer (g) (see 4.2.3.1).
   ii. Dilute heat-treated plasma stock samples 1:10 with sample preparation buffer (g).
   iii. Ethanol-treated plasma stock samples may be applied almost undiluted. Only add 1% CHAPS, 5% glycerol and 0.01% bromophenol blue (final concentrations in sample).
b. Skeletal muscle protein and parvalbumin samples:
   i. Dilute whole muscle protein and parvalbumin stock samples 1:5 with sample preparation buffer (g) (see 4.2.3.1). Again, 25 µl of each sample are required for subsequent separations using gels with 20 wells.

4.2.3.4 Procedures for Gradient Gel Electrophoresis

The procedures are summarized here in stages for convenience, based on information given in the *Pharmacia LKB 2001 vertical electrophoresis laboratory manual* (Pharmacia LKB 1982) and the *Hoefer instructions for vertical slab gel electrophoresis units* (Hoefer 1989). These publications offer more detailed and illustrated instructions.

4.2.3.4.1. Gel mould assembly

a. Place one glass plate flat on a clean even surface (e.g., flat table).
b. Position both spacers along both short length edges of the glass plate.
c. Cover the spacers with a second glass plate forming a sandwich of the spacers.
d. Slide clamps over the edges of the glass plates with spacers.
e. Tighten one screw on each clamp.
f. Stand the clamped glass plates on one of their long sides.
g. Loosen the screws to allow the clamps to slide down onto the table surface.
h. Align spacers to flush with the upper, lower and side edges of glass plates.
i. Gently tighten all clamp screws (Fig. 4.2a).
j. Smear a thin film of Cello-seal compound on the lower edges of gel mould (glass plates and spacers).
k. Place the assembly in the gel casting stand.
l. Insert a cam (gel stand screw) into each side of the stand and turn 180°. The gel mould is now sealed to the silicon rubber base of the stand to avoid leakage of gel solution during the next phase (Fig. 4.2e).
m. Precool the gel chamber to about 5°C, in a refrigerator.

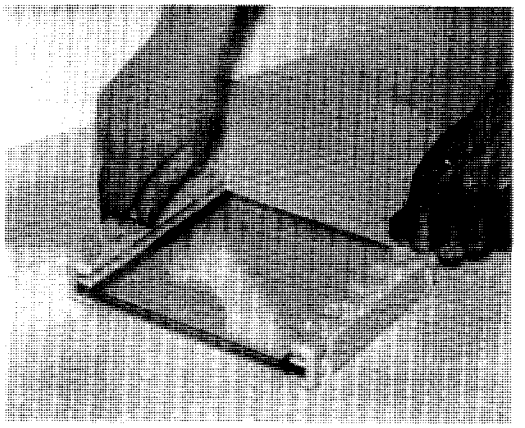
Important:

Look for white-colored spots (air bubbles) at the base of the glass plates which indicate that sealing is not sufficient. If these spots are seen, repeat the process from j above.

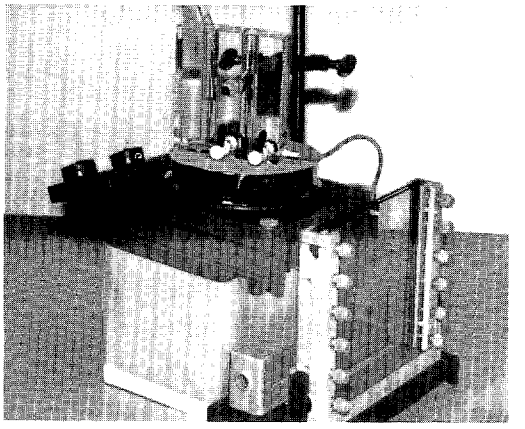
Gradient gel casting

A complete, ready-to-use gel consists of two portions: the separation gel (gradient gel) and the sample collection or loading gel (above the separation gel). The separation gel is cast and allowed to polymerize before casting the collection gel.

A linear acrylamide gradient within a separation gel is obtained by mixing equal volumes of a dense (here, 30%) and a light (here, 5%)



a.



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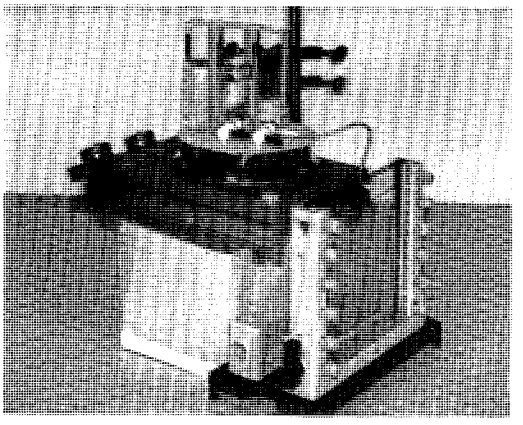

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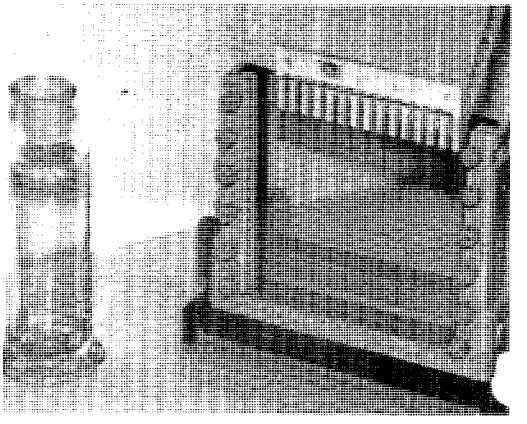
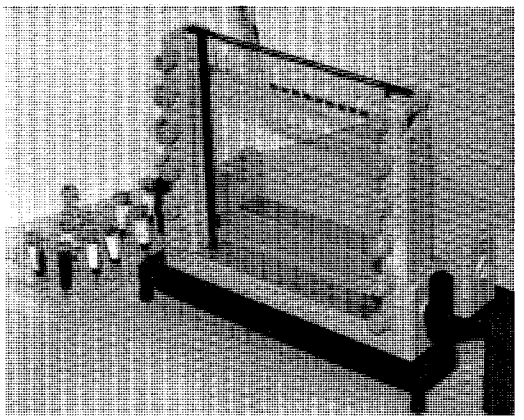


d.

Fig. 4.2. Gradient PAGE procedure (for explanation of the apparatus and steps illustrated, see sections 4.2.3.4.1-4.2.3.4.4).

continued...

Fig. 4.2 continued



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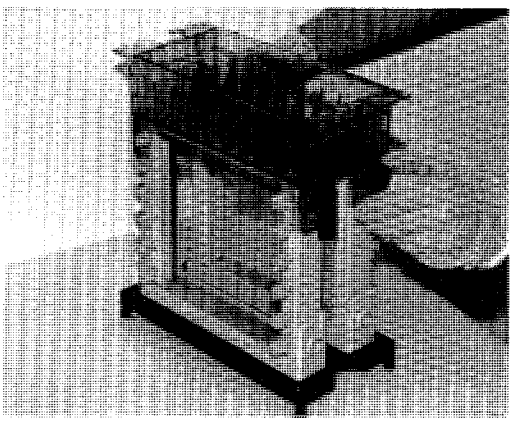

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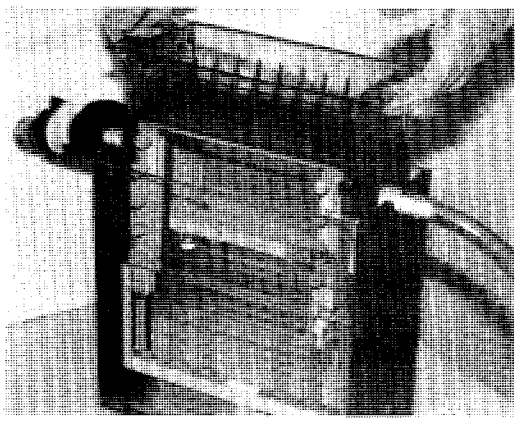

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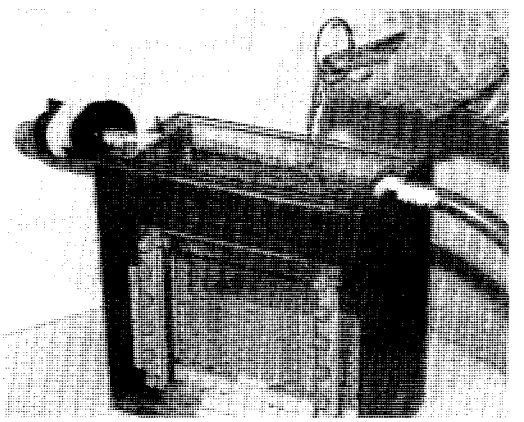

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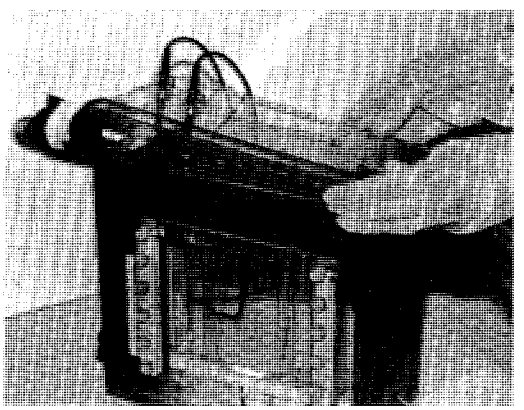


i.

acrylamide solution using a gradient mixer with two compartments, the mixing chamber nearest to the outlet and the reservoir.

4.2.3.4.2 *Preparation of the separation gel*

a. Prepare dense (30%) and light (5%) acrylamide solutions as stipulated in Table 4.4.
b. Degas solutions for 15 min and cool them to about 5°C.
c. Stand the gradient mixer with the mixing chamber (nearest to outlet) centered on a level magnetic stirring stand (Fig. 4.2b).
d. Put an appropriate magnetic stirrer into mixing chamber.
e. Place the precooled gel mould on a level surface, near and just below the level of the gradient mixer: the difference in height between the outlet of the gradient maker and the top of the gel mould should be about 5 cm (Fig. 4.2b).
f. Fit fine silicon rubber tubing (inner diameter 1.3 mm, length 8 cm) to the outlet of the gradient mixer and connect the tube to top of the gel mould centered between the glass plates (Fig. 4.2b).
g. Close both valves of the gradient maker.
h. Fill the reservoir chamber of the gradient mixer with precooled light acrylamide solution (5%), by pipette (Fig. 4.2b).
i. Open the valve of the reservoir chamber for about a second to fill the channel between both chambers.
j. Pipette excess light solution from the mixing chamber back into reservoir.
k. Fill the mixing chamber (nearest to outlet) with precooled dense acrylamide solution (30%).
l. Start the magnetic stirrer in the mixing chamber (about 250 rpm).
m. Ensure that the connecting channel is free from air bubbles.
n. Add appropriate amounts of temed and ammonium persulfate to each solution in the chambers (Table 4.4).
o. Simultaneously, open both valves of gradient maker: ensure that both solutions are flowing and mixing, and that the mixture is filling the gel mould (Fig. 4.2c).
p. After gel casting, remove and detach gradient mixer from the gel mould.

Table 4.4. Formulations for native 5-30% gradient gels (modified after Laemmli 1970). These formulations provide solutions sufficient for one gel; a-d refer to section 4.2.3.1.

Gel components	Volumes of the different gel components required		
	Collection gel	5-30% gradient separation gel	
		Light solution	Dense solution
Acrylamide/bis (a)	0.833 ml	1.25 ml	7.50 ml
Separation gel buffer(c)		3.75 ml	3.75 ml
Collection gel buffer(d)	2.500 ml		
Glycerol (60%)			3.75 ml
Distilled water	6.667 ml	10.00 ml	
Total volumes	10.000 ml	15.00 ml	15.00 ml
Temed	7.500 µl	10.00 µl	10.00 µl
Ammonium persulfate (b)	15.000 µl	20.00 µl	20.00 µl

Add temed and ammonium persulfate directly to the acrylamide solutions in the gradient maker before the gel casting starts.

q. Carefully fill up ("top off") the gel solution in the mould with distilled water (0.5 ml) by pipette.
r. Allow the gel to stand for polymerization: 1-3 hours at room temperature.
s. Rinse the gradient maker with distilled water after use.
t. The collection gel is then cast on top of the polymerized separation gel.

4.2.3.4.3 Casting of the collection gel

a. Prepare collection gel solution (Table 4.4).
b. Degas the solution for 15 min.
c. Carefully remove the water on top of separation gel by carefully pouring.
d. Insert a sample well comb (15 or 20 wells) between the glass plates of the mould (Fig. 4.2d).
e. Add appropriate amounts (Table 4.4) of temed and ammonium persulfate to collection gel solution and mix gently.
f. Using a 10 ml pipette, transfer the collection gel solution onto polymerized separation gel from one end of the mould (Fig. 4.2d).
g. Ensure complete filling of the mould without air bubbles.
h. Allow the collection gel to stand for 1-2 hours to polymerize. Gels should be used immediately.

4.2.3.4.4 Sample application (loading) and electrophoresis

a. Fill the lower tank, including the heat exchanger, with electrode buffer (e) (see section 4.2.3.1).
b. Start cooling the lower electrode buffer (10°C).
c. Carefully remove the comb from the polymerized collection gel.
d. Rinse the sample wells, created by the comb, with electrode buffer (e) (see section 4.2.3.1).
e. Repeat this rinsing of wells with buffer twice.
f. Completely fill all sample wells with electrode buffer (e) (see section 4.2.3.1).
g. Using special long pipette tips for sample loading (one per sample), transfer 25-40 µl volumes of each sample prepared into the wells as close as possible to the bottom *without the tips touching the gel* (Fig. 4.2e). Avoid air bubbles in the pipette loading tips. From the loading of the gel until the beginning of the run, all steps should be done as precisely and as fast as possible to avoid diffusion of samples in the gel.
h. Take the upper buffer tank and ensure that both silicon rubber gaskets are in place.
i. Apply a trace of Cello-seal compound to the surfaces of the rubber gaskets.
j. Mount the top tank chamber onto the gel mould (Fig. 4.2f).
k. Loosen the four screws on the gel mould stand and insert them on both sides of the upper buffer tank.
l. Turn each screw 180° (Fig. 4.2f).
m. Take the gel cassettes attached to upper buffer tank (by upper tank) and insert in the lower tank with cooled buffer (Fig. 4.2g). If only one gel is used, close the second opening of the upper buffer tank. Ensure that both electrodes (from the upper tank and the heat exchanger) are on the same side.
n. Fill the upper tank with electrode buffer (e) (see section 4.2.3.1) without disturbing the samples (Fig. 4.2h).
o. Fit the lid of the electrophoresis unit onto the upper tank (Fig. 4.2i).

p. Connect the electrodes of electrophoresis unit correctly and firmly to the power supply unit: positive electrode at the bottom of the gel.
q. Start the gel run under the following conditions: voltage set to 350 V, milliamperage to 30 mA/gel and power to 100 W.
r. Continue the run for 24 hours at 10 °C. Starting voltage should be between 150 and 180 V; voltage will increase during the run (upto 350 V).

4.2.3.5 Sample preparation for acidic urea and acidic urea triton PAGE

Use hemolysate solutions as stock samples (see section 3.2.6).

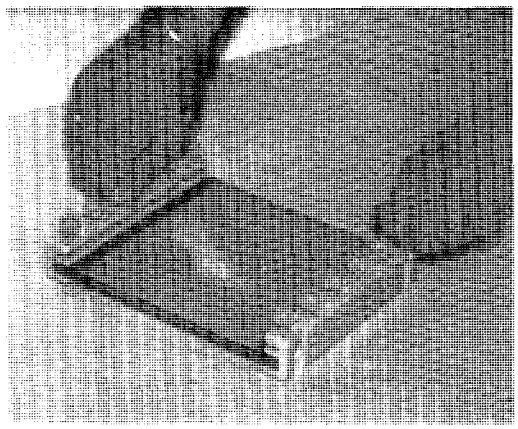
a. Dilute the hemolysate solution 1:10 with sample preparation buffer (m) (see section 4.2.3.2).
b. Incubate the diluted samples for 15 min at 5°C.
c. To 100 µl of incubated sample, add 40 µl of methylene green/ glycerol solution (k) (see section 4.2.3.2) and mix well.
d. For a run using gels with 15 wells, 5-8 µl of sample are required.

4.2.3.6 Procedure for Acidic Urea and Acidic Urea Triton PAGE

The major operational procedures are as outlined above (section 4.2.3.3). The stock solutions (see section 4.2.3.2), the gel and buffer compositions are modified from those given by Alter et al. (1980).

Gel casting

a. Prepare the separation and collection gel solutions (Table 4.5).
b. Degas both solutions for 15 min.
c. Assemble the gel mould(s) (*without* pre-cooling) (Figs. 4.3a-b).
d. Add appropriate volumes of temed and ammonium persulfate to the separation gel solution (Table 4.5).
e. Mix gently and immediately start casting the gel using a 10 ml pipette to transfer the gel solution (Fig. 4.3b).
f. Carefully overlay the solution in the mould with 0.5 ml distilled water (Fig. 4.3c).
g. Allow at least 1 hour for polymerization.



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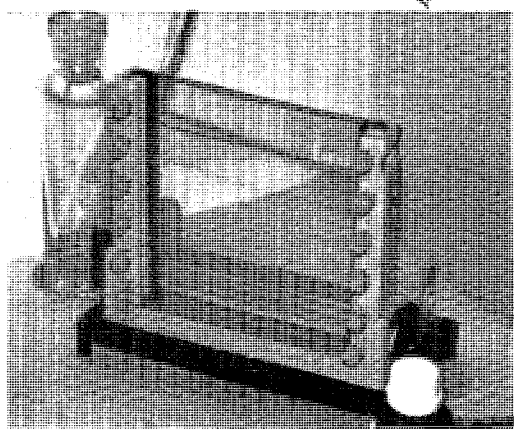

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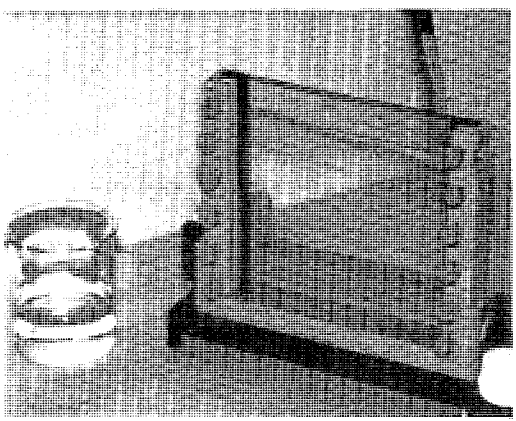

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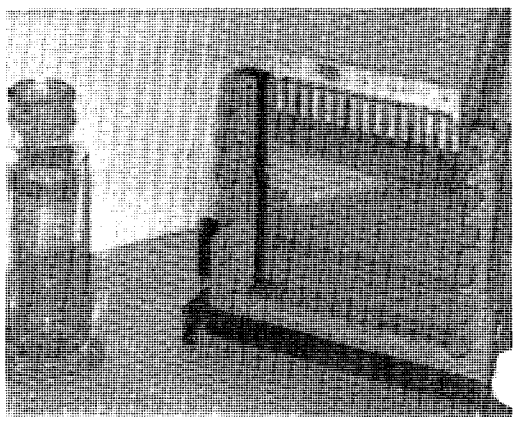

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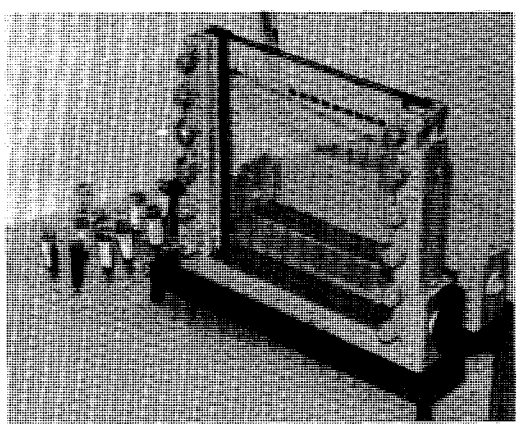

e.

Fig. 4.3. AU and AUT-PAGE procedures (for explanation of the apparatus and steps illustrated, see section 4.2.3.6).

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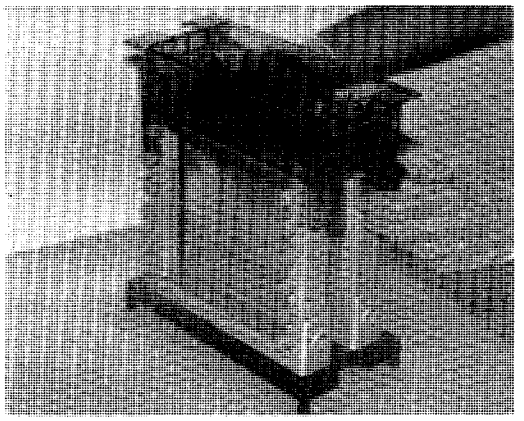
Fig. 4.3 continued



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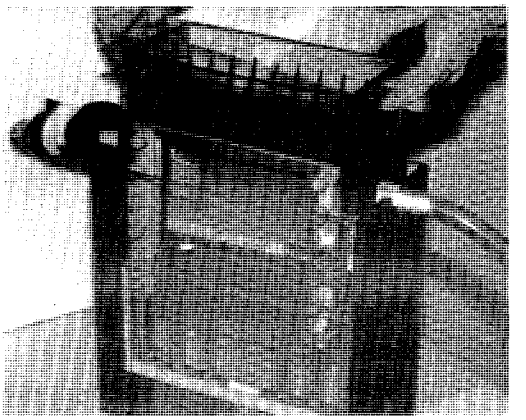

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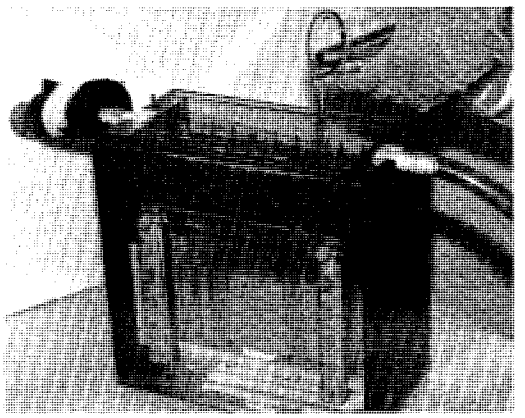

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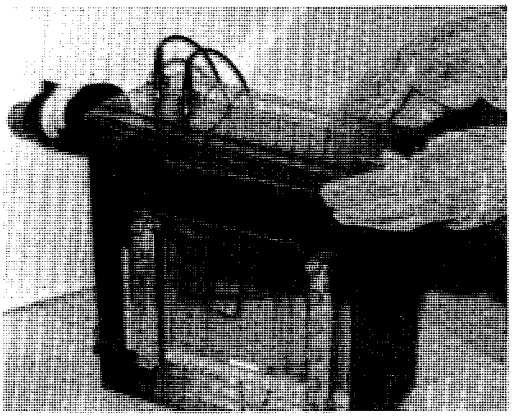


i.

Table 4.5. Formulations for acidic 8 M urea 12% polyacrylamide gels; modified after Alter et al. (1980). These formulations provide solutions sufficient for one gel; h-j refer to section 4.2.3.2.

Gel components	Volumes of the different gel components required	
	3.8% collection gel	12% separation gel
Acrylamide/bis (60%, 0.6%) (h)	0.95 ml	6.00 ml
Urea solution 12 M (j)	11.0 ml	20.0 ml
Acetic acid (glacial)	0.75 ml	2.50 ml
Distilled water	2.30 ml	1.50 ml
Distilled water/triton x 100[a]	*2.0 ml + 0.3 ml Triton*	*0.9 ml + 0.6 ml Triton*
Total volumes	15.0 ml	30.0 ml
Temed	75.0 µl	75.0 µl
Ammonium persulfate (i)	150 µl	70 µl

[a]Use these components instead of distilled water when preparing 2% triton X-100 gels (AUT-PAGE). Add temed and ammonium persulfate solutions directly before gel casting starts.

h. Remove water from top of gel and insert a comb (15 wells)(Fig. 4.3d).
i. Add appropriate amounts of temed and ammonium persulfate to the collection gel solution (Table 4.5).
j. Mix gently and cast the collection gel on top of the separation gel (Fig. 4.3d).
k. Allow 2 hours for polymerization at room temperature.
l. Use the gel immediately after polymerization.

Important:

i. Acidic urea gels are more sensitive to handling pressure due to their low content of the cross-linkage reagent (bisacrylamide).
ii. The separation gel should reach 12 cm in the mould.
iii. Polymerization of the collection gel can be enhanced by gentle heating. Put a 100 W lamp near the top of the gel for the first hour of polymerization.

Sample application and electrophoresis

a. Fill the lower buffer tank, including the heat exchanger, with electrode solution (l) (see section 4.2.3.2).
b. Start precooling the electrode solution to about 15°C.
c. Remove the comb from gel mould very carefully.

d. Rinse (three times) and fill the sample wells with electrode solution.
e. Load 5-8 µl of each sample prepared (Fig. 4.3e).
f. Assemble the electrophoresis unit and fill the upper buffer tank with electrode solution (l) (Figs. 4.3f-h).
g. Fit the lid of the unit onto the upper buffer tank and connect the unit to the power supply. *Important:* the *negative* terminal must connect to the bottom of the gel assembly (Fig. 4.3i).
h. Start electrophoresis under the following conditions: voltage set to 280 V; milliamperage, 30 mA/gel; power, 100 W.
i. Continue electrophoresis for 15 hours.

4.2.3.7 Protein Detection in Acrylamide Gels

For evaluation of electrophoretic separations, the protein bands have to be fixed within the gel and stained. Afterwards, gels have to be destained. The following solutions are used:

a. Fixing solution: 12.5% trichloroacetic acid: 125 g.l^{-1} distilled water.
b. Staining solution: Coomassie brilliant blue (CBB).
   Stock solution A: 1 g Coomassie G 250 in 1 l of 90% methanol.
   Stock solution B: 200 ml glacial acetic acid + 800 ml distilled water.
   Final staining solution: 1 volume stock solution A + 1 volume stock solution B.
   Destaining solution: 10% glacial acetic acid containing 5% methanol.

Fixing, staining and destaining of acrylamide gels are done following electrophoresis

a. Remove the gel mould and carefully separate the glass plates.
b. Transfer the gel into the fixing solution (300 ml/gel).
c. Allow the gel to fix for one hour.
d. Put the gel into the final staining solution.
e. Allow the gel to stain on a shaker: for 5-30% gradient gels, stain for about 16 hours; for 12% gels, stain for about 4 hours.

f. Transfer the gel into the destaining solution (300 ml/gel).
g. Destain on a shaker until background dye of gel gets clear (the destaining solution has to be changed twice).
h. Store the gel in 5% acetic acid.

4.2.4 RESULTS AND INTERPRETATION OF GRADIENT GEL ELECTROPHORESIS OF TILAPIA PLASMA PROTEINS

The examples of comparative electrophoretic analyses outlined here are from attempts to find discriminatory protein markers among four tilapia species: *Oreochromis niloticus* (ONi), *Sarotherodon galilaeus* (SGa), *S. melanotheron* (SMe) and *Tilapia guineensis* (TGu).

Figs. 4.4, 4.5 and 4.6 show electrophoretic separations (5-30% gradient gels) of their plasma proteins. In all the figures, column A depicts calibration proteins from which the molecular weights of plasma proteins were extrapolated. Fig. 4.4 compares electropherograms of untreated (B-E) and heat-treated tilapia plasma proteins (B*-E*). Fig. 4.5 shows electrophoretic separations of untreated (B-E) and ethanol-treated (B*-E*) plasma proteins. Fig. 4.6 compares untreated (E), heat-treated (E*) and ethanol-treated (E**) samples of TGu.

It is evident from the three figures that electrophoretic separations of untreated tilapia plasma proteins were barely readable. Hence, plasma protein markers were identified from heat- and ethanol-treated plasma samples. From heat-treated samples, it was possible to identify species-specific plasma proteins in the region indicated in Fig. 4.4. The molecular weights of the proteins in this region ranged from 90 to 114 kD for all four species. Table 4.6 shows their molecular weights.

Protein markers identified from ethanol-treated samples are presented in Table 4.7 (compare Fig. 4.5). In both cases, no remarkable individual variation was observed for the heat- or ethanol-resistant plasma proteins of these tilapias. As may be expected, Tables 4.6 and 4.7 also show proteins that are common to more than one species in addition to species-characteristic components.

Notes:

From the studies that led to the preparation of this manual and those of earlier works (e.g., Avtalion and Wodjani 1971; Badawi 1971; Avtalion et al. 1976), it is evident that the taxonomic value of

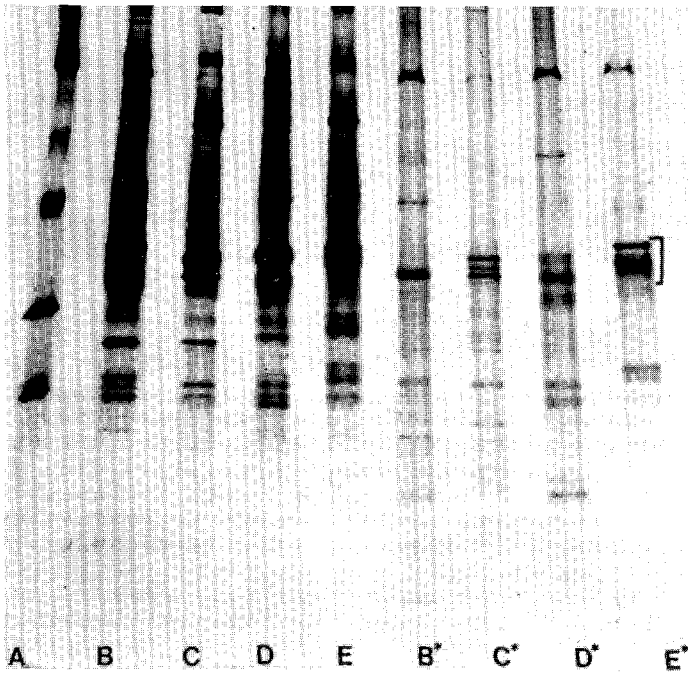


Fig. 4.4. Electrophoretic separation of untreated and heat-treated tilapia plasma proteins in a 5-30% gradient gel. The species diagnostic protein range is marked. A, calibration proteins; B-E, untreated plasma proteins of *O. niloticus* (B), *S. galilaeus* (C), *S. melanotheron* (D) and *T. guineensis* (E), respectively; B*-E*, heat-treated plasma proteins of corresponding species (Oberst et al. 1993).

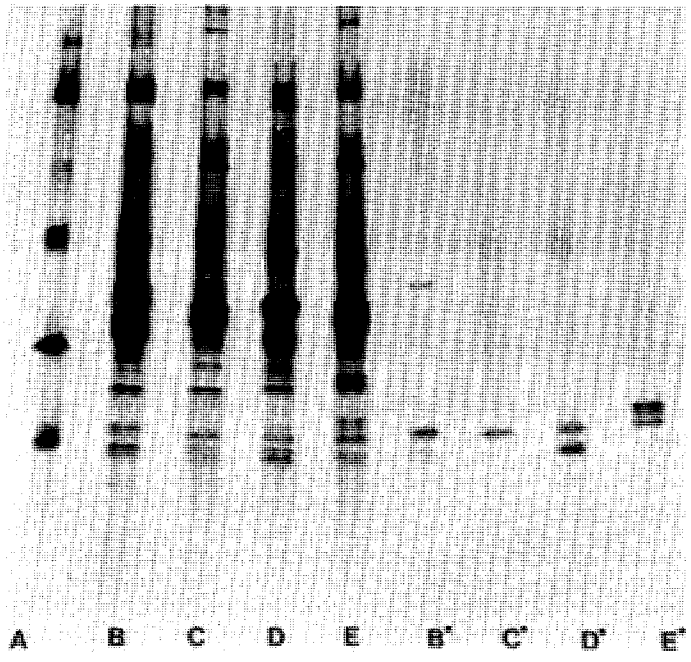


Fig. 4.5. Electropherogram of untreated and ethanol-treated tilapia plasma proteins (5-30% gradient gel). A, calibration proteins; B-E, untreated plasma proteins; and B*-E*, ethanol-treated plasma proteins of *O. niloticus, S. galilaeus, S. melanotheron* and *T. guineensis*, respectively (Oberst et al. 1993).

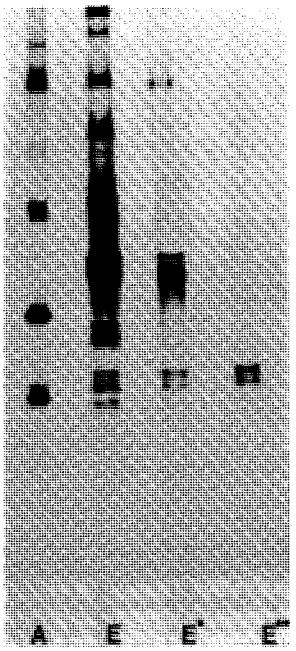


Fig. 4.6. Electropherogram of *T. guineensis* plasma proteins (5-30% gradient gel): A, calibration proteins; E, untreated plasma; E*, heat-treated plasma, E**, ethanol-treated plasma (Oberst et al. 1993).

Table 4.6. Molecular weights (kD) of species-specific proteins from heat-treated plasma of four tilapias (modified after Oberst et al. 1993). (See Fig. 4.4 for explanation of B*-E*).

O. niloticus (B*)	*S. galilaeus* (C*)	*S. melanotheron* (D*)	*T. guineensis* (E*)
		90 kD	
	92 kD		
94 kD			
	100 kD	100 kD	100 kD
	105 kD		
	108 kD	108 kD	108 kD
			114 kD

Table 4.7. Molecular weights (kD) of major proteins from ethanol-treated plasma of four tilapias (modified after Oberst et al. 1993). See Fig. 4.5 for explanation of B*-E*.

O. niloticus (B*)	*S. galilaeus* (C*)	*S. melanotheron* (D*)	*T. guineensis* (E*)
		41 kD	
46 kD	45 kD	46 kD	46 kD
			49 kD
			52 kD
116 kD			

electrophoresis of whole plasma samples from tilapias is poor. Thus, reduction of the number of plasma protein components prior to electrophoresis, by heat treatment or especially by ethanol treatment as used here, is recommended.

Comparative electrophoresis of tilapia plasma proteins, aimed at the identification of species- or hybrid-specific markers, has been described by Sanders (1964); Badawi (1971); Avtalion and Wodjani (1971); Avtalion et al. (1975, 1976); and Avtalion (1982).

The taxonomic value of enzyme electrophoresis in particular, and of immuno- and lectin blot analysis has been demonstrated by Galman et al. (1988); Oberst (1990); and Oberst et al. (1992). These techniques have similar advantages compared to the electrophoresis of plasma proteins after heat or ethanol treatment as outlined here. Basically, all these techniques reduce the number of proteins stained and thus, make gels more readable.

However, intraspecific variation in the protein profiles of untreated plasma from different fish species, including some tilapias, has been indicated mainly by the different staining intensities of bands (Saito 1957; Lysak and Wojcik 1960; Badawi 1968; Avtalion and Wodjani 1971, Avtalion et al. 1975, 1976; Avtalion and Mires 1976). These variations have been attributed to one or more of the following: seasonal variation, change in diet, some pathological states, and sex differences or sexual maturity stages.

4.2.5 RESULTS AND INTERPRETATION OF GRADIENT GEL ELECTROPHORESIS OF TILAPIA SKELETAL MUSCLE PROTEINS INCLUDING PARVALBUMINS

Within several groups of teleost fishes, skeletal muscle proteins, especially low molecular weight parvalbumins and muscle enzymes, are useful for distinguishing taxa above or at the species level (e.g., Buth 1982; McAndrew and Majumdar 1983, 1984; Sakaizumi 1985; Whitmore 1986; Abban 1988; Rehbein and Van Lessen 1989; Eknath et al. 1991; Sodsuk and McAndrew 1991; Focant et al. 1994; Pouyaud and Agnèse 1995). Our findings from electrophoretic studies on tilapia parvalbumins are similar.

Fig. 4.7 shows electropherograms of water-soluble skeletal muscle proteins of six tilapias (*O. niloticus, S. galilaeus, S. melanotheron, T. zillii, T. dageti* and *T. guineensis*) separated in a 5-30% polyacrylamide gradient gel. Each profile shows a high number of separated and

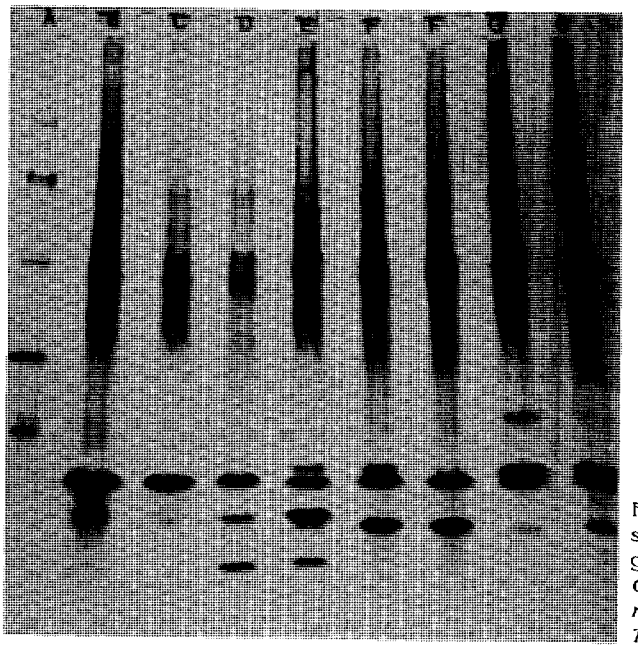


Fig. 4.7. Electropherogram of untreated skeletal muscle extracts of tilapias (5-30% gradient gel). A, calibration proteins; B, *O. niloticus*; C, *S. galilaeus*; D, *S. melanotheron*; E, *T. zillii*; F, *T. dageti*; G, *T. guineensis* (Oberst et al. 1996).

Coomassie-stained proteins. The estimated molecular weights of resolvable components range from 13 to 200 kD. However, clear species or species group characteristic bands were only detectable in the 13-25 kD range. These were considered to be parvalbumins because they were abundant in the white skeletal muscle of fish and were of low molecular weight.

Estimated molecular weights of the putative parvalbumin components of tilapias are presented in Table 4.8. The 22 kD parvalbumin component was common to all species studied and is probably characteristic of tilapias. Species of the genus *Tilapia* were characterized by the 24/24.5 kD component, which was absent in the *Sarotherodon* and *Oreochromis* species studied. A 13 kD parvalbumin component was specific to *S. melanotheron*.

Although there appear to be no unique parvalbumin markers for most of the species studied here (*O. aureus, O. niloticus, S. galilaeus, T. zillii, T. busumana, T. dageti and T. guineensis*), the first three species on this list are characterized by the expression of only one major parvalbumin component (22 kD). Moreover, the 13.5 kD component is characteristic only for *T. zillii* and *T. busumana*, whereas the 18 kD component occurs only in *T. guineensis* and *T. dageti*. These results indicate that the taxonomic value of parvalbumins, based on their

Table 4.8. Estimated molecular weights (kD) of major muscle parvalbumin components of tilapias. B-G refer to Fig. 4.7 (modified after Oberst et al. 1996, with results for *O. aureus* and *T. busumana* added).

O. aureus, O. niloticus, S. galilaeus	*S. melanotheron*	*T. zillii, T. busumana*	*T. dageti*	*T. guineensis*
		24.5 kD	24.5 kD	24 kD
22 kD	22 kD	22 kD	22 kD	22 kD
	20 kD	20 kD		
			18 kD	18 kD
		13.5 kD		
	13 kD			

molecular weight differences, is limited, among closely related fishes such as tilapias.

Notes:

Parvalbumins are also heat-resistant (upto 80° C), and have highly acidic isoelectric points and high affinities for calcium binding (Lehky et al. 1974; Blum et al. 1977; Lehky and Stein 1979; Buth 1982; Whitmore 1986). Therefore, parvalbumins can be obtained by subjecting crude muscle extracts, for example, to heat treatment (70-75°C) (Rehbein and Van Lessen 1989). This precipitates heat-sensitive muscle proteins (see Fig. 4.8).

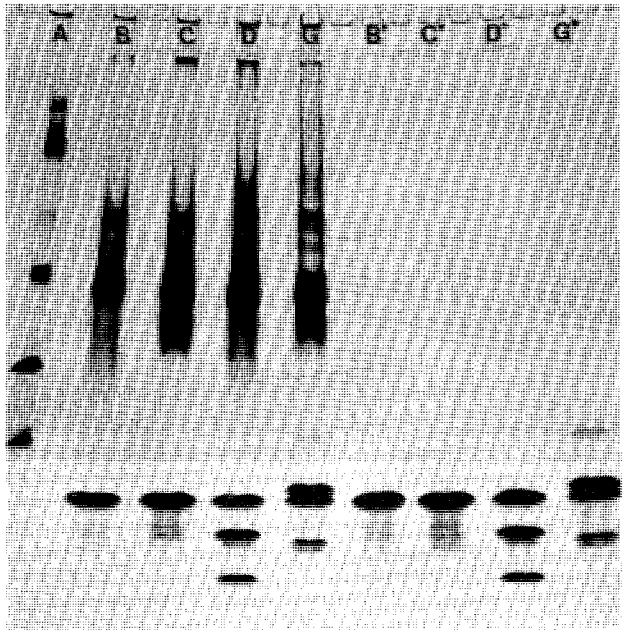


Fig. 4.8. Electropherogram of untreated and heat-treated tilapia muscle extracts (5-30% gradient gel). A, calibration proteins; B-D, crude muscle extracts of *O. niloticus* (B), *S. galilaeus* (C), *S. melanotheron* (D), *T. guineensis* (G), respectively; B*-G*, heat-treated muscle extracts of the corresponding species (Oberst et al. 1993).

Complementary to the results presented here, Focant et al. (1994) analyzed tilapia parvalbumins (10% polyacrylamide gels, pH 8.6) and found that, whereas *O. mossambicus* had two parvalbumin components, *O. aureus, O. niloticus, O. macrochir* and *S. galilaeus* had only a single common component and *T. guineensis, T. mariae* and *T. rendalli* had four common *T. guineensis* components.

4.2.6 RESULTS AND INTERPRETATION OF ACIDIC UREA AND ACIDIC UREA TRITON PAGE OF TILAPIA GLOBIN CHAINS

Vertebrate hemoglobin molecules are usually tetrameric and consist of two different types of polypeptide chains, designated as α-like and ß-like. In tilapias, isolated hemoglobin tetramers have been found to consist of doublets of identical α- and ß-chains (known as $\alpha_2\beta_2$), two different α-type and two different ß-type chains ($\alpha\alpha^*\beta\beta^*$) or combinations of these; e.g., $\alpha\alpha^*\beta_2$ (Falk 1994). Based on this general genetic variability, tilapia globin chains were subjected to electrophoresis aimed at identifying species-characteristic globin chain variants.

Globin chain electrophoresis was performed either with (AUT-PAGE) or without (AU-PAGE) 2% triton X 100 (Figs. 4.9-4.11). With reference to the chain nomenclature of mammalian hemoglobins, the more cathodal group of chains was designated as α-like and the less cathodal group as ß-like. Major chain variants within each group were numbered (Tables 4.9 and 4.10).

Table 4.9. Globin chain composition of tilapias, elucidated by AU-PAGE. A-H refer to Figs. 4.9 and 4.10 (Falk et al., in press).

O. niloticus (A)	*O. aureus* (B)	*O. andersonii* (C)	*S. melanotheron* (D)	*S. galilaeus* (E)	*T. zillii* (F)	*T. guineensis* (G) *T. dageti* (J) *T. busumana* (I)	*T. discolor* (H)
ß2	ß2	ß2	ß2	ß1	ß2	ß1	ß1
					ß3	ß3	ß3
ß4	ß4		ß4	ß4			
		ß5					
ß6	ß6						
							α1
α2	α2		α2				
		α3		α3			α3
	α4				α4	α4	
α5							
		α6					
			α7				
				α8			

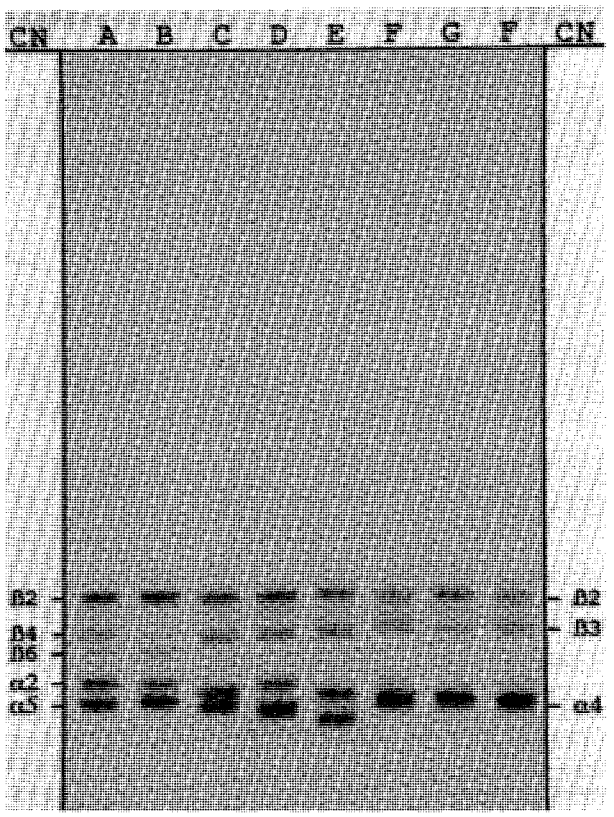


Fig. 4.9. Separation of tilapia globin chains by AU-PAGE: A, *O. niloticus*; B, *O. aureus*; C, *O. andersonii*; D, *S. melanotheron*; E, *S. galilaeus*; F, *T. zillii*; G, *T. guineensis*; CN, chain nomenclature of lanes A and F, respectively (Falk et al., in press).

Table 4.10. Globin chain composition of tilapias elucidated by AUT-PAGE. I-J refer to Fig. 4.11 (Falk et al., in press).

T. busumana (I)	*T. zillii* (F)	*T. guineensis* (G)	*T. dageti* (J)
ß1			
	ß2		
		ß3	ß3
	ß4		
ß5			
		ß6	ß6
α1	α1	α1	α1
α2	α2	α2	α2
α3	α3	α3	α3
α4	α4	α4	α4

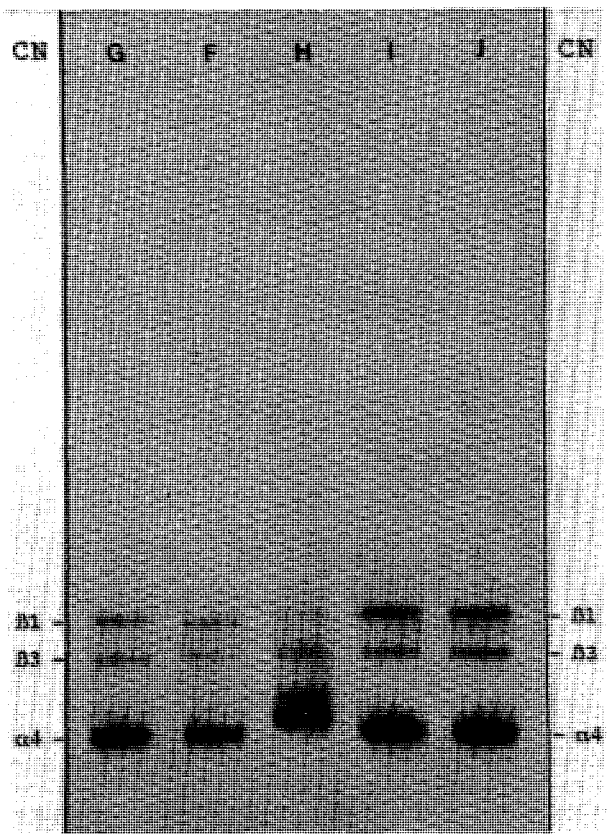


Fig. 4.10. Separation of tilapia globin chains by AU-PAGE: F, *T. zillii*; G, *T. guineensis*; H, *T. discolor*; I, *T. busumana*; J, *T. dageti*; CN, chain nomenclature (Falk et al., in press).

Results indicated that tilapia globin chains are heterogeneous within both the α-type and ß-type chains. Eight major α-chains and six major ß-chains were identified. For taxonomic purposes, our results (Figs. 4.9 and 4.10; Table 4.9) show that all the mouthbrooding species studied (*Oreochromis* and *Sarotherodon* spp.) together with *T. discolor,* can be distinguished by AU-PAGE systems. For example, α5 is specific to *O. niloticus,* α6 to *O. andersonii,* α7 to *S. melanotheron* and α8 to *S. galilaeus*. Moreover, some species are distinguishable by their combinations of chains;

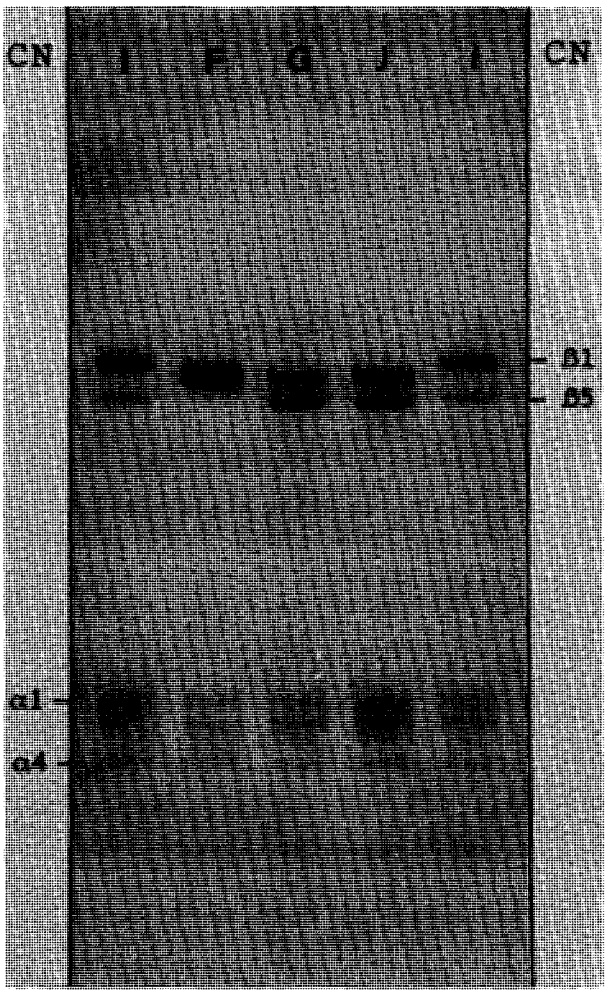


Fig. 4.11. Separation of tilapia globin chains by AUT-PAGE: F, *T. zillii*; G, *T. guineensis*; I, *T. busumana*; J, *T. dageti*; CN, chain nomenclature (Falk et al., in press).

e.g., the α2/α4 combination is specific to *O. aureus* and the α1/α3 to *T. discolor*.

Among the *Tilapia* species, *T. busumana, T. dageti, T. guineensis* and *T. zillii* were nearly indistinguishable on the basis of the AU-PAGE results (Fig. 4.10). They showed almost identical ß-chains and a single and common major α-chain (α4). Therefore, globin chain samples of these species were subjected to the AUT-PAGE system for possible further discrimination. Based on this, species characteristic ß-globin chains were identified for *T. busumana* and *T. zillii*. However, *T. dageti*

and *T. guineensis* remained indistinguishable (Fig. 4.11, Table 4.10). Moreover, the AUT-PAGE procedure also separates the single major α-chain of these four *Tilapia* species (AU-PAGE results) into four different ones.

Therefore, by combination of the AU-PAGE and AUT-PAGE results, all the *Oreochromis, Sarotherodon* and *Tilapia* species studied here can be discriminated, except *T. dageti* and *T. guineensis.*

Notes:

Comparable electrophoretic studies on fish globin chains of different species have been described by Tsuyuki and Ronald (1970, 1971); Wilkins (1970); Ronald and Tsuyuki (1971); Powers and Edmundson (1972a); Perez and Maclean (1976); Mied and Powers (1978); Panara and Puccetti (1986); and Ohkubo et al. (1993).

The identity of all three major ß-globin chains of *O. niloticus* (Fig. 4.9: ß2, ß4, ß6) was confirmed by isolation and N-terminal amino acid sequence analysis (amino acid positions: 1 to 40). The N-termini of both major α-globins of *O. niloticus* were blocked (Falk 1994).

Comparative immunological assays, using polyclonal anti-tilapia hemoglobin-antisera raised in rabbits, did not improve or facilitate the identification of tilapias based on their globin chain characteristics (Falk 1994).

4.3 Allozyme electrophoresis (starch gel electrophoresis)

4.3.1 PRINCIPLES AND OBJECTIVES

The general principles and objectives of electrophoresis (see section 4.2.1) apply here. Allozyme electrophoresis is used to determine:

a. how many loci are responsible for the coding of a protein (usually an enzyme in species, or isozymes in the different organs of a species);
b. how many variants of a protein (allozymes) are coded by a given locus; and
c. the genotype(s) of the specimens studied with respect to the genes coding for these enzymes.

During electrophoresis, different isozymes and allozymes migrate to different positions in the medium (usually starch gel) based on

charge differences. Hence, different phenotypes of the enzyme can be observed, after appropriate "staining mixtures" have been added to the gel. This process will result in a specific enzyme reaction, finally producing colored bands which indicate active enzyme locations.

The process can be summarized as follows:

				+ Salt		
Enzyme	+	Substrate	—> Colorless product	———>		Colored product
(in sample)		(in reagent)		(in reagent)		(stained enzyme band)

4.3.2 EQUIPMENT AND REAGENTS

a. Electrophoretic assembly
b. Power supply, PS 250-2, Sigma
c. Bunsen burner or other gas burner
d. Vacuum pump
e. Buckner flask
f. Glass plates (25 x 25 cm)
g. Gel mould fitting with glass plates
h. Electrode wicks
i. Sample applicator strips (Wattmans filter paper no. 3)
j. Spacers
k. Gel cutter
l. Staining trays
m. Ice cooling packs
n. Scalpel
o. Plastic film (thin)
p. Hydrolyzed potato starch, Sigma
q. Tris (Trihydroxymethylaminomethane), Sigma
r. Citric acid, anhydrous, Sigma
s. Boric acid 99%, Sigma
t. Acetic acid, glacial, Sigma
u. Methanol, Sigma

The reagents required for staining specific enzymes are described in section 4.3.3.5 below.

Buffer solutions:

a. Continuous tris-citrate-buffer (CTC) (Ward and Beardmore 1977)

Electrode buffer: 0.25 M Tris, 0.057 M citric acid, pH 8.0:

Tris	30.29 g
Citric acid	11.98 g

Make upto a final volume of 1 000 ml with distilled water.

Gel buffer: add 8.5 ml electrode buffer (above) to 211.5 ml distilled water.

b. Tris-HCl-buffer: 0.2 M Tris, pH 8.0 or 9.0:

Tris	24.65 g
HCl, 0.1M	30 ml

Make upto a final volume of 1 000 ml with distilled water and adjust pH to 8.0 or 9.0 as required.

Fixing solution:

Acetic acid (glacial)	10%
Methanol	20%
Distilled water	70%

4.3.3 PROCEDURE FOR ALLOZYME ELECTROPHORESIS

4.3.3.1 Preparation of Starch Gel

a. Set the gel mould on a glass plate on a flat surface.
b. Weigh enough hydrolyzed potato starch to make a 12-12.5% mixture with the appropriate gel buffer (here CTC-buffer) in a Buckner flask.
c. Mix the starch and buffer thoroughly.
d. Heat the mixture over a Bunsen burner with constant rotation of the flask by hand (Fig. 4.12b).
e. Stop heating when the first big bubble appears.
f. Degas immediately for about 2 min (Fig. 4.12c).
g. Pour the contents of the flask into the gel mould on the glass plate (Fig. 4.12d).
h. Cover the starch with the second glass plate.
i. Allow the gel to set at room temperature for a minimum of 3-4 hours.
j. Leave the gel for about 4 hours at 4°C or in a refrigerator overnight.

4.3.3.2 Preparation of Electrophoretic Assembly

a. Fill the buffer tanks of the unit with the appropriate electrode buffer.

b. Soak the electrode wicks in buffer.
c. Store the prepared assembly in a refrigerator (about 4°C).

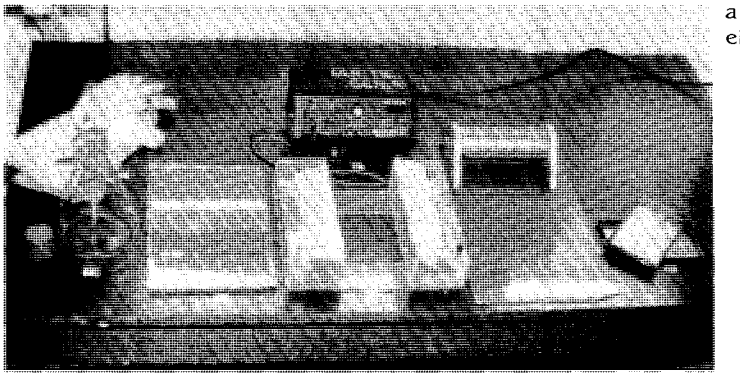
4.3.3.3 Loading the Samples; Procedures for an Electrophoresis Run

a. Arrange samples on the bench in the order that they will be loaded on the gel.
b. Record this arrangement.
c. Insert the sample strips (3 x 10 mm) into the samples.
d. Allow the sample strips to soak up the samples.
e. Remove the starch gel from the refrigerator.
f. Remove the glass plate covering the starch gel.
g. Make a cut through the gel about 5-6 cm from one end using a scalpel.
h. Remove the mould from the gel.
i. Push apart the two parts of the gel.
j. Remove the sample strips and blot off any excess liquid.
k. Arrange the sample strips on the inner surface of bigger portion of the gel (Fig. 4.12e).
l. Push the smaller gel portion back into place.
m. Replace the gel in the former as before.
n. Insert spacer(s) into the gel former at the end of the smaller part of the gel to maximize contact between the two parts and the sample strips.
o. Mount the gel on the electrophoresis unit.
p. Connect the electrode wicks to the two ends of the gel.
q. Cover the gel with a plastic film.
r. Put the second glass plate onto the top of the gel.
s. Put cooling packs (-20°C) on top of the second glass plate.
t. Place the unit in a refrigerator (4°C) and connect with the power supply unit.
u. Connect the assembly to the power supply.
v. Set the milliamperage to 50 mA and the voltage to 200 V.
w. Run the gel for 4-5 hours at constant voltage (Fig. 4.12g).

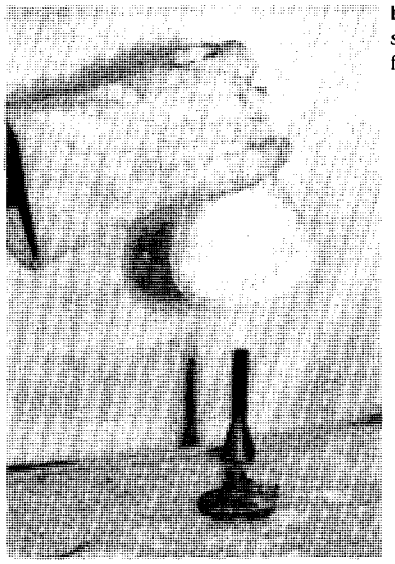
4.3.3.4 Slicing and Staining the Gel

After the run:

a. Using the gel cutter, slice the gel into 2 mm thick slices (Fig. 4.12h).
b. Place each slice in a staining tray.

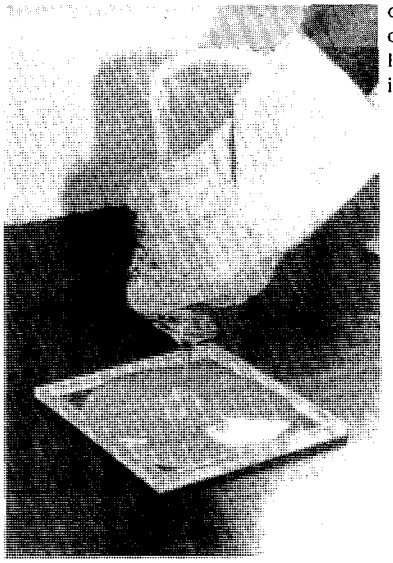


a. Basic equipment used in electrophoretic studies.

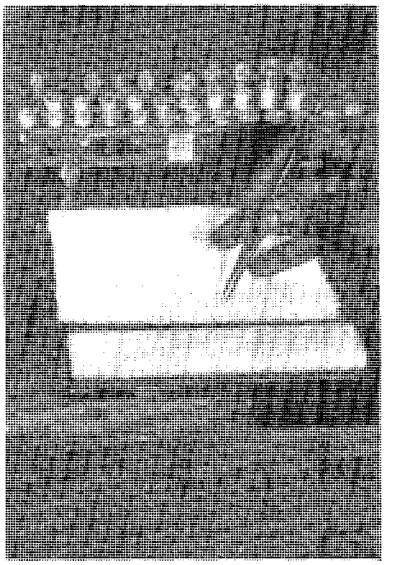


b. Dissolving starch in buffer solution.

c. Degassing liquid starch solution.

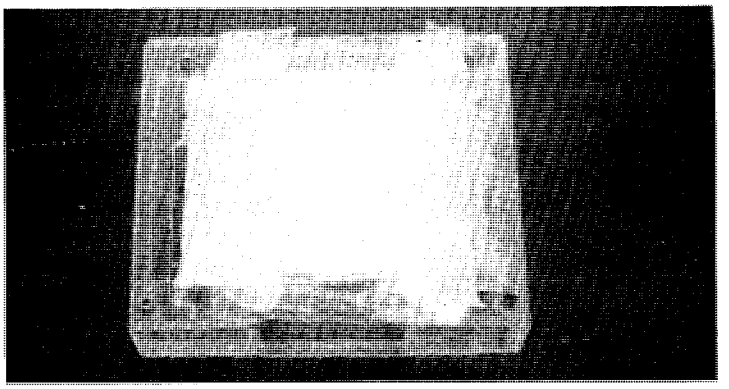


d. Pouring degassed liquid starch into mould.

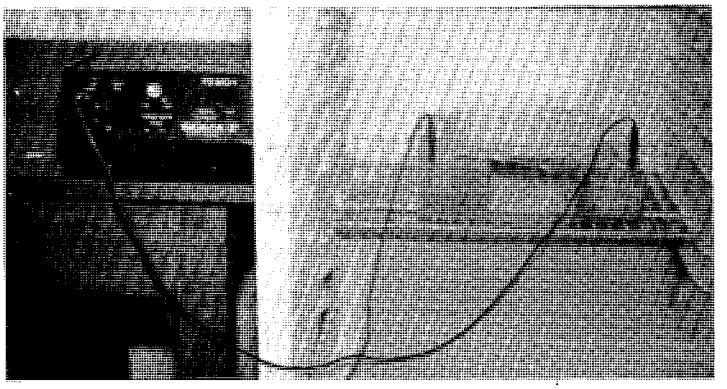


e. Loading of samples into starch gel.

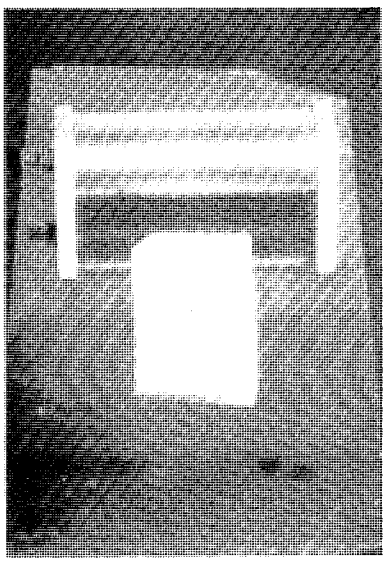
Fig. 4.12. Allozyme electrophoresis procedure.



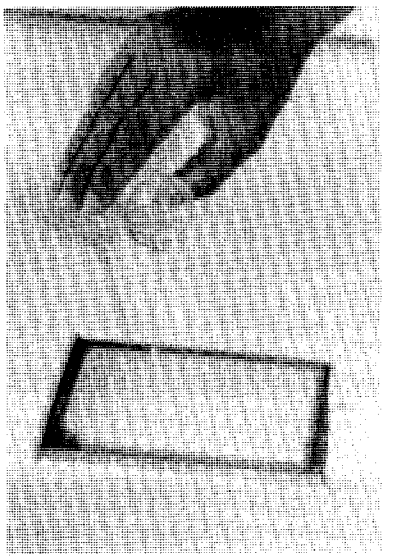
f. Electrophoretric assembly ready for run.



g. Running samples in a buffer system for a specified period of time.



h. Slicing starch gel slab into 2 mm thickness.



i. Pouring staining solution onto 2 mm thick starch gel to stain for particular enzymes.

c. Label each tray according to enzyme to be stained.
d. Prepare appropriate staining mixtures (4.3.3.5) and pour these onto the gels (Fig. 4.12i).
e. Incubate the gels for a few minutes at room temperature until the bands appear. Some reactions are light-sensitive, and these gels must be incubated in the dark.
f. Wash off the staining mixtures with distilled water.
g. Add fixing solution to the gel.

4.3.3.5 Enzyme Staining Recipes

Abbreviations:

NAD	ß-nicotinamide adenine dinucleotide
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyl-tetrazoliumbromide
PMS	N-methyldibenzopyrazine methyl sulfate salt, phenazine methosulfate
EDTA	Ethylenediaminetetra-acetic acid

a. For alcohol dehydrogenase (ADH)

NAD, Sigma	15 mg
MTT, Sigma	6 mg
PMS, Sigma	10 mg
0.2 M Tris-HCl pH 9.0	30 ml
Isopropanol	0.75 ml

b. For glycerol-3-phosphate dehydrogenase (GPDH)

NAD	15 mg
MTT	7 mg
a-glycerophosphate, disodium salt pentahydrate, Sigma	20 mg
EDTA, Sigma	60 mg
PMS	10 mg
0.2 M Tris-HCl pH 8.0	30 ml

c. For lactate dehydrogenase (LDH)

NAD	15 mg
MTT	7 mg
Sodium lactate solution, 50% extrapure, Merck	1 ml

PMS	10 mg
0.2 M Tris-HCl pH 8.0	30 ml

d. For malate dehydrogenase (MDH)

NAD	10 mg
MTT	6 mg
L-malic acid, mono-sodium salt, Sigma	150 mg
PMS	10 mg
Distilled water	30 ml

e. For sorbitol dehydrogenase (SDH)

NAD	15 mg
MTT	7 mg
D-sorbitol, Sigma	150 mg
$MgCl_2$, hexahydrate, Merck	10 mg
PMS	10 mg
0.2 M Tris-HCl pH 8.0	30 ml

f. For phosphoglucoisomerase (PGI)

NADP, Sigma	4 mg
MTT	7 mg
Fructose-6-phosphate, Sigma	20 mg
Glucose-6-phosphate dehydrogenase, Sigma (500 units dissolved in 500 µl distilled water)	50 µl
$MgCl_2$, hexahydrate, Merck	20 mg
PMS	10 mg
0.2 M Tris-HCl pH 8.0	30 ml

4.3.4 RESULTS AND INTERPRETATION OF STARCH GEL ELECTROPHORESIS OF TILAPIA SKELETAL MUSCLE ENZYMES

After starch gel electrophoresis, the stained bands in a gel represent different isozymes. In the tilapias studied for the preparation of this manual, most of the enzymes were coded for by more than a gene locus. For a beginner, this poses a problem of identifying those bands that represent allelic forms of the same locus. Resolution of this

requires some knowledge of the enzymes and how they will appear on gels from samples of homozygous and heterozygous individuals. Once this issue has been resolved, all the bands for each locus are considered as variants (alleles) of the protein coded for at the locus.

For taxonomic purposes at the species level, it is necessary to find fixed alleles with different electrophoretic mobilities. This means samples from all the members of the species, homozygotes and heterozygotes, show the discriminatory allelic band at the locus.

For example, Fig. 4.13 (represented diagrammatically in Fig. 4.14) shows alleles of various tilapia species at the MDH*2 and MDH*3 loci. At MDH*2, *O. niloticus* (ONi) and *S. galilaeus* (SGa) express the same fixed allele (b), which differs from that fixed for the other species (a) (*S. melanotheron* (SMe), *T. guineensis* (TGu), *T. zillii* (TZi), *T. discolor* (TDi) and *T. busumana* (TBu)). No individual variation was observed at MDH*2. A slightly more complex situation is shown at the MDH*3 locus. This is because at this locus, individuals of all species were apparently heterozygotes (as indicated by the presence of three bands for each, because MDH is a dimeric protein). For species discrimination, a basic interpretation is as follows. Alleles b and d have unique electrophoretic mobilities and are apparently fixed for all *O. niloticus* specimens studied. Therefore, either of these could be used to discriminate *O. niloticus* specimens from all the other species represented here. Similarly, alleles c and e are unique for *S. melanotheron* and *S. galilaeus*, respectively. Among the other species, alleles a and f are shared.

Fig. 4.15 (represented diagrammatically in Fig. 4.16) shows alleles of the same species at the PGI*1 and PGI*2 loci. With reference to PGI*1, five alleles (a-e) are distinguishable. Alleles a, b and d are unique and are fixed for *T. guineensis*, *T. zillii* and *T. discolor*, respectively. However, specimens of *O. niloticus*, *S. galilaeus* and *T. busumana* share a common allele (c). Note that the two *S. melanotheron* specimens show two different alleles (c and e) in the homozygote state. Therefore, allele e, which occurs only in *S. melanotheron*, is not discriminatory for all specimens of the species because it may not be expressed by some individuals. At PGI*2, there is no unique allele for specimens of any of the species studied here.

Other important enzyme loci for the discrimination of tilapia species investigated include LDH*1, GPDH*1, ADH*1 and SDH*1.

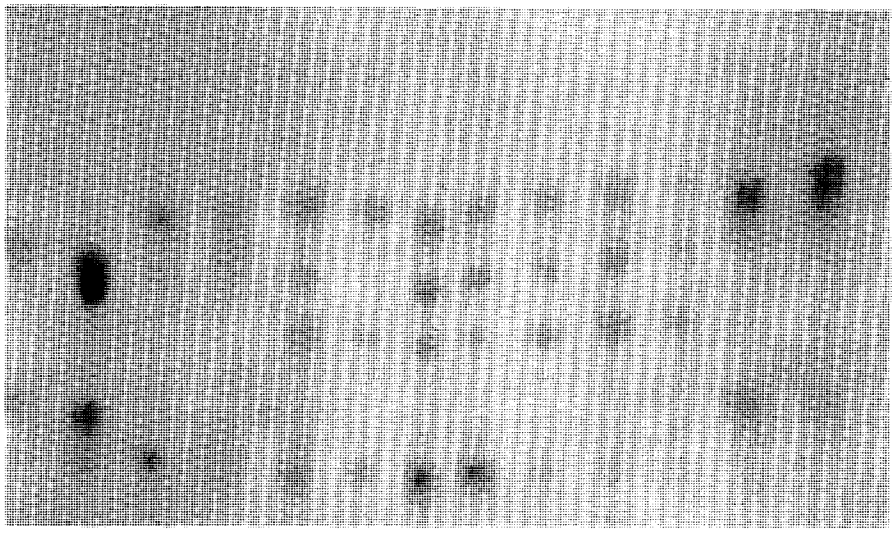


Fig. 4.13. Starch gel of tilapia skeletal muscle samples, after staining for MDH.

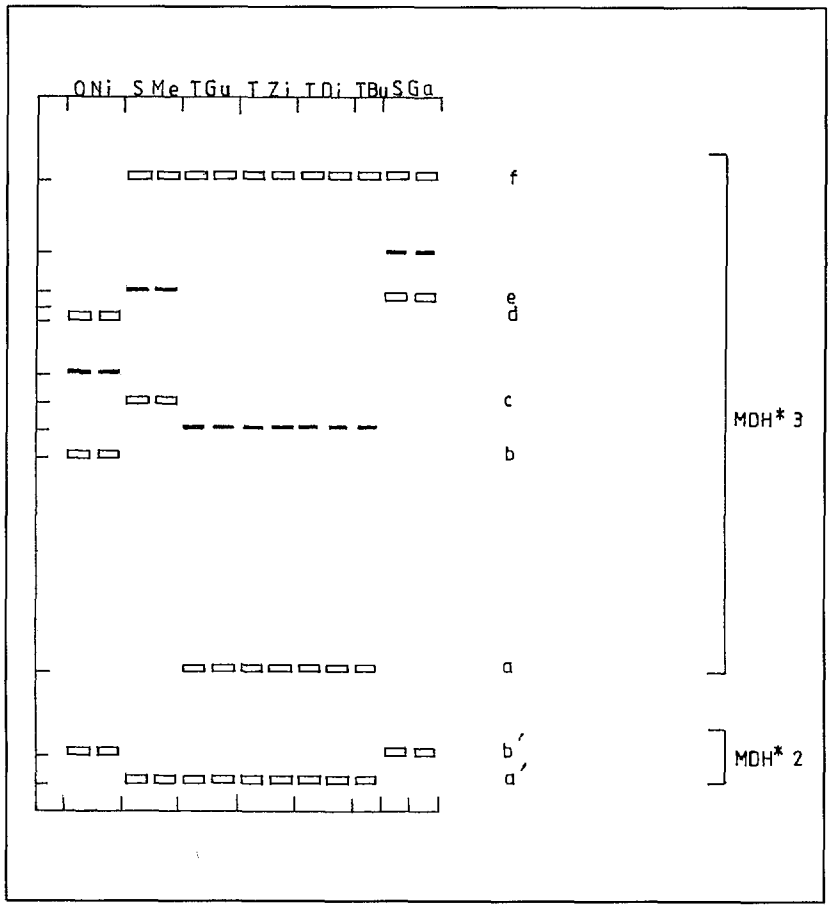


Fig. 4.14. Diagrammatic representation of the starch gel (Fig. 4.13) of tilapia skeletal muscle MDH*2, MDH*3, showing two alleles (a', b') at MDH*2 and six alleles (a-f) at MDH*3.

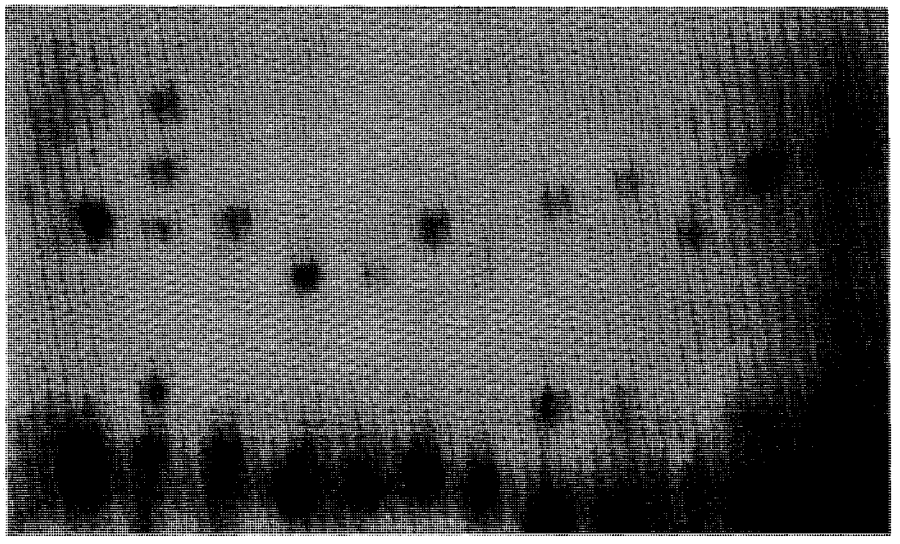


Fig 4.15. Starch gel of tilapia skeletal muscle samples after staining for PGI.

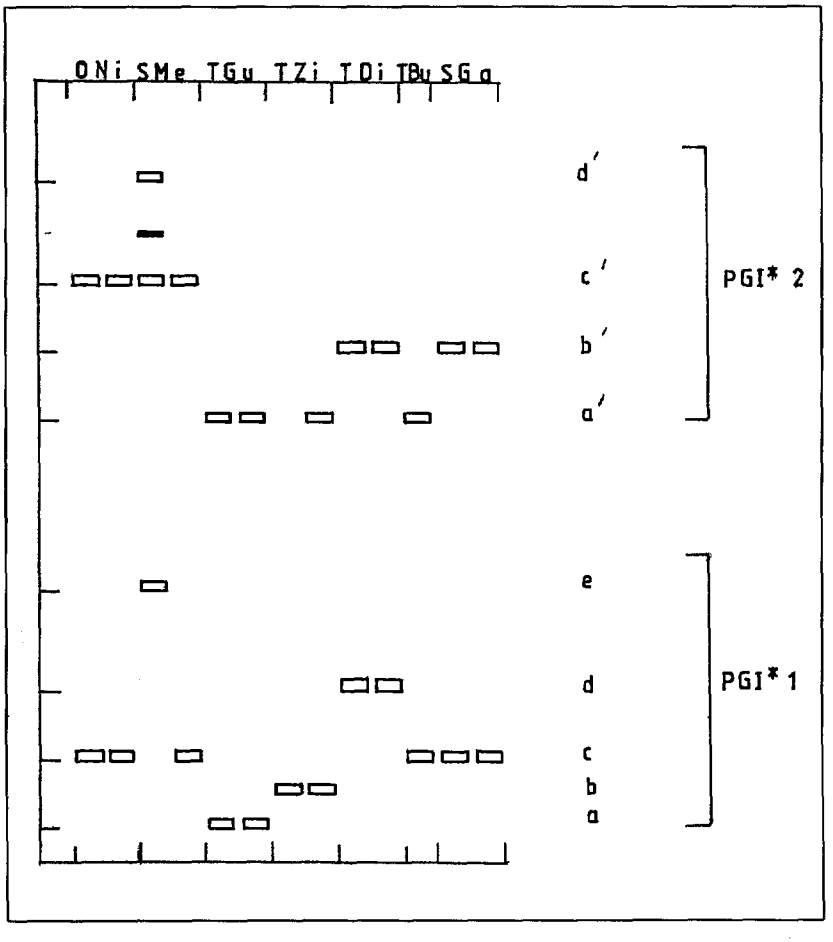


Fig. 4.16. Diagrammatic representation of the starch gel (Fig. 4.15) of tilapia skeletal muscle PGI*1, PGI*2, showing five alleles (a-e) at PGI*1.

Note:

Readers can consult the following significant allozyme studies on tilapias and other fishes: McAndrew and Majumdar (1983, 1984); Seyoum (1989); Shaklee et al. (1990); Sodsuk and McAndrew (1991); Ward et al. (1992); and Pouyaud and Agnèse (1995).

4.4 Isoelectric focusing

4.4.1 BACKGROUND

IEF is a powerful technique for separating proteins according to their isoelectric points (pI). In comparison to other electrophoretic procedures such as gradient gel or starch gel electrophoresis, IEF offers opportunities to separate and to characterize proteins, which may not be separable by other electrophoretic approaches.

The separation medium used for IEF is a pH gradient polyacrylamide gel through which protein molecules migrate mainly on the basis of their individual net surface charges. Put simply, as a protein migrates through a pH gradient, it progressively loses surface charge, until this becomes zero, and then it stops migrating. The pH value in the gradient at which point the charge of a protein becomes zero is designated as the isoelectric point (pI) of the protein. Hence, IEF can be considered as an "end-point" technique, with or without minimal diffusion problems. The pI's of unknown proteins are estimated by comparison with co-focused standard pI marker proteins.

The following sections describe IEF procedures for separating tilapia parvalbumins and hemoglobins. For standardization, consistency and comparison of results within and among different laboratories, industrially produced precast IEF gels, "Precotes", are preferred to pH gradient gels prepared in individual laboratories.

4.4.2 EQUIPMENT AND REAGENTS

a. Horizontal multiphor II electrophoresis unit 2117, Pharmacia LKB or equivalent
b. Macrodrive 5 power supply, Pharmacia LKB or equivalent
c. Thermostatic circulator

Precast IEF polyacrylamide gels

a. Servalyt Precotes, Serva, pH range 3-10 for hemoglobin separations
   Dimensions: 125 x 125 mm; gel layer, 150 µm; polymer concentration (T), 5%; cross linkage (C), 3%; ampholyte concentration, 5%
b. Servalyt Precotes, Serva, pH range 3-6 for skeletal muscle parvalbumin separations
   Dimensions: 125 x 125 mm; gel layer, 150 µm; polymer concentration (T), 5%; cross linkage (C), 3%; ampholyte concentration, 5%
c. Electrode wicks, Serva, 115 mm long for use with 125 x 125 mm IEF gels
d. Sample applicator strips, Serva, 10 slots (7 x 1 mm/slot)

Electrode fluids and chemicals

a. Anode fluid 3, Serva: 3.3 g L-aspartic acid and 3.7 g L-glutamic acid in 1 l distilled water
b. Cathode fluid 10, Serva: 4 g L-arginine, 4 g L-lysine, 120 ml ethylenediamine in 1 l distilled water
c. n-decan, Merck
d. Glycerol, Serva
e. 2-mercaptoethanol, Serva

Protein fixation and staining reagents

a. Trichloroacetic acid, Merck
b. CBB G-250, Serva
c. 4-chloro-1-naphthol, Serva

Marker proteins for pI determination

a. Serva protein test mixture 9 on IEF gels, pH range 3-10: myoglobin-wale, pI 8.3; myoglobin-horse, pI 7.3 and 6.9; ovotransferrin, pI 5.9 and -lactoglobulin, pI 5.34
b. Single marker proteins (Sigma) on IEF gels, pH range 3-6: amyloglucosidase, pI 3.6; glucose oxidase, pI 4.2; trypsin inhibitor, pI 4.6; and -lactoglobulin A, pI 5.1

4.4.3 PROCEDURES

The procedures are outlined here in sequence, following the instructions supplied with Servalyt Precotes, Serva, Heidelberg, Germany.

4.4.3.1 Preparation of Samples for IEF

Hemolysate samples

a. Dilute hemolysate stock samples (see section 3.2.6) 3:1 with distilled water containing 9% 2-mercaptoethanol (ME) (2 volumes of stock solution + 1 volume of 9% mercaptoethanol/distilled water).
b. Precool the samples at 5°C for 15 min.
c. 10 µl of each precooled ME-reduced sample is required per IEF gel application.

Parvalbumin samples

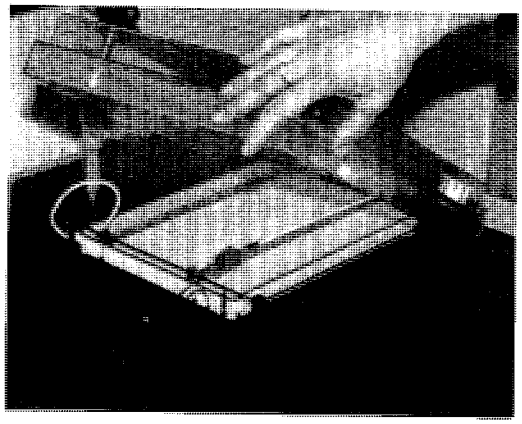
a. Dilute stock parvalbumin samples (see section 3.3.3) 1:4 with distilled water.
b. Precool the samples at 5°C for 15 min.
c. 10 µl of each precooled sample is required per IEF gel application.

4.4.3.2 IEF of Hemoglobins and Parvalbumins

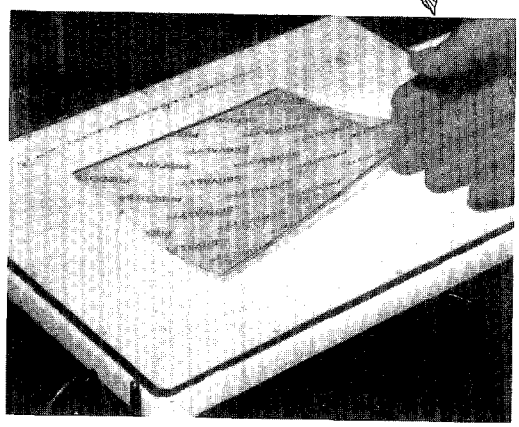
Using the horizontal IEF system specified above, two IEF gels can be run simultaneously. This allows the comparative separation of 20 samples in a single run. The Precotes are very thin and are sensitive to prolonged air exposure and handling. Therefore, all procedures should be performed very carefully and within a minimum of time.

Placement of IEF Precotes

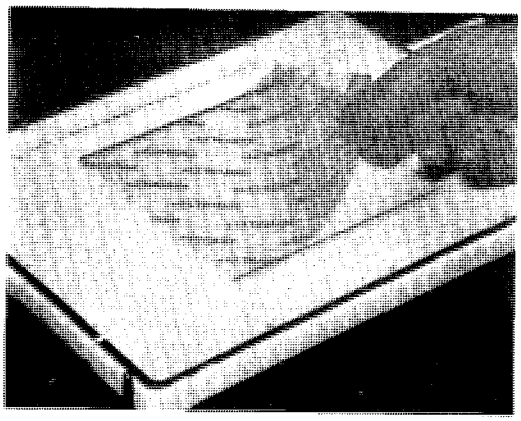
a. Assemble the horizontal multiphor II electrophoresis unit correctly and connect the cooling plate of the system with the thermostatic circulator (Fig. 4.17a).



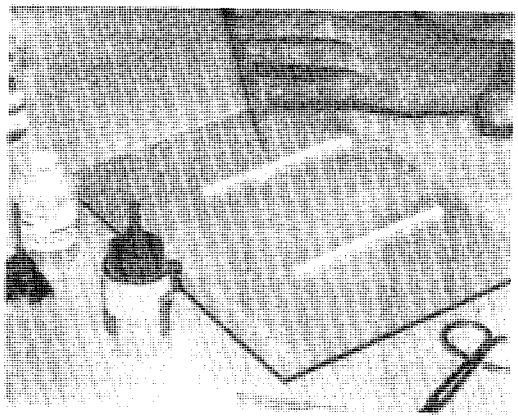
a.



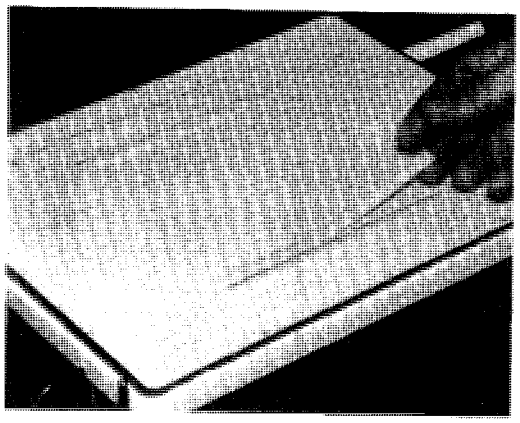
b.



c.



d.

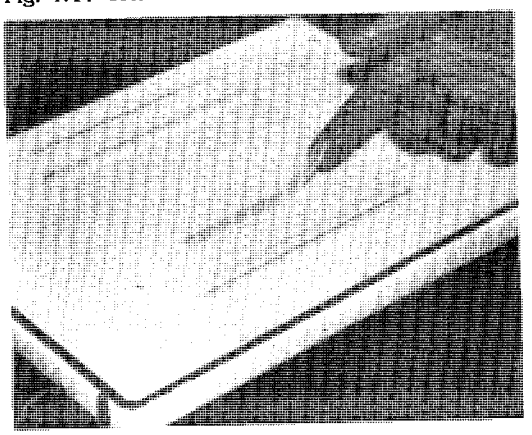


e.

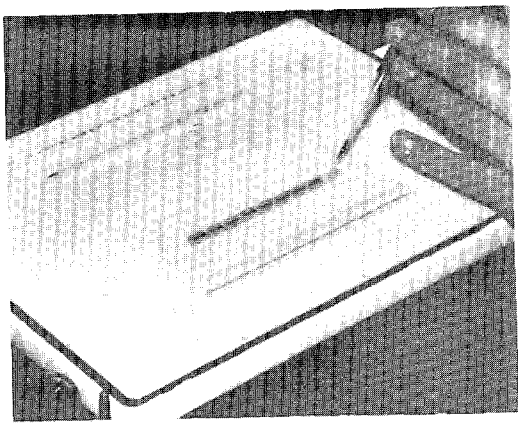
Fig. 4.17. IEF procedure (for explanation of the apparatus and steps illustrated, see section 4.4.3.2).

continued...

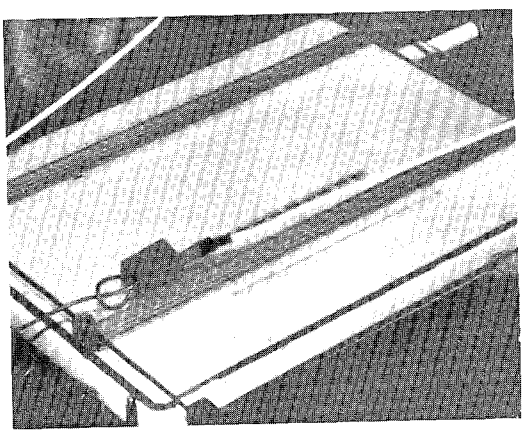
Fig. 4.17 continued



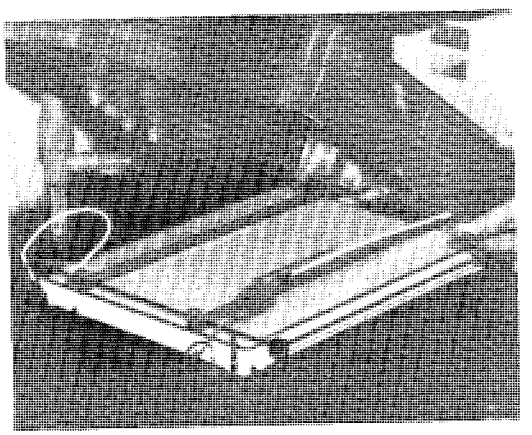
f.



g.



h.



i.

b. Set thermostatic circulator to 10°C and start precooling the unit for 30 min.
c. Open the IEF chamber and remove the electrode holder (the glass plate with both electrodes).
d. Spread the surface of cooling plate with n-decan (about 0.7 ml per gel).
e. Roll or lay the IEF gel with its cover sheet on the cooling plate (with red line of the gel towards the red electrode (+) of the unit) avoiding trapping any air bubbles. The pH range of the gel required for parvalbumins is pH 3-6. For hemoglobins, the range is pH 3-10 (Fig. 4.17b).
f. Peel off the cover sheet of the precooled IEF gel (Fig. 4.17c).

Preparation of the electrode wicks and the applicator strips

a. Two electrode wicks are required per gel.
b. Cut both wicks to exactly 115 mm.
c. Place the wicks on a clean, even glass plate.
d. Saturate one wick evenly with the appropriate anode fluid and the other with cathode fluid: about 0.1 ml electrode fluid is required per 1 cm of wick (Fig. 4.17d).
e. Leave the wicks in their fluids on the glass plate for 10 min. to ensure even saturation.
f. Before placing wicks onto the Precote blot off excess fluid with filter paper.
g. Wash the sample applicator strip with distilled water and allow it to dry on filter paper.

Placement of the electrode wicks and the sample applicator strip

a. Place the cathode fluid-soaked wick carefully along the red line on the gel.
b. Place the anode fluid-soaked wick carefully along the black line on the gel. *Important:* The wicks should *not* protrude beyond the edges of the gel (Fig. 4.17e).
c. Position the sample applicator strip. For hemoglobin separations, using a pH 3-10 gradient gel, place the strip 2.75 cm from the red line (+) of the gel parallel to the wicks. For parvalbumin separations, using a pH 3-6 gradient gel, place the strip 3.75 cm from the black line (-) of the gel (Fig. 4.17f).
d. Center the corresponding electrodes of the IEF apparatus on the wicks (see Fig. 4.17h).
e. Apply electrode pressure (900 g) using pressure bars.
f. Check all electrical connections.
g. Close the IEF chamber (see Fig. 4.17i).
h. Connect the IEF system to the power supply for the next step: prefocusing of IEF gel.

Important:

Accuracy in placement of the wicks, centering of the electrodes on wicks and placement of the sample applicator strip are absolutely

essential for good results. Electrode wicks cannot be moved on Precotes without damaging the gel layer.

Prefocusing of IEF gel with a programmable power supply

a. Switch on the power supply unit.
b. Set the voltage to 230 V and the power to 4 W/gel (use 8 W for two gels).
c. Start this prefocusing run.
d. Adjust the initial voltage to 200 V by setting the current to 5-6 mA.
e. Now set the voltage to 500 V and lower the temperature of the thermostatic circulator to 5°C.
f. Allow 40-50 min for prefocusing of the gel, until the voltage has reached 500 V.

Sample application and electrofocusing

a. Turn off the power supply and open the IEF chamber.
b. Remove the electrode holder.
c. Pipette 10 µl of each sample (parvalbumin or hemoglobin) into the slots of the applicator, without leaving any unfilled slots between filled slots (Fig. 4.17g).
d. Apply the appropriate mixture of pI standards into the first and last slots.
e. Center the electrodes on the wicks as before (Fig 4.17h).
f. Check all electrical connections and close the IEF chamber (Fig. 4.17i).
g. Set voltage at 1 700 V and start the IEF run proper.
h. Continue IEF for about 3.5 hours.
i. The run is complete when the current has reduced to 1.5 to 2 mA per gel (minimum time is 3 hours).

4.4.3.3 Protein Detection in IEF Gels

For parvalbumins

a. After the IEF run, carefully remove the wicks and the applicator strip.

b. Place the gel(s) into the fixing solution (12.5% trichloroacetic acid; i.e., 125g/1 000 ml) and incubate for 15 min.
c. Stain the gels in 0.05% CBB G-250 (250 ml/gel), 30 min (for making up the staining solution, see section 4.2.4).
d. Destain the gels in 10% acetic acid containing 5% methanol (2 X 10 min).
e. Store the gels in 5% acidic acid or dry at room temperature.

For hemoglobins (after Miribel and Arnaud 1988)

a. Stock solutions required:
Stock solution A: 120 mg of 4-chloro-1-naphthol + 60 ml methanol.
Stock solution B: PBS pH 7.4 (300 mOsm/kg, see erythrocyte sample preparation, section 3.2.5).
b. Final staining solution (prepare fresh, immediately before use): 60 ml stock solution A + 340 ml of stock solution B + 1 ml of 30% H_2O_2. This is sufficient for one gel.
c. Place the gel in the final staining solution and stain *in the dark* for about 30 to 60 min.
d. Rinse three times in distilled water.
e. Photograph the stained gels within an hour from staining.
f. Store the gels in distilled water or dry them at room temperature in the dark. In either case, the dye will fade.

4.4.4 RESULTS AND INTERPRETATION OF IEF OF TILAPIA PARVALBUMINS AND HEMOGLOBINS

4.4.4.1 Parvalbumin Analysis

The taxonomic value of fish parvalbumins (major heat-resistant proteins from the dorso-lateral white skeletal muscle) is fairly well documented (e.g., Sakaizumi 1985; Whitmore 1986; Rehbein and Van Lessen 1989: Focant and Vandewalle 1991; Focant et al. 1994). However, for the tilapias studied here, the molecular weight differences of their major parvalbumins obtained after gradient PAGE (see section 4.2) were not sufficiently discriminative. Thus, parvalbumin samples were subjected to IEF to access probable differences among their isoelectric points as a basis for species discrimination.

Fig. 4.18 presents IEF parvalbumin profiles, mostly of mouthbrooding tilapias (*Oreochromis* and *Sarotherodon* spp.). Results

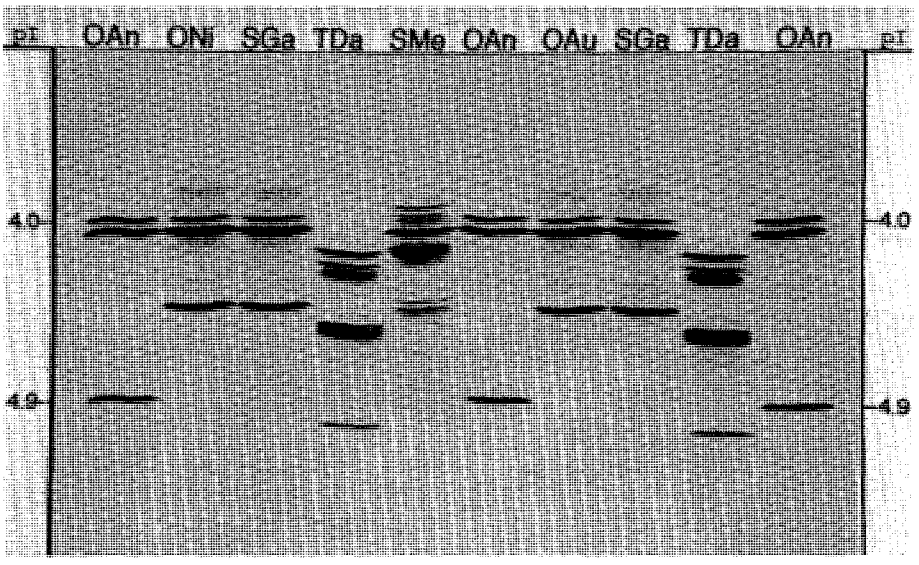


Fig. 4.18. IEF of tilapia parvalbumins (pH range 3.0-6.0): OAn, *O. andersonii*; OAu, *O. aureus*; ONi, *O. niloticus*; SGa, *S. galilaeus*; SMe, *S. melanotheron*; and TDa, *T. dageti*.

indicated that the banding patterns of *O. niloticus*, *O. aureus* and *S. galilaeus* were almost identical. However, *S. melanotheron* and *O. andersonii* showed species-characteristic profiles. For example, the overall combination of pI's 4.00, 4.08 and 4.87 is unique for *O. andersonii* whereas, two groups of bands, within the pI ranges of 3.95-4.00 and 4.17-4.21, are characteristic for *S. melanotheron* (Table 4.11, Fig. 4.18).

Fig. 4.19 shows IEF parvalbumin profiles for *Tilapia* spp. Based on the prominent components, *T. dageti* could be identified by a three-band set around pI 4.5, whereas a three-band set around pI 4.4 was characteristic for *T. guineensis*. The remaining species, *T. zillii*, *T. busumana* and *T. discolor*, all shared a distinctive three-band set around pI 4.25. However, *T. discolor* possessed another prominent component (pI 3.97) which distinguished it from *T. zillii* and *T. busumana* (see also Table 4.11).

Notes:

Comparative IEF analyses of muscle extracts, including parvalbumins, have been used successfully to identify various fish species and hybrids (e.g., Lundstrom and Roderick 1979; Lundstrom 1981; Monaco et al. 1982; Whitemore 1986; Macaranas et al. 1986; Girija and Rehbein 1988; Whitmore and Hellier 1988).

Table 4.11. Isoelectric points of tilapia parvalbumins. The major diagnostic components are framed. The minor components are in brackets (modified after Oberst et al. 1996).

T. dageti	*T. guineensis*	*T. zillii*	*T. busumana*	*T. discolor*	*O. niloticus/ O. aureus S. galilaeus*	*O. andersonii*	*S. melanotheron*
				3,97			3,95
					4,00	4,00	3,97
		4,06	(4,06)	(4,06)			4,00
					4,08	4,08	
	4,12	4,12	(4,12)	4,12			4,08
4,16	4,16	4,16	4,16	(4,16)			
							4,17
							4,19
							4,21
(4,22)		4,22	4,22	4,22			
(4,25)		4,25	4,25	4,25			
		4,30	4,30	4,30			
	4,35						
	4,40				4,40		(4,40)
	4,43						
4,49							
4,52							
4,57		4,55	4,55	4,55			
	4,86					4,87	
4,98		(4,98)	(4,98)				

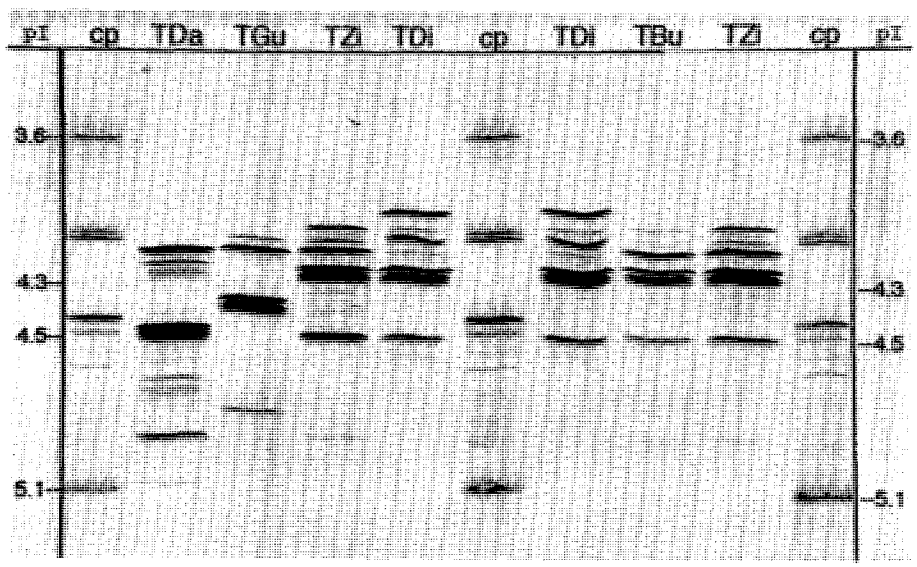


Fig. 4.19. IEF of tilapia parvalbumins (pH range 3.0-6.0): cp, calibration proteins; TBu, *T. busumana*; TDa, *T. dageti*; TDi, *T. discolor*; TGu, *T. guineensis*; TZi, *T. zillii* (Oberst, unpubl.).

Focant and Vandewalle (1991) analyzed skeletal muscle parvalbumins of *O. aureus, O. niloticus, O. macrochir, O. mossambicus, T. guineensis* and *T. rendalli* by IEF using pH gradients from pH 4 to 6. With the exception of *O. mossambicus*, parvalbumin profiles observed by their studies were only useful in discriminating between the two genera. Focant et al. (1992) and Huriaux et al. (1990, 1991) found variations in the parvalbumin profiles of *Barbus barbus* with different developmental stages and muscle sample locations in the dorso-lateral white muscle used. Our results, however, have shown no qualitative or quantitative differences in parvalbumin IEF profiles observed from different parts of the dorso-lateral white muscle of *O. niloticus* or among individuals ranging from 10 to 30 cm in standard length.

Oberst et al. (1996) found tilapia parvalbumins to be immunologically distinct: antisera raised against *T. guineensis* parvalbumins produced partly species-specific precipitation lines using techniques like Ouchterlony tests and immuno-electrophoresis. The species studied were: *O. niloticus, S. galilaeus, S. melanotheron, T. dageti, T. guineensis* and *T. zillii.*

4.4.4.2 Hemoglobin Analysis

The heterogeneity of hemoglobins of freshwater and marine teleost fishes has been well demonstrated electrophoretically (e.g., Bonaventura et al. 1975; Weber et al. 1976; Fyhn et al. 1979; Perez and Rylander 1985; Val et al. 1987; Di Prisco and Tamburrini 1992).

In our studies, hemoglobin samples (hemolysates) of 10 tilapia species (*O. andersonii, O. aureus, O. niloticus, S. galilaeus, S. melanotheron, T. busumana, T. dageti, T. discolor, T. guineensis* and *T. zillii*) and of one hybrid (F1 *O. niloticus* x *O. andersonii* (Hy)) were subjected to IEF using pH gradients from pH 3 to 10. Samples were freshly prepared as indicated in section 3.2.6. The objective was to identify species-characteristic hemoglobin patterns.

Figs. 4.20-4.22 show IEF separations of the hemoglobins of all these samples and demonstrate the high degree of heterogeneity within tilapia hemoglobin molecules. Estimated isoelectric points of prominent tilapia hemoglobins are ranged between pH 5.88 and pH 8.06. On average, 24 different hemoglobins have been detected per species (OAn: 22; OAu: 22; ONi: 26; SGa: 21; SMe: 22; TBu: 25; TDa: 26; TDi: 25; TGu: 26; TZi: 22), indicating the occurrence of multiple

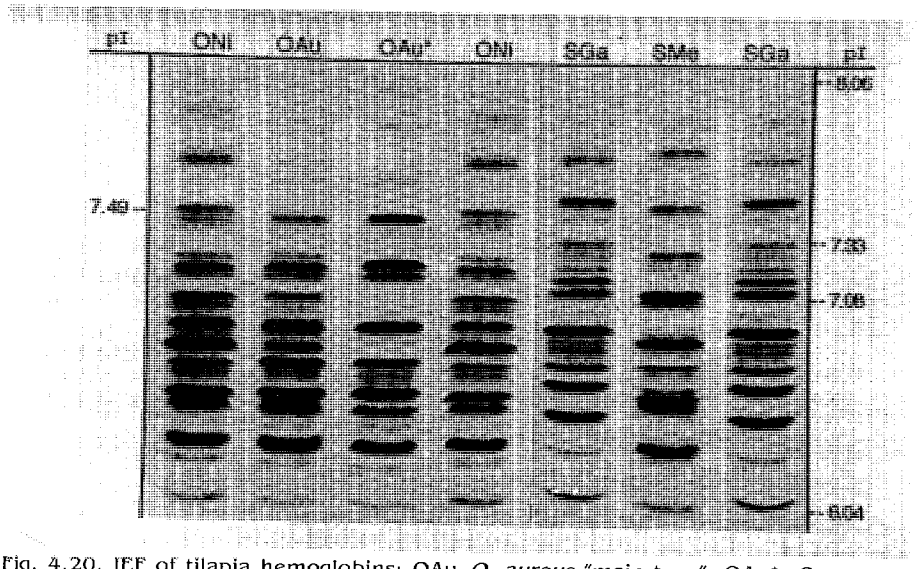


Fig. 4.20. IEF of tilapia hemoglobins: OAu, *O. aureus* "main type"; OAu*, *O. aureus* "odd type"; ONi, *O. niloticus*; SGa, *S. galilaeus*; and SMe, *S. melanotheron* (Falk et al., in press).

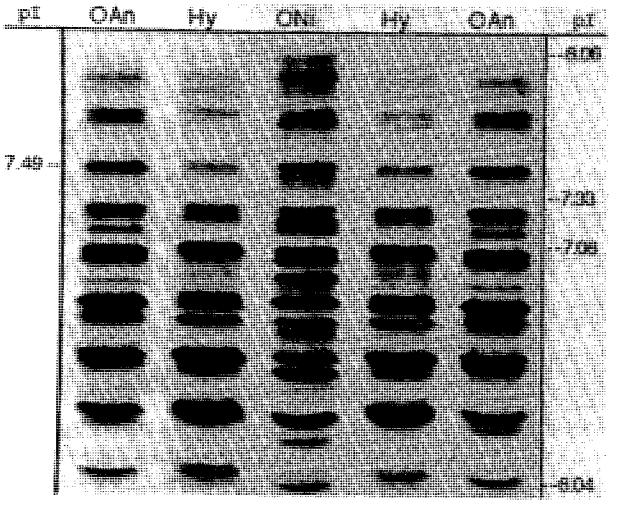


Fig. 4.21. IEF of tilapia hemoglobins: OAn, *O. andersonii*; ONi, *O. niloticus*; and Hy, ONi x OAn hybrid (Falk et al., in press).

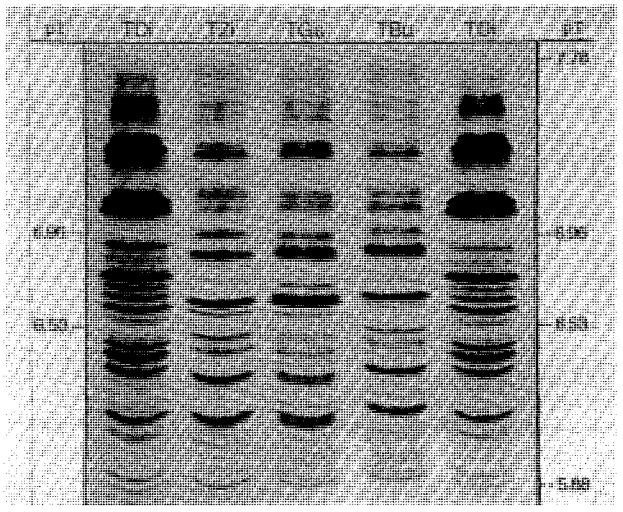


Fig. 4.22. IEF of tilapia hemoglobins: TBu, *T. busumana*; TDi, *T. discolor*; TGu, *T. guineensis*; and TZi, *T. zillii* (Falk et al., in press).

globin chain variants involved in the composition of different tetrameric hemoglobin types. Moreover, variations of hemoglobin types among tilapia species have been observed resulting in the identification of species characteristic hemoglobin band patterns. These species characteristics have been obtained constantly after isoelectric focusing of mercaptoethanol-treated hemolysates from 10 to 52 specimens each of one species. Only within the *O. aureus* specimens investigated did intraspecific variation occur. One individual obtained from the University of Stirling (see Fig. 4.20, OAu*) has been characterized by lacking four prominent hemoglobins compared to the patterns observed for all other *O. aureus* specimens studied.

Considering the pI ranges of hemoglobins observed for the tilapias investigated, genera characteristic differences have been detected. As a common feature of the species of the genus *Tilapia*, their most alkaline hemoglobins reached pI values only up to pH 7.78. By comparison, those of *Oreochromis* and *Sarotherodon* spp. reached pI values up to pH 8.06 and pH 7.96, respectively. Within the three genera species characteristic, hemoglobin patterns could be identified for almost all the species studied (Figs. 4.20-4.22), only *T. dageti* and *T. guineensis* remained indistinguishable. Tables 4.12 and 4.13 summarize some characteristic pI values of hemoglobin molecules leading to the identification of individual species. These pI ranges are also indicated in Figs. 4.20-4.22. Furthermore, ONi x OAn hybrid specimens could be distinguished from their parents by unique IEF patterns composed of individual parental hemoglobins within the pI

Table 4.12. Species-characteristic pI range of hemoglobin profiles of some mouthbrooding tilapias, *Oreochromis* and *Sarotherodon* spp. (Falk et al., in press).

O. niloticus	*O. aureus*	*O. andersonii*	*S. galilaeus*	*S. melanotheron*
			7.08	7.08
7.13	7.13	7.13	7.13	
7.18	7.18		7.18	7.18
		7.23		
7.25	7.25	7.25	7.25	7.25
			7.33	
7.49		7.49		

Table 4.13. Species-characteristic pI range of hemoglobin profiles of substrate spawning tilapias (*Tilapia* spp.) (Falk et al., in press).

T. zillii	*T. dageti, T. guineensis*	*T. busumana*	*T. discolor*
		6.96	
6.93	6.93		
		6.90	6.90
6.88	6.88		
			6.86
		6.73	
6.70	6.70	6.70	6.70
6.68	6.68		6.68
	6.62	6.62	6.62
6.60	6.60	6.60	
6.57	6.57		6.57
			6.53

range from pH 6.04 to 7.08 (Fig. 4.21). However, some hemoglobins present in one or even both parental species remained absent in all hybrid specimens studied.

Notes:

Other reports of multiple hemoglobins and species-specific hemoglobin patterns in fish include those of Chen and Tsuyuki (1970); Hines et al. (1971); Fyhn et al. (1979); Ermolenko and Viktorovskij (1983); Grigorjeva and Sideleva (1985); Perez and Rylander (1985); Val et al. (1987); Arefjev and Karnauchov (1989); Oberst et al. (1989); Falk (1992, 1994); and Macaranas et al. (1996).

Ontogenetic variations in hemoglobin profiles have been documented in both oviparous and viviparous fish. The salmonids

especially display a general increase of cathodic hemoglobins from early life history stages to adulthood (Giles and Vanstone 1976; Ingermann and Terwilliger 1982; Weber and Hartvig 1984; Wilkins 1985; Giles and Rystephanuk 1989; Korsgaard and Weber 1989; and Fyhn and Whitler 1991).

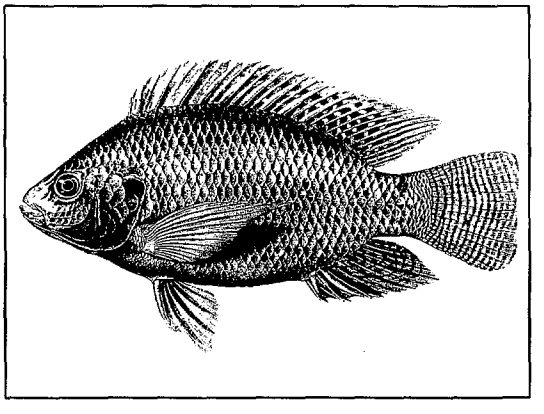
There are also several examples, mainly in catadromous and anadromous species of fish that show striking differences in the functional properties of their various hemoglobin components (e.g., Hashimoto et al. 1960; Binotti et al. 1971; Powers 1972; Powers and Edmundson 1972a, 1972b; Gillen and Riggs 1973; Brunori 1975; and Weber and de Wilde 1976).

5.0 Illustrated Summary and Matrix of Diagnostic Biochemical Techniques for Tilapia Characterization

The taxonomic value of the various biochemical techniques described in this manual is clearly indicated. By combining them, unambiguous identification of tilapia species can be achieved even when striking phenotypic species markers are missing. Such "protein-based" characterization can be supported and combined with classical descriptive taxonomy (morphology, morphometrics, meristics, color, etc.). This chapter therefore provides some illustrations of tilapia species and a diagnostic matrix for biochemical techniques for species discrimination. Using combinations of at least two different diagnostic biochemical techniques helps to resolve problems with samples for which a single technique gives unclear results.

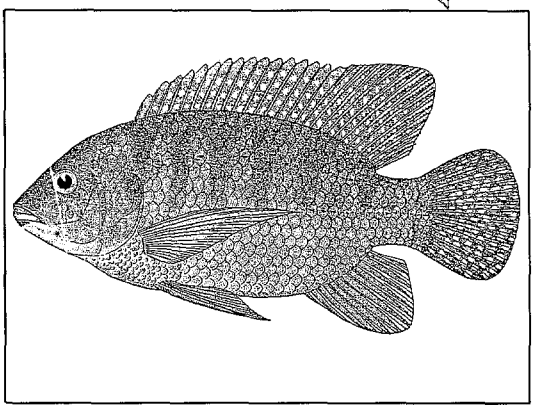
a. *Oreochromis niloticus*
(after Boulenger 1909)
Recommended diagnostic tests:

1. Agglutination assays with enzyme-treated erythrocytes
2. Allozyme electrophoresis of muscle extracts (e.g., MDH)
3. Globin chain electrophoresis (AU-PAGE)
4. IEF of hemoglobins



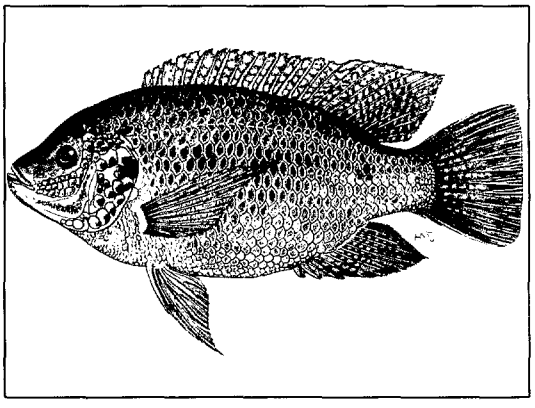
b. *Oreochromis aureus*
(after Daget 1954)
Recommended diagnostic tests:

1. Agglutination assays with enzyme-treated erythrocytes
2. Globin chain electrophoresis (AU-PAGE)
3. IEF of hemoglobins



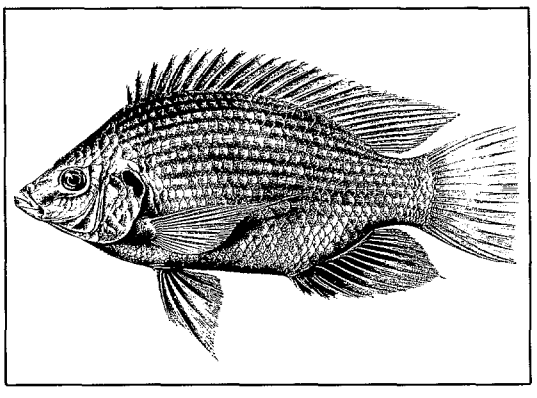
c. *Oreochromis andersonii*
(after Jubb 1967)
Recommended diagnostic tests:

1. Globin chain electrophoresis (AU-PAGE)
2. IEF of hemoglobins
3. IEF of parvalbumins



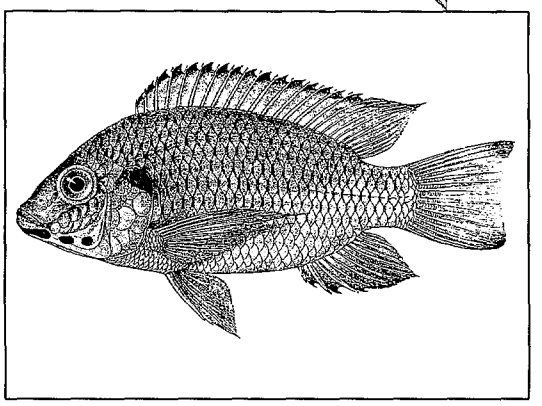
d. *Sarotherodon galilaeus*
(after Boulenger 1909)
Recommended diagnostic tests:

1. Agglutination assays with enzyme-treated erythrocytes
2. Allozyme electrophoresis of muscle extracts (e.g., MDH, PGI)
3. Globin chain electrophoresis (AU-PAGE)
4. IEF of hemoglobins



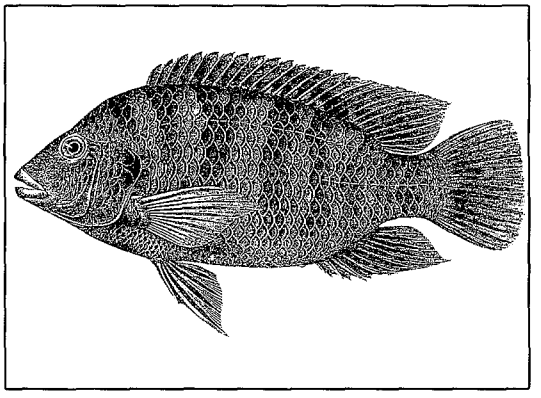
e. *Sarotherodon melanotheron*
(after Boulenger 1915)
Recommended diagnostic tests:

1. Allozyme electrophoresis of muscle extracts (e.g., MDH)
2. Globin chain electrophoresis (AU-PAGE)
3. Gradient PAGE of parvalbumins
4. IEF of parvalbumins
5. IEF of hemoglobins



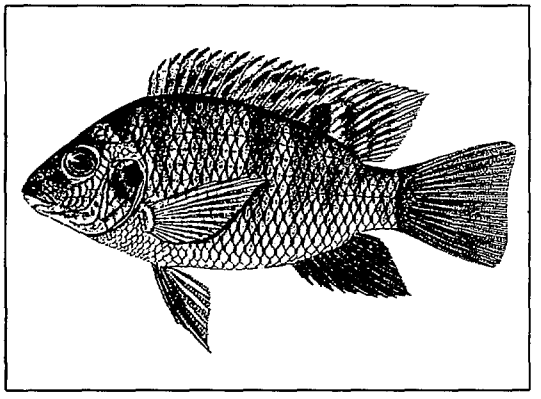
f. *Tilapia zillii*
(after Boulenger 1901)
Recommended diagnostic tests:

1. Agglutination assays with untreated erythrocytes
2. Allozyme electrophoresis of muscle extracts (e.g., PGI, LDH)
3. Globin chain electrophoresis (AUT-PAGE)
4. IEF of hemoglobins

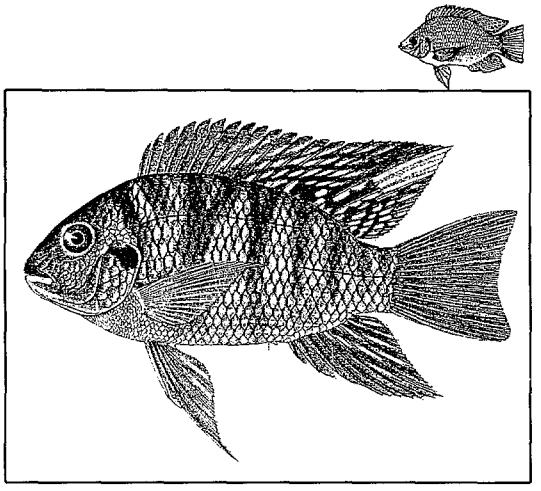


g. *Tilapia guineensis*
(after Teugels and Thys van den Audenaerde 1992)
Recommended diagnostic tests:

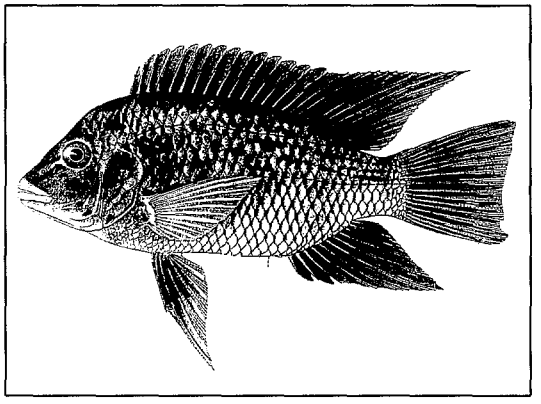
1. Allozyme electrophoresis of muscle extracts (e.g., PGI)
2. IEF of parvalbumins



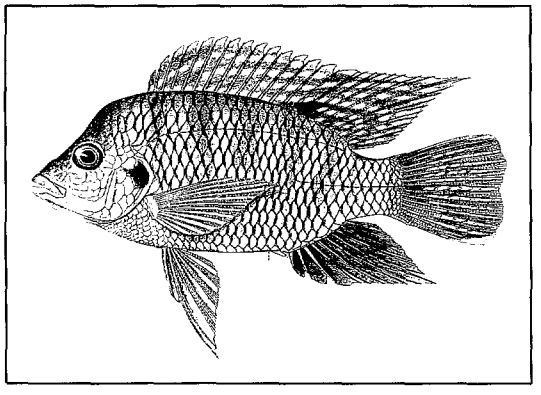
h. *Tilapia dageti*
(after Teugels and Thys van den Audenaerde 1992)
Recommended diagnostic tests:
1. Allozyme electrophoresis of muscle extracts (e.g., LDH)
2. IEF of parvalbumins



i. *Tilapia discolor*
(after Teugels and Thys van den Audenaerde 1992)
Recommended diagnostic tests:
1. Allozyme electrophoresis of muscle extracts (e.g., PGI)
2. Globin chain electrophoresis (AU-PAGE)
3. IEF of hemoglobins
4. IEF of parvalbumins



j. *Tilapia busumana*
(after Teugels and Thys van den Audenaerde 1992)
Recommended diagnostic tests:
1. Globin chain electrophoresis (AUT-PAGE)
2. IEF of hemoglobins



Matrix of the diagnostic biochemical tests and assays for the discrimination of some tilapia species.

	OAu	OAn	SGa	SMe	TZi	TGu	TDa	TDi	TBu
ONi	1b; 3a; 4a	3a; 4a; 4b	1b; 2; 3a; 4a	1a; 2; 3a; 4a,b	1a; 2; 3a; 4a,b	1a; 2; 3a; 4a,b	1a; 3a; 4a,b	2; 3a; 4a,b	2; 3a; 4a,b
OAu		3a; 4a,b	1b; 3a; 4a	1a; 3a 4a,b	1a; 3a; 4a,b	1a; 3a; 4a,b	1a; 3a; 4a,b	3a; 4a,b	3a; ra,b
OAn			3a; 4a,b	3a; 4a,b	3a; 4a,b	3a; 4a,b	3a; 4a,b	3a, 4a,b	3a; 4a,b
SGa				1a; 2; 3a; 4a,b	1a; 2; 3a; 4a,b	1a; 2; 3a; 4a,b	1a; 3a; 4a,b	2; 3a; 4a,b	2; 2a; 4a,b
SMe					1a; 2; 3a; 4a,b	1a; 2; 3a; 4a,b	1a; 3a 4a,b	2; 3a; 4a,b	2; 3a; 4a,b
TZi						1a; 2; 3b; 4a,b	1a; 2; 3b; 4a,b	2; 3a; 4a,b	2; 3b 4a
TGu							2; 4b	2; 3a; 4a,b	2; 3b; 4a,b
TDa								3a; 4a,b	3b; 4a,b
TDi									2; 3a; 4a,b

ONi: *O. niloticus*
OAu: *O. aureus*
OAn: *O. andersonii*
SGa: *S. galilaeus*
SMe: *S. melanotheron*
TZi: *T. zillii*
TGu: *T. guineensis*
TDa: *T. dageti*
TDi: *T. discolor*
TBu: *T. busumana*

1a: Agglutination assay with untreated erythrocytes.
1b: Agglutination assay with enzyme-treated erythrocytes.
2: Allozyme electrophoresis using different enzyme-staining recipes.
3a: Acidic urea PAGE of globin chains.
3b: Acidic urea triton PAGE of globin chains.
4a: Isoelectric focusing of hemoglobins.
4b: Isoelectric focusing of parvalbumins.

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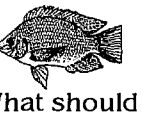

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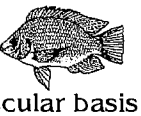

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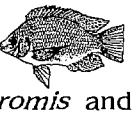

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