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TECHNIQUES OF STARCH-GEL ELECTROPHORESIS OF PENAEID PRAWN ENZYMES (*PENAEUS* SPP. AND *METAPENAEUS* SPP.)

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ABSTRACT

Standard starch-gel electrophoretic and histochemical staining techniques suitable for penaeid prawns are documented including modifications to increase productivity and reduce cost. A new technique for histochemical staining of 1-pyrroline dehydrogene (1-Pydh) is described.

INTRODUCTION

During the past fifteen years there has been a proliferation of studies on the genetic variation in species (Ayala 1976; Selander and Johnson 1973). This major advancement in our knowledge of the genetic structure of populations and species is directly related to the development of electrophoretic techniques and histochemical staining (Smithies 1959; Hunter and Markert 1957).

When we began our studies on genetic variation in penaeid prawns, a variety of electrophoretic procedures were initially attempted. Few of these procedures were described adequately for use with prawns or other crustaceans. We therefore had to develop a set of techniques suitable for broad scale genetic variation studies of prawns. Technique development attempted to increase gel resolution, speed and efficiency of procedures while reducing the cost of staining. Standardisation of buffers and stain solutions was an important factor in our streamlining of laboratory techniques. This paper summarises our experience and the techniques are detailed so that future users may avoid the difficulties we encountered. Although these techniques were developed for penaeid prawns, they should be applicable, with a minimum of testing, to other Crustacea. The major sources consulted were Giblett (1969), Smith (1968), Brewer (1970), Manwell and Baker (1970), Ayala *et al.* (1972, 1973) and Selander *et al.* (1969). Chemicals used in staining are given in Table 1, and Table 2 lists enzyme proteins examined.

TABLE 1. Chemicals Used in Staining

Abbreviation	Name
ADP	Adenosine 5′-diphosphate
ATP	Adenosine 5′-triphosphate
EDTA(Na_2)	Ethylenediamine tetraacetic acid, disodium salt
EDTA	Ethylenediamine tetraacetic acid, free acid
F-1,6-DP	D-fructose-1,6-diphosphate
F-6-P	D-fructose-6-phosphate
G-1-P	α-D-glucose-1-phosphate
HCl	Hydrochloric acid
LiOH	Lithium hydroxide
$MgCl_2$	Magnesium chloride
$MnCl_2$	Manganese chloride
M-6-P	D-Mannose-6-phosphate
MTT	3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide
β-NAD	β-nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
PMS	Phenazine methosulphate
P-5′-P	Pyridoxyl-5′-phosphate
Na_3AsO_4	Sodium arsenate
NaOH	Sodium hydroxide
Tris	Tris(hydroxymethyl) aminomethane

TABLE 2. Enzyme Proteins Examined

Abbreviation	E.C. No.	Name
Acph	3.1.3.2	Acid phosphatase
Adk	2.7.4.3	Adenylate kinase (myokinase)
Ald	4.1.2.13	Aldolase
Ao	1.2.3.1	Aldehyde oxidase
Aph	3.1.3.1	Alkaline phosphatase
Est	3.1.1.	Esterase
Fum	4.2.1.2	Fumerase
Gdh	1.4.1.3	Glutamate dehydrogenase
Got	2.6.1.1	Glutamic oxalacetic transaminase
G-3-pdh	1.2.1.12	Glyceraldehyde-3-phosphate dehydrogenase
α-Gpdh	1.1.1.8	α-Glycerophosphate dehydrogenase
Hk	2.7.1.1	Hexokinase
Lap	3.4.1.1	Leucine amino peptidase
Ldh	1.1.1.27	L-Lactic dehydrogenase
Mdh	1.1.1.37	Malate dehydrogenase
Me	1.1.1.40	Malic enzyme
Mpi	5.3.1.8	Mannose phosphate isomerase
Odh	1.1.1.1	Octanol (alcohol) dehydrogenase
Pep	3.4.4.	Peptidase
Pgi	5.3.1.9	Phosphoglucoisomerase
Pgm	2.7.5.1	Phosphoglucomutase
6-Pgdh	1.1.1.44	6-Phosphogluconate dehydrogenase
Pt		General (total) protein
1-Pydh	1.5.1.12	1-Pyrroline dehydrogenase
Sdh	1.1.1.14	Sorbitol dehydrogenase
To		Tetrazolium oxidase
Tr		Tetrazolium reductase
Tyr	1.14.18.1	Tyrosinase
Tpi	5.3.1.1	Triosephosphate isomerase

Note: Esterase and peptidase refer to groups of enzymes. Tetrazolium oxidase and tetrazolium reductase are non-specific enzymes whose function is unknown.

PROCEDURES

Sample Collection and Storage

All prawns were collected in remote tropical Australia, mostly from trawls off the R.V. *Kalinda*, a CSIRO research vessel based in the Gulf of Carpentaria. Once prawns were caught, they were frozen in an ultra-low temperature freezer (-80°C). On later cruises, prawn samples were frozen in the holds of a commercial prawn trawler. For larger species, the cephalothorax and first abdominal segment only were collected.

Frozen samples were transported to the Cleveland laboratory on dry ice, and were stored at -90°C until the day of running. No dehydration of samples has been found and the development of black-spot (Ruello 1975) is non-existent if prawns are kept at this temperature. Prawns have been stored for up to six months with no apparent deterioration in resolution.

Sample Preparation

Abdominal muscle and cephalothorax tissue were routinely tested. A small quantity (1 cc) of tissue was cut from the prawn (Figure 1) and mixed with a grinding buffer (1.21 g Tris: 0.37 g EDTA(Na_2): 0.00153 g NADP per 1 H_2O, pH adjusted to 6.8 with HCl). Muscle samples were quite viscous unless two volumes

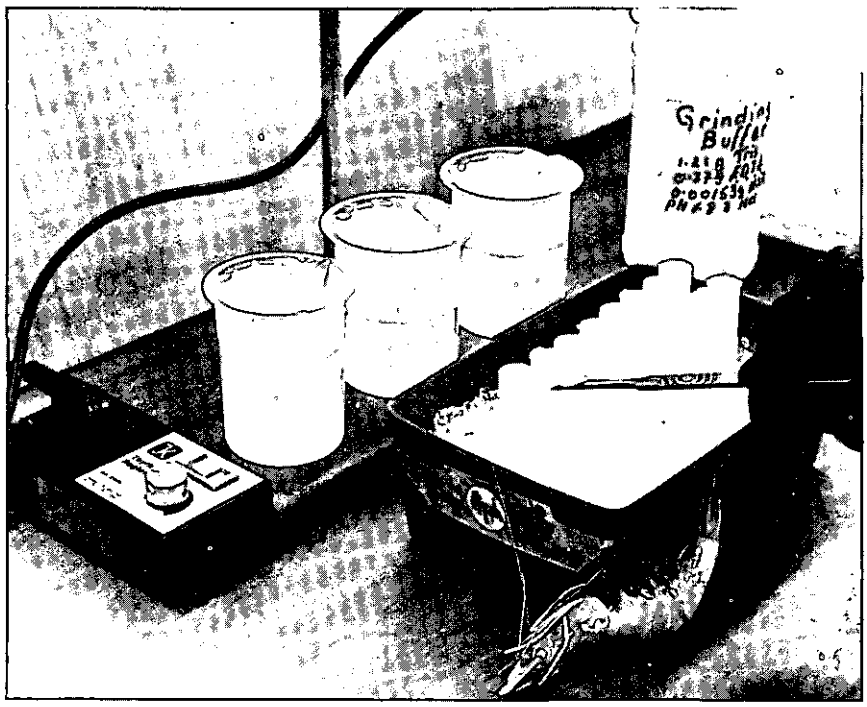


Figure 1. Approximately 1 cc of tissue was placed into centrifuge tubes with 1 or 2 cc of grinding buffer for cephalothorax or muscle respectively.

of grinding buffer were added; the cephalothorax tissue could be ground with only a single volume of grinding buffer.

Tissues were ground with an Ultra-Turrax tissue grinder (Figure 2). We favoured this grinder because of its speed, ease of cleaning, and safety.

Macerated samples were spun at 27,000 G in a high speed refrigerated centrifuge (Sorvall RC5, Superspeed) at 4°C for 20 minutes. The supernatant was decanted into a separate container for storage and later electrophoresis.

Gel Buffers

A variety of buffers currently used in electrophoresis of vertebrate and invertebrate tissues were tried to find the best buffers for each enzyme. We found that a good buffer for a given enzyme in one species may not be a good buffer for that enzyme in another species. Buffers fall into two categories: continuous, where the buffer in the gel is of the same chemical composition as in the electrode; and discontinuous, (Poulik 1957) where the gel and electrode buffers are of different chemical composition. We give the composition of different buffers tried in our laboratory, their running conditions, and comment on their suitability.

A. Tris-citric-boric-NaOH (Poulik 1957)

Gel, pH 8.65	*Electrode, pH 8.1*
9.20 g/l Tris	18.55 g/l Boric acid
1.05 g/l Citric acid	2.40 g/l NaOH

Running conditions: 200 V, 4 hours.

Comments: A discontinuous buffer. We recommend the next buffer, A', in preference to this one.

A'. Tris-citric-boric-LiOH (Ferguson and Wallace 1961)

Gel, pH 8.31	*Electrode, pH 8.26*
3.63 g/l Tris	2.51 g/l LiOH
1.05 g/l Citric acid	18.54 g/l Boric acid
10 ml/l electrode buffer	

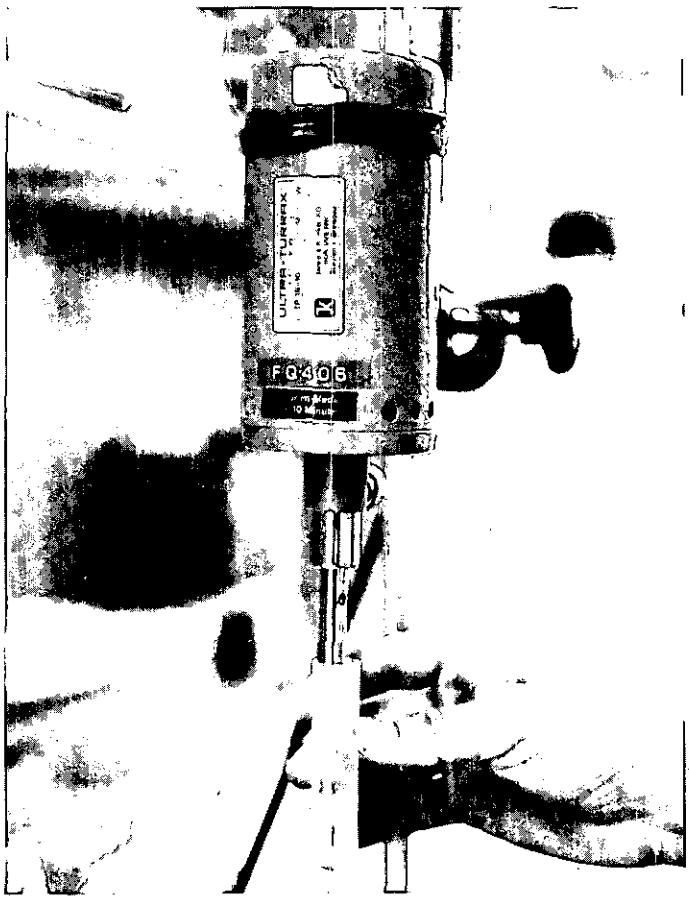


Figure 2. The tissue samples were quickly and efficiently macerated between samples.

Running conditions: 175 V, 4 hours, or until visible borate front has moved 7 cm beyond the insertion point.

Comments: Discontinuous buffer. This buffer is superior to A because the resolution is sharper and separation is better.

Enzymes: Ald, Got, G-3-pdh, Ldh, Mdh, Me, Mpi, Pgi, Pt, Tpi, Tr.

B. Tris-boric-EDTA (Ayala *et al.* 1972)

Gel and Electrode, pH 9.0

10.53 g/l Tris
0.54 g/l Boric acid
0.39 g/l EDTA(Na_2)

Running conditions: 300 V, 6 hours

Comments: A continuous buffer. This is a very good buffer for a variety of dehydrogenases, but it can mask polymorphism in some systems.

Enzymes: Ald, Gdh, Got, Me, Mpi, Odh, Pt, 1-Pydh, Sdh, Tpi

C. Tris-citric-EDTA (Ayala *et al.* 1972)

Gel, pH 7.0	***Electrode, pH 7.0***
1.09 g/l Tris	16.35 g/l Tris
0.63 g/l Citric acid	9.45 g/l Citric acid
0.47 g/l EDTA(Na_2)	0.47 g/l EDTA(Na_2)

Running conditions: 150 V, 5 hours.

Comments: A continuous buffer. We use this buffer in preference to D. below. pH is adjusted to 7.0 with 2M Tris.

Enzymes: Adh, Ald, Est, Got, G-3-pdh, α-Gpdh, Ldh, Mdh, Me, Mpi, 6-Pgdh, Pgm, Pgi, Pt, Tpi.

D. Tris-citric (Ayala *et al.* 1972)

Gel, pH 7.0	*Electrode, pH 7.0*
1.09 g/l Tris	16.34 g/l Tris
0.63 g/l Citric acid	9.45 g/l Citric acid

Running conditions: 150 V, 5 hours

Comments: See buffer C.

E. Tris-HCl (Selander *et al.* 1971)

Gel, pH 8.5	*Electrode, pH 8.1*
1.21 g/l Tris	18.55 g/l Boric acid
HCl to pH 8.5	2.4 g/l NaOH

Running conditions: 300 V, 4 hours

Comments: A discontinuous buffer.

Enzymes: Est (muscle), Gdh, Ldh, Mdh, Me, Mpi, Odh, Pgm, Pt, 1-Pydh, To, Tpi, tr.

Gel Preparation

Two sizes of gel mould were used, 20 cm x 20 cm x 1 cm, and 20 cm x 20 cm x 0.6 cm. The 1 cm thick gel needs 450 ml of buffer and the 0.6 cm thick gel needs 300 ml of buffer. The technique for making the gels was virtually identical to that described by Smith (1968). The relevant amount of buffer in a 1 l sidearm flask was combined with the 12.5 g (per 100 ml of buffer) of hydrolyzed potato starch (Connaught Laboratories, Toronto) and thoroughly mixed. This solution was heated while continuously swirling over an open bunsen burner flame (Figure 3). While heating, care was taken to neither scorch the gel solution nor break the flask by heating too quickly. Once this solution was heated to 80°C (a point at which the solution becomes translucent and viscous) the sidearm flask was attached to an aspirator and excess air was removed under reduced pressure (Figure 4). The degassed solution was quickly poured into the mould and allowed to set for 30 minutes before covering with plastic film (Glad-wrap). The gels were allowed to set at room temperature for at least 2 hours or left overnight in the refrigerator before loading.

Sample Application and Running

When gels had cooled sufficiently, a series of 20 or 24 slots were cut in the gel using a specially made slit-slicer (Figure 5). An extract of muscle or cephalothorax was absorbed onto a small (6 mm x 10 mm, or 6 mm x 6 mm) piece of Whatman #3 CHR filter paper and the filter paper was inserted into a slit in the gel. After the samples were applied to a gel, it was put into a specially designed gel holder (Figure 6). The gel-electrode contact was via sponge wicks to the buffer trays and from there a power source (Auditec Power Supply 1012-500, Sydney).

It was desirable to keep the current of an individual gel below 70 mA (Figure 7). The Auditec power supply was divided between ten different output modules through a distribution box making it possible to run up to ten gels from a single power source.

Gels were run in a cold room or refrigerator at 4°C to prevent overheating. Running in a cold room was far superior to a refrigerator because all of the heat generated could be more easily dissipated. After the proper running time, the gel was disconnected from the power source, removed from the cold room, removed from the mould, and prepared for multiple slicing.

Gel Slicing for Multiple Staining

The gel was prepared for slicing by cutting and discarding end portions of the gel. The insertion point was marked by clipping the corner. The gel was placed on a plexiglass sheet and sliced into 1.0 or 1.5 mm thin slabs. Practice is required in order to obtain uniform slices.

We sliced the gel by stacking two series of 1.0 mm (or 1.5 mm) x 10 mm x 250 mm plexiglass strips on either side of the prepared gel and drawing a tight 'E' violin string through the gel. The top slice was discarded and one strip on each side of the gel removed (Figure 8). A new cut was made and the slice was covered with a sheet of GAF matt finish drafting film (Type U.F.2003) slightly larger than the slice. By carefully lifting the edge of the gel, the slice adhered to the film and was easily lifted from the gel. This process was repeated until the desired number of slices were obtained. The drafting film was essential to the successful multiple slicing of gels. Gel slices were placed on a large clear plastic sheet (5 mm thick) for

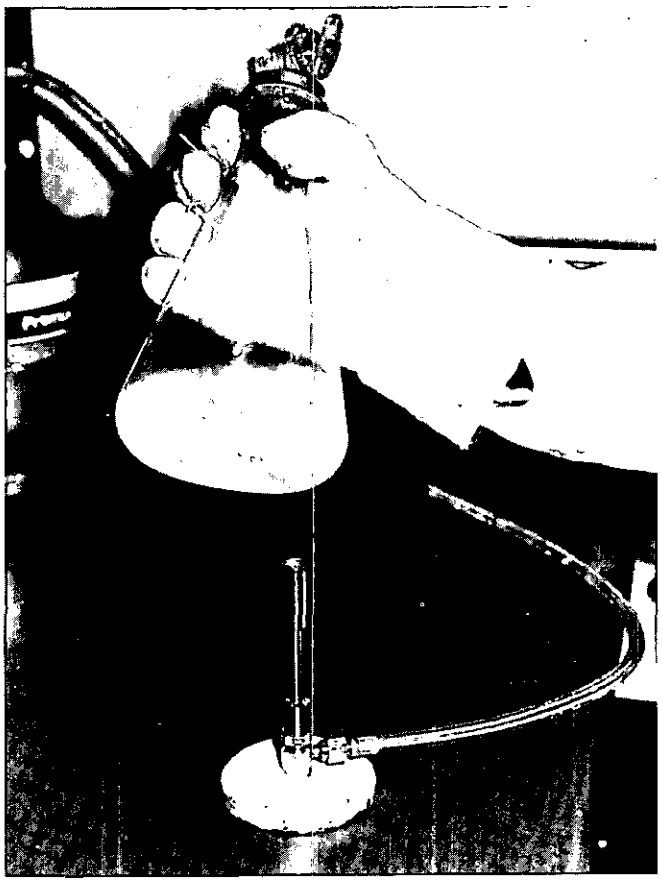


Figure 3. Heating of the starch and gel buffer suspension over a gas flame required continuous agitation.

agar overlay staining or put into a staining tray. Information regarding the gel was written directly on the drafting film.

Staining Procedures

Two methods of staining were used, one using a staining bath and the other using agar overlay. Virtually all of the following stains have been tried with agar overlay, but some cannot be done with the overlay. Therefore, the staining procedures given below are divided into those using a staining bath and those using agar overlay. Before the specific method for each stain is given, an outline of the chemical composition of the stock staining buffers and solutions used is provided.

A. Stock Staining Buffers

1. Tris-HCl, 0.2M (pH variable from 7.0 to 8.5).

24.2 g Tris
900 ml water

Adjust pH to desired level with *concentrated HCl. Top up to 1000 ml with* water.

Stock Tris-HCl buffers at the following pH's were used: 7.0, 7.5, 8.0 and 8.5. Care must be taken as pH 7.0 is approached, because this is near the limit of the buffering capacity of Tris.

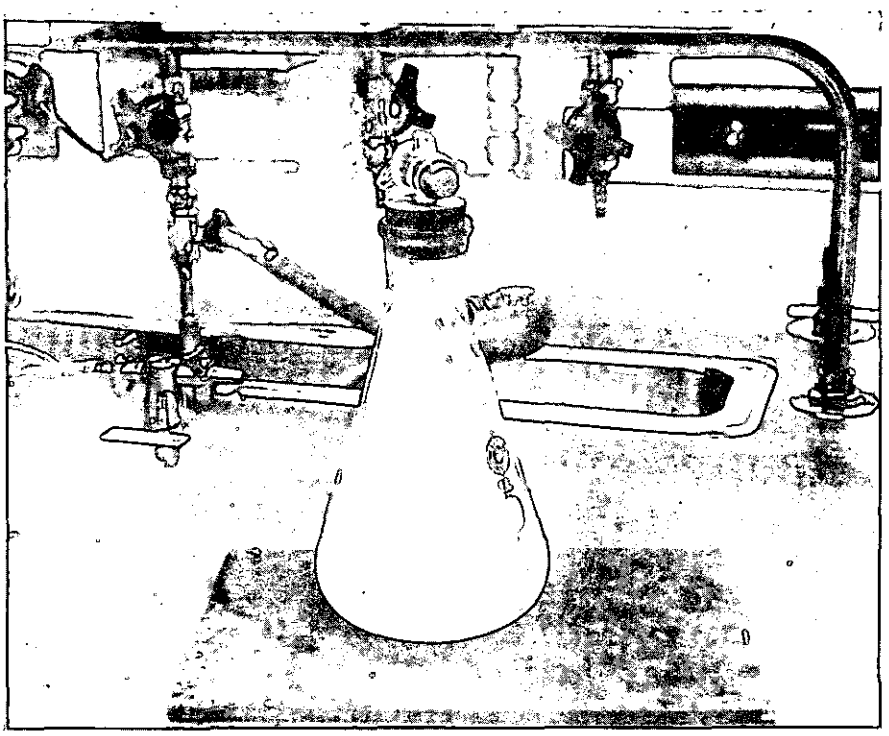


Figure 4. Air bubbles were eliminated, before pouring the gel, under vacuum (degassing).

2. Acetate, 0.2M, pH 5.0

3.3 ml glacial acetic acid
950 ml water
19.2 g sodium acetate

Adjust pH with glacial acetic acid. Top up to 1000 ml with water.

3. Tris-maleate, 0.2M, pH 5.2

24.2 g Tris
20.0 g maleic acid
950 ml water

Adjust pH to 5.2 with 1M maleic acid. Top up to 1000 ml with water.

B. Stock Solutions used in Staining

Many of the staining methods require very small amounts of expensive chemicals or biochemicals and we found it was difficult to measure these small amounts consistently and rapidly. Most of the chemicals are stable in solution, and solutions of all of them were made. Volumes of the solution were substituted for weighed amounts. All these solutions should be stored cold in a refrigerator (4°C). When gels were being run, occasionally it was more effective to prepare the stains from fresh material. Solutions of MTT, PMS α- and β-naphthyl acetate, however, are stable and very useful even for small amounts of staining.

Stock solutions of the reagents, cofactors and substrates were prepared as follows:

Reagents and Cofactors:

1. Tetrazolium Salt (10 mg/ml) : 1 g of MTT in 100 ml water (store in the dark.)
2. β-nicotinamide adenine dinucleotide (20 mg/ml) : 2 g β-NAD in 100 ml water.
3. Nicotinamide adenine dinucleotide phosphate (4 mg/ml): 400 mg NADP in 100 ml water.
4. Phenazine methosulphate (2 mg/ml): 200 mg PMS in 100 ml water. (Store in a black bottle in the dark.)
5. Adenosine triphosphate (20 mg/ml): 1 g ATP in 50 ml water.
6. Adenosine diphosphate (20 mg/ml): 500 mg ADP in 25 ml water.

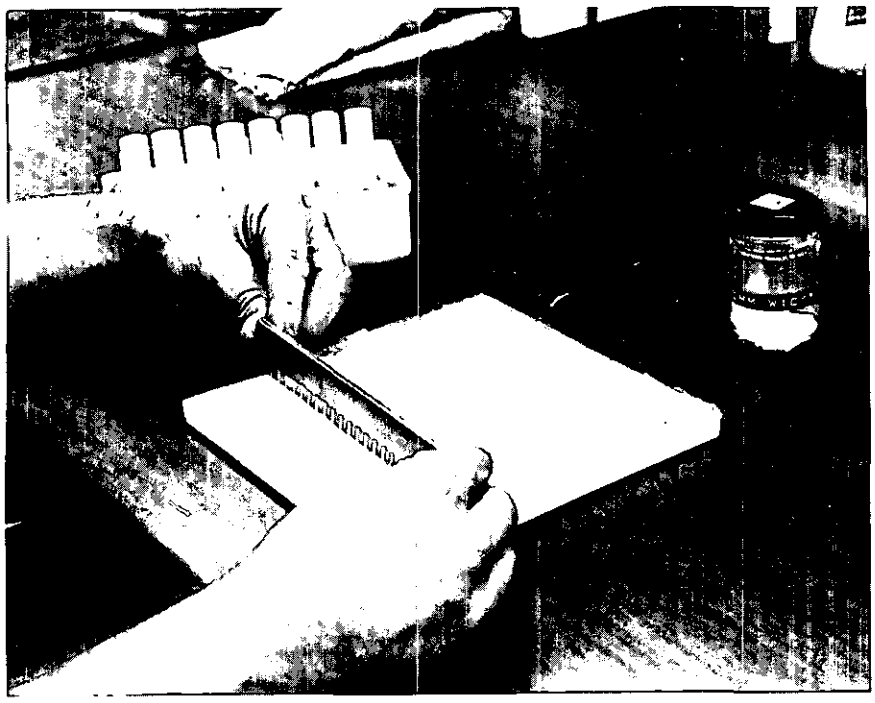


Figure 5. The toothed slicer used for making insertion slots for the supernatant soaked filter paper wicks.

7. Pyridoxyl-5'-phosphate (20 mg/ml): 1 g P-5'-P in 50 ml water.
8. Magnesium chloride (20 mg/ml): 20 g $MgCl_2$ in 1000 ml water.
9. Manganese chloride (20 mg/ml): 20 g $MnCl_2$ in 1000 ml water.
10. Sodium arsenate (15 mg/ml): 1.5 g Na_3AsO_4 in 100 ml water.
11. Disodium ethylene diamine tetra-acetic acid (20 mg/ml): 200 mg EDTA(Na_2) in 10 ml water.
12. α-Naphthyl acetate (10 mg/ml): 1 g Na-α-N-A dissolved in 50 ml acetone; 50 ml water then added.
13. β-naphthyl acetate (10 mg/ml): 1 g Na-β-N-A dissolved in 50 ml acetone; 50 ml water then added.
14. 0.5M boric acid: 30.9 g in 1000 ml water.

Substrates:

(Add 1 drop of chloroform to all of these at the start of each week.)

15. 6-phosphogluconic acid (100 mg/ml): 1 g of 6-PGA in 10 ml water.
16. Fructose-6-phosphate (20 mg/ml): 200 mg F-6-P in 10 ml water.
17. Glucose-1-phosphate (20 mg/ml): 200 mg G-1-P in 10 ml water.
18. Fructose-1,6-diphosphate (55 mg/ml): 550 mg F-1,6-DP in 10 ml water.
19. Mannose-6-phosphate (10 mg/ml): 100 mg M-6-P in 10 ml water.
20. Potassium-fumarate (100 mg/ml): 1 g in 10 ml water.
21. Sodium-glutamate (500 mg/ml): 10 g in 20 ml water.
22. α-glycerophosphate (80 mg/ml): 800 mg in 10 ml water.
23. Glucose (900 mg/ml): 90 g in 100 ml water.
24. Malic acid (10 mg/ml): 100 mg in 10 ml water.
25. Pyroglutamic acid (100 mg/ml): 1 g in 10 ml water.
26. Catechol (2 mg/ml): 200 mg in 10 ml water.

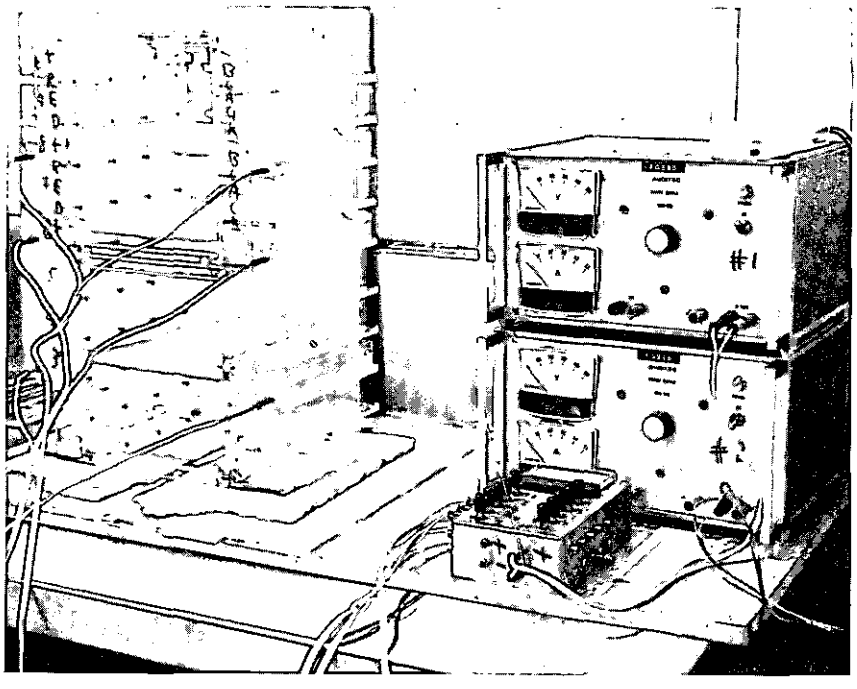


Figure 6. The cold room arrangement for running the gels. Note the power supply units feeding into the distribution box which distributes current to the buffer tanks flanking the gels in the gel holder. Generally, proteins migrated from negative to positive, or right to left in the photograph.

27. L-proline (2 mg/ml): 200 mg in 100 ml water.

C. Staining procedures

As a standard operating procedure, a supply of staining solution (minus the unstable or coloured reactants) is made up weekly. In the procedures outlined below, both a single staining method and a method using the pre-mixed solutions is given. Using the pre-mixed methods, the time needed to make up stains has been reduced by at least 75%.

I. STAINS USING AGAR OVERLAY

In these procedures, 5 ml of premixed staining solution is mixed with 15 ml of 2% agar solution (50° to 60°C) and the appropriate amount of coloured reactants, and poured directly over the gel. As the agar cools, the solution solidifies on the surface of the gel. To make the agar solution, we boil the desired quantity of distilled water, add the dry agar, and mix the solution on a magnetic stirrer until the temperature drops to 50°C. All of these procedures require the gel to be incubated in darkness at 30-37°C for 10 minutes or longer. Where PMS is used in these solutions, it should be added immediately prior to staining and out of direct sunlight.

1. Adenylate Kinase (Adk)

Tissue: Muscle

Single Stain Method

1 ml ADP solution
200 mg glucose
4 ml $MgCl_2$ solution
2 ml NADP solution
2 ml MTT solution
10 units G-6-pdh
14 units hexokinase
1 ml PMS solution
5 ml Tris-HCl, pH 7.0
15 ml 2% agar

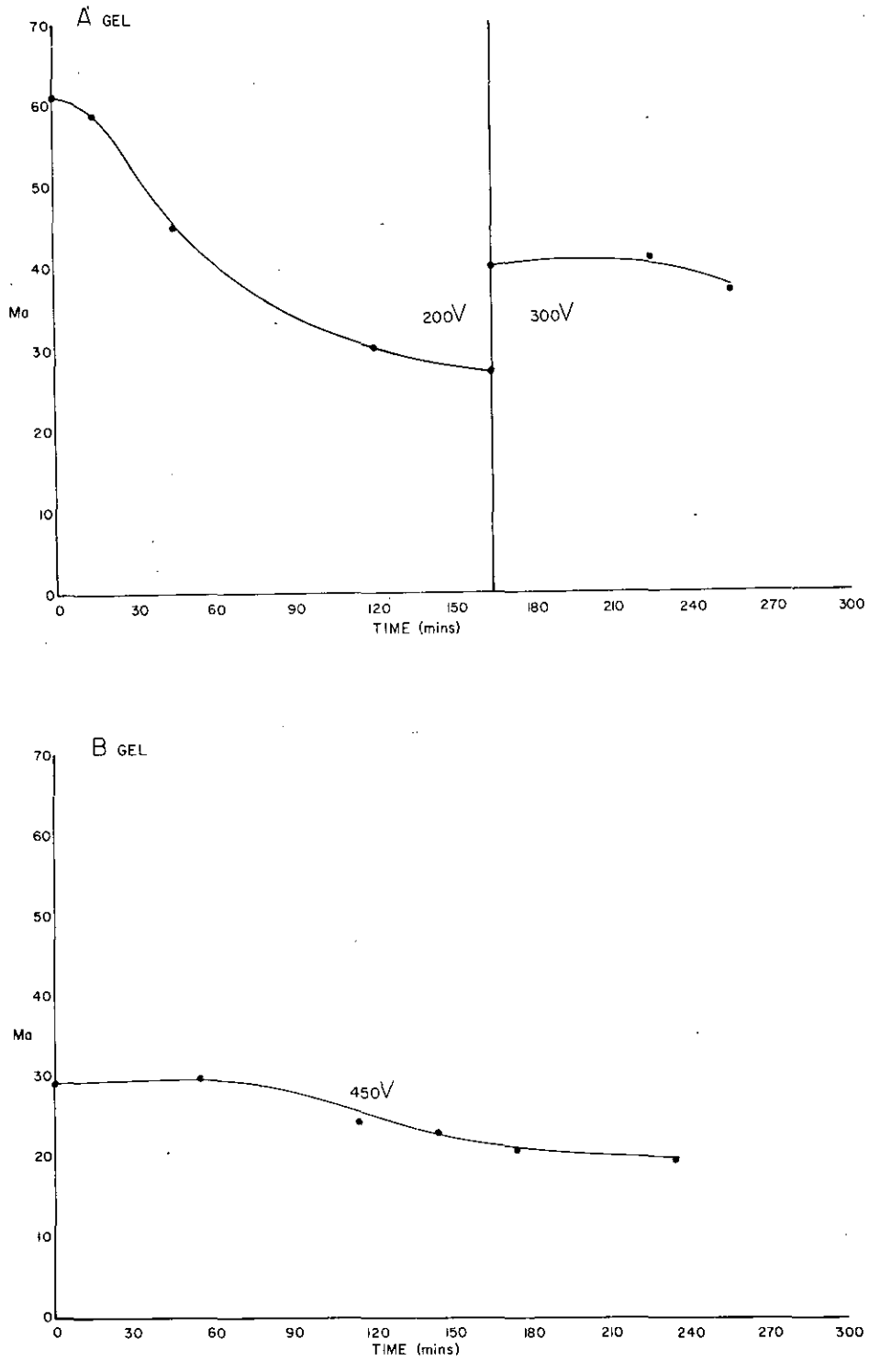
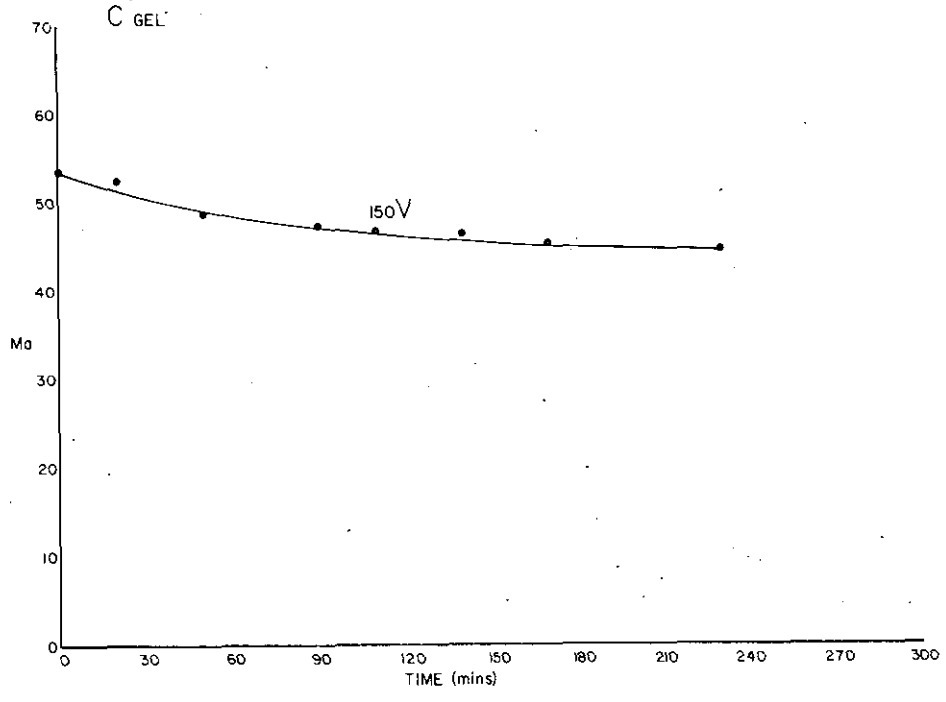


Figure 7. (A) and (B). Some examples of voltage and current characteristics of various 1 cm thick gels.



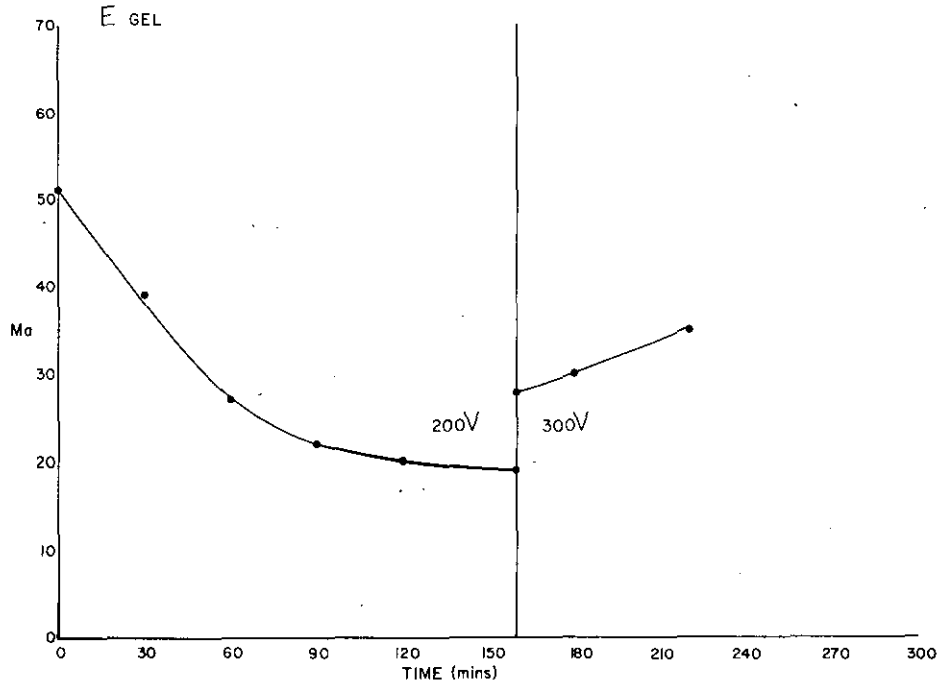


Figure 7. (C) and (D). Further examples of voltage and current characteristics of various 1 cm thick gels.

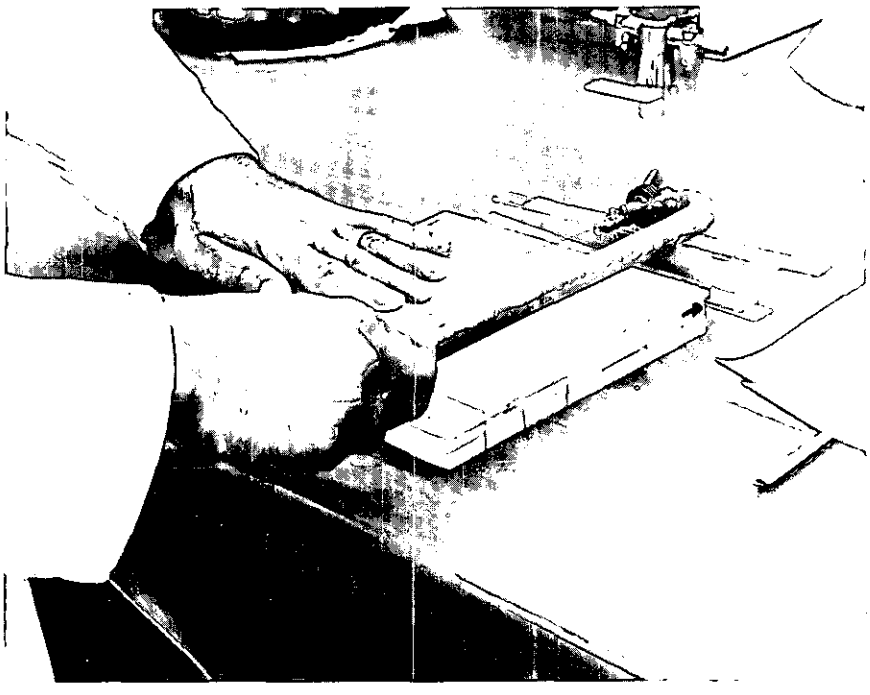


Figure 8. Method used for multiple slicing of the run gel. The section sliced is 7-9 cm forward of the insertion line. Note the drafting film over the gel.

Pre-Mixed Method

5 ml Adk solution
1 ml PMS solution
10 units G-6-pdh
14 units hexokinase
2 ml MTT solution
15 ml 2% agar

Adk Solution

100 mg ADP (5 ml ADP solution)
2.0 g glucose
400 mg $MgCl_2$
10 ml NADP solution
40 ml Tris-HCl, pH 8.0
(35 ml if ADP solution used)

Comments: Resolution is often poor and varies markedly between prawn genera. Buffers C and E have given the best results.

2. Aldolase (Ald)

Tissue: Muscle

Single Stain Method

6 ml fructose-1,6-diphosphate solution
1 ml NAD solution
2 ml MTT solution
10 units G-3-pdh
1 ml PMS solution
5 ml Tris-HCl, pH 7.0
15 ml 2% agar

Pre-Mixed Method

5 ml Ald solution
10 units G-3-pdh
1 ml PMS solution
2 ml MTT solution
15 ml 2% agar

Ald Solution

1.5 g fructose-1,6-diphosphate
4 ml NAD solution
46 ml Tris-HCl, pH 7.0

Comments: Staining intensity is strong; resolution often blurred. Buffer C gives the most consistently good resolution.

3. Fumerase (Fum)

Tissue: Muscle

Single Stain Method

4 ml potassium fumarate solution
1 ml NAD solution
2 ml MTT solution
1 ml PMS solution
50 units Mdh
5 ml Tris-HCl, pH 7.0
15 ml 2% agar

Pre-Mixed Method

5 ml FUM solution
50 units Mdh
1 ml PMS solution
2 ml MTT
15 ml 2% agar

FUM Solution

2 g potassium fumarate
5 ml NAD solution
45 ml Tris-HCl, pH 7.0

Comments: Staining intensity is very light; resolution often blurred. Buffer E gives good resolution.

4. Glutamate Dehydrogenase (Gdh)

Tissue: Muscle

Single Stain Method

2 ml sodium glutamate solution
1 ml NAD solution
1 ml MTT solution
1 ml PMS solution
5 ml Tris-HCl, pH 8.5
15 ml 2% agar

Pre-Mixed Method

5 ml Gdh solution
1 ml PMS solution
1 ml MTT solution
15 ml 2% agar

Gdh Solution

500 mg sodium glutamate
5 ml NAD solution
45 ml Tris-HCl, pH 8.5

Comments: Usually very good resolution; staining intensity is strong. Buffers A', B and E have given sharp resolution with various species.

5. Glyceraldehyde-3-Phosphate Dehydrogenase (G-3-pdh)

Tissue: Muscle

Single Stain Method

5 ml fructose-1,6-diphosphate solution
0.5 ml NAD solution
1 ml MTT solution
1 ml Na_3AsO_4 solution
1 ml PMS solution
1.0 unit aldolase
5 ml Tris-HCl, pH 7.0
15 ml 2% agar

Pre-Mixed Method

5 ml G-3-pdh solution
1 ml PMS solution
1.0 unit aldolase
1 ml MTT solution
15 ml 2% agar

G-3-pdh Solution

275 mg fructose-1,6-diphosphate
2.5 ml NAD solution
75 mg Na_3AsO_4
47.5 ml Tris-HCl, pH 7.0

Comments: Very good resolution, staining intensity is strong. Buffer C gives good resolution for all species.

6. α-Glycerophosphate Dehydrogenase (α-Gpdh)

Tissue: Muscle

Single Stain Method

1 ml α-glycerophosphate solution
1 ml EDTA (free acid) solution
0.5 ml NAD solution
1 ml MTT solution
1 ml PMS solution
5 ml Tris-HCl, pH 8.5
15 ml 2% agar

Pre-Mixed Method

5 ml α-Gpdh solution
1 ml PMS solution
1 ml MTT solution
15 ml 2% agar

α-Gpdh Solution

400 mg α-glycerophosphate
100 mg EDTA (free acid)
2.5 ml NAD solution
47.5 ml Tris-HCl, pH 8.5

Comments: Good resolution, strong staining intensity. Buffer C is usually the best buffer, but with one species Buffer E was better.

7. Hexokinase (Hk)

Tissue: Muscle

Single Stain Method

900 mg glucose
4 ml $MgCl_2$ solution
1 ml ATP solution
4 ml NADP solution
2 ml MTT solution
1 ml PMS solution
10 units G-6-pdh
5 ml Tris-HCl, pH 7.0
15 ml 2% agar

Pre-Mixed Method

5 ml Hk solution
1 ml PMS solution
10 units G-6-pdh
2 ml MTT
15 ml 2% agar

Hk Solution

4.5 g glucose
1.6 g $MgCl_2$
5 ml ATP solution
20 ml NADP solution
25 ml Tris-HCl, pH 7.0

Comments: Poor resolution, a difficult enzyme to work with in prawns. Buffer B usually gives the best results, but most often no activity is found.

8. Lactate Dehydrogenase (Ldh)

Tissue: Muscle

Single Stain Method

1 ml sodium lactate syrup
0.5 ml NAD solution
1 ml MTT solution
1 ml PMS solution
5 ml Tris-HCl, pH 7.0
15 ml 2% agar

Pre-Mixed Method

5 ml Ldh solution
1 ml PMS solution
1 ml MTT
15 ml 2% agar

Ldh Solution

5 ml sodium lactate syrup
2.5 ml NAD solution
42.5 ml Tris-HCl, pH 7.0

Comments: Good resolution, a very strong staining enzyme. All buffers have given good resolution for different species. The buffer that gives the best resolution for a particular species needs to be empirically determined.

9. Malate Dehydrogenase (Mdh)

Tissue: Muscle

Single Stain Method

1 ml malic acid solution
0.5 ml NAD solution
1 ml MTT solution
1 ml PMS solution
5 ml Tris-HCl, pH 8.5
15 ml 2% agar

Pre-Mixed Method

5 ml Mdh solution
1 ml PMS solution
1 ml MTT solution
15 ml 2% agar

Mdh Solution

5 ml malic acid solution
2.5 ml NAD solution
42.5 ml Tris-HCl, pH 8.5

Comments: Very good resolution, very strong and rapid staining. Buffer C always gives good resolution. For one species, buffers A', B, C and E gave good resolution. With all other species buffers A', B and E had very bad resolution or no activity.

10. Malic Enzyme (Me)

Tissue: Muscle

Single Stain Method

1 ml malic acid solution
1 ml NADP solution
1 ml MTT solution
1 ml $MgCl_2$ solution
1 ml PMS solution
5 ml Tris-HCl, pH 8.5
15 ml 2% agar

Pre-Mixed Method

5 ml Me solution
1 ml PMS solution
1 ml MTT solution
15 ml 2% agar

Me Solution

5 ml malic acid solution
5 ml NADP solution
100 mg $MgCl_2$
40 ml Tris-HCl, pH 8.5

Comments: Sharp resolution, staining intensity is strong.

11. Mannose Phosphate Isomerase (Mpi)

Tissue: Muscle

Single Stain Method

1 ml D-mannose-6-phosphate solution
1 ml NADP solution
1 ml MTT solution
10 units Pgi
10 units G-6-pdh
1 ml PMS solution
5 ml Tris-HCl, pH 8.0
15 ml 2% agar

Pre-Mixed Method

5 ml Mpi solution
10 units Pgi
10 units G-6-pdh
1 ml PMS solution
1 ml MTT solution
15 ml 2% agar

Mpi Solution

5 ml D-mannose-6-phosphate solution
5 ml NADP solution
40 ml Tris-HCl, pH 8.0

Comments: Very sharp resolution; staining intensity is strong. Buffers A' and C give the best resolution with Buffer C the most consistent.

12. 6-Phosphogluconate Dehydrogenase (6-Pgdh)

Tissue: Muscle

Single Stain Method

0.5 ml 6-phosphogluconic acid solution
1 ml $MgCl_2$ solution
5 ml NADP solution
1 ml MTT solution
1 ml PMS solution
5 ml Tris-HCl, pH 8.0
15 ml 2% agar

Pre-Mixed Method

5 ml 6-Pgdh solution
1 ml PMS solution
1 ml MTT solution
15 ml 2% agar

6-Pgdh Solution

250 mg 6-phosphogluconic acid
100 mg $MgCl_2$
100 mg NADP
50 ml Tris-HCl, pH 8.0

Comments: Sharp resolution, staining intensity is strong. Either Buffer B or C give very good results, but not both with the same species. The best buffer for a particular species will need to be empirically determined.

13. Phosphoglucoisomerase (Pgi)

Tissue: Muscle

Single Stain Method

0.5 ml fructose-6-phosphate solution
1 ml NADP solution
0.5 ml MTT solution
1 ml $MgCl_2$ solution
0.5 ml PMS solution
10 units G-6-pdh
15 ml 2% agar

Pre-Mix Method

5 ml Pgi solution
0.5 ml PMS solution
0.5 ml MTT solution
10 units G-6-pdh
15 ml 2% agar

Pgi Solution

2.5 ml fructose-6-phosphate solution
5 ml NADP solution
100 mg $MgCl_2$
42.5 ml Tris-HCl, pH 8.0

Comments: Very sharp resolution; stains very quickly and pattern must be read within 5 minutes. Buffer A' is the best one for this system. Buffers C and E give very sharp resolution, but both can mask some known variants.

14. Phosphoglucomutase (Pgm)

Tissue: Muscle

Single Stain Method

1 ml glucose-1-phosphate solution
0.5 ml $MgCl_2$ solution
0.5 ml NADP solution
1 ml MTT solution
1 ml PMS solution
10 units G-6-pdh
5 ml Tris-HCl, pH 8.0
15 ml 2% agar

Pre-Mixed Method

5 ml Pgm solution
1 ml PMS solution
1 ml MTT solution
10 units G-6-pdh
15 ml 2% agar

Pgm Solution

100 mg glucose-1-phosphate
5 ml $MgCl_2$ solution
5 ml NADP solution
40 ml Tris-HCl, pH 8.0

Comments: Very sharp resolution; stains very quickly and the zymogram should be read within 30 minutes. Buffer C gives the best resolution, but all other buffers can give adequate results. With Buffer C homozygotes appear as two or three banded, and heterozygotes are three or four banded. For the other buffers, one or more of all bands do not appear.

15. Triosephosphateisomerase (Tpi)

Tissue: Muscle

Single Stain Method

200 mg α-glycerophosphate
100 mg sodium pyruvate
0.5 ml NAD solution
10 units α-glycerophosphate dehydrogenase
15 units Ldh
5 ml Tris-HCl, pH 8.0
Incubate 2 hours; adjust pH to 2.0 with 1M HCl, adjust to pH 7.0 with 1M Tris.
0.5 ml NAD solution
1 ml MTT solution
2 ml Na_3AsO_4 solution
10 units G-3-pdh
1 ml PMS solution
15 ml 2% agar

Pre-Mix Method

5 ml Tpi solution
10 units G-3-pdh
1 ml PMS solution
1 ml MTT solution
15 ml 2% agar

Tpi Solution

1 g α-glycerophosphate
0.5 g sodium pyruvate
2.5 ml NAD solution
100 units α-glycerophosphate dehydrogenase
150 units Ldh
40 ml Tris-HCl, pH 8.0
Incubate 2 hours; adjust pH to 2.0 with HCl, adjust pH to 7.0 with 1M Tris
2.5 ml NAD solution
125 mg Na_3AsO_4

Comments: Sharp resolution; staining intensity is strong. Buffer E gives the best resolution for all species.

II. STAINS USING SOLUTIONS

1. Acid Phosphatase (Acph)

Tissue: Cephalothorax

Single Stain Method

Pre-soak gel 1 hour in acetate buffer, pH 5.0
500 mg polyvinylpyrrolidone
100 mg α-naphthyl acid phosphate
100 mg fast blue BB salt, or fast garnet GBC salt
50 ml acetate buffer, pH 5.0
50 ml water

Pre-Mix Method

Pre-soak gel 1 hour in acetate buffer, pH 5.0
50 ml Acph solution
50 ml water
100 mg fast blue BB salt, or fast garnet GBC salt

Acph Solution

2.5 g polyvinylpyrrolidone
200 mg α-naphthyl acid phosphate
250 ml acetate buffer, pH 5.0

Comments: Sharp resolution and strong intensity. However, this enzyme must be treated carefully as it seems to produce poor resolution if handled improperly. Buffer B gives the best resolution.

2. Aldehyde Oxidase (Ao)

Tissue: Cephalothorax

Single Stain Method

1 ml benzaldehyde
1 ml NAD solution
1 ml MTT solution
1 ml PMS solution
50 ml water
50 ml Tris-HCl, pH 8.5

Pre-Mix Method

Same as Single Stain Method

Comments: Very sharp resolution and strong staining intensity on Buffer A'.

3. Alkaline phosphatase (Aph)

Tissue: Cephalothorax

Single Stain Method

100 mg α-naphthyl acid phosphate
100 mg fast blue BB salt, or fast garnet GBC salt
50 ml water
50 ml Tris-HCl, pH 8.5

Pre-Mix Method

50 ml Aph solution
50 ml water
100 mg fast blue BB salt, or fast garnet GBC salt

Aph Solution

500 mg α-naphthyl acid phosphate
250 ml Tris-HCl, pH 8.5

Comments: Sharp resolution and strong intensity, but the enzyme must be treated carefully. Buffers A′ and B give best resolution.

4. Esterase (Est)

Tissue: Cephalothorax and Muscle

Single Stain Method

1.5 ml α-naphthyl acetate solution
100 mg fast blue RR salt
50 ml water
50 ml Tris-HCl, pH 7.0

Pre-Mix Method

Same as Single Stain Method

Comments: As a useful variation, substitute 1.5 ml α-naphthyl acetate with 1.5 ml β-naphthyl acetate. Stains very quickly from cephalothorax and very light from muscle. Very sharp resolution. On overstaining, a series of white areas (Ng locus(?) (Birdsall *et al.*1970)) appears. All buffers can give adequate resolution.

5. Glutamateoxaloacetate Transaminase (Got)

Tissue: Cephalothorax

Single Stain Method

1 ml pyridoxyl-5′-phosphate solution
200 mg L-aspartic acid
100 mg α-ketoglutaric acid
150 mg fast blue BB salt
50 ml water
50 ml Tris-HCl, pH 8.5

Pre-Mix Method

50 ml Got solution
1 ml pyridoxyl-5′-phosphate solution
150 mg fast blue BB salt
50 ml water

Got solution

1 g L-aspartic acid
500 mg α-ketoglutaric acid
250 ml Tris-HCl, pH 8.5

Comments: Sharp resolution, rapid staining. On overstaining a series of white bands appears which are identical to the major protein component of the tissue. All four buffers give good resolution.

6. Leucine Amino Peptidase (Lap)

Tissue: Cephalothorax

Single Stain Method

Pre-soak gel 1 hour in 0.5M boric acid
50 mg L-leucyl-β-naphthylamide-HCl
25 mg fast black K salt
1 ml $MgCl_2$ solution
100 ml Tris-maleate, pH 5.0

Pre-Mix Method

Same as Single Stain Method

Comments: Sharp resolution; light staining intensity. Buffer B gives the most consistent resolution.

7. Octanol Dehydrogenase (Odh)

Tissue: Muscle

(In prawns analagous to alcohol dehydrogenase.)

Single Stain Method

3 ml l-octanol
1 ml NAD solution
1 ml MTT solution
1 ml PMS solution
47 ml water
50 ml Tris-HCl, pH 8.5

Pre-Mix Method

Same as Single Stain Method

Comments: Sharp resolution; strong staining intensity. Buffers B and E give the best resolution.

8. Peptidase (Pep)

Tissue: Cephalothorax

Single Stain Method

10 mg D,L-valyl-leucine
1 mg horseradish peroxidase
5 mg O-dianisidine in 10 ml acetone
1 ml $MnCl_2$ solution
1 mg *Bothrops atrox* venom
100 ml phosphate buffer, pH 7.5

Pre-Mix Method

Same as Single Stain Method

Comments: Adequate resolution, poor intensity and fades rapidly. Buffers A′ and C give the best resolution.

9. 1-Pyrroline Dehydrogenase (1-Pydh)

Tissue: Muscle

Single Stain Method

50 mg L-Pyroglutamic acid
1 ml NAD solution
1 ml MTT solution
1 ml PMS solution
47 ml water
50 ml Tris-HCl, pH 8.0

Pre-Mix Method

Same as Single Stain Method

Comments: This technique was developed in our laboratory. Sharp resolution and strong staining. Buffers A′, B and E all give good resolution.

10. Sorbitoldehydrogenase (Sdh)

Tissue: Muscle

Single Stain Method

1 g D-sorbitol
1 ml NAD solution
1 ml MTT solution
1 ml PMS solution
47 ml water
50 ml Tris-HCl, pH 8.0

Pre-Mix Method

Same as Single Stain Method

Comments: Adequate resolution and staining intensity. Buffers B and E give adequate results.

11. Tyrosinase (Tyr)

Tissue: Cephalothorax

Single Stain Method

1 ml catechol solution
1 ml l-proline solution
100 ml phosphate buffer, pH 7.0

Pre-Mix Method

Same as Single Stain Method

Comments: Fades very quickly. Fast staining and gives adequate resolution. Buffer C gives best resolution.

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