A SIMPLIFIED GEL-ISOELECTRIC FOCUSING TECHNIQUE FOR STUDYING ISOENZYMES IN MOSQUITOES

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ABSTRACT. A simplified technique of gel isoelectric focusing suitable for separating and characterizing isoenzymes of mosquito has been described. Each ampholyte possesses a characteristic isoelectric point, a pH at which it is electrically neutral. This technique has a far superior fractionation power than the routine electrophoretic techniques. Under appropriate experimental conditions, molecules with a difference of isoelectric points as low as 0.01 pH unit can be separated and characterized easily. Electrophoretically similar proteins (with similar migration rates) can often be further fractionated by their isoelectric points. The great operational simplicity makes this technique an ideal tool for protein separation and characterization in routine research.

INTRODUCTION

The combination of starch gel electrophoresis (Smithies, 1955, 1959) with the zymogram technique (Hunter and Markert, 1957) has greatly facilitated the study of isoenzymes (Markert and Møller, 1959). This technique has been very useful in the study of genetic variation of natural populations (Hubby and Lewontin, 1966). Generally isoenzymes are characterized by their relative mobilities with respect to a standard tracking dye, or a standard reference strain if one is available. The overlap of migration of different molecules can obscure their identities unless they are further distinguished by genetic analysis (Narang and Kitzmiller, 1971) or other physico-chemical parameters (Narang and Narang 1974a & b).

The simple technique of gel-isoelectric focusing, described in this paper, is suitable for separating and characterizing isoenzymes by their isoelectric points. This technique is at present being used for the characterization of isoenzymes of various gene-enzyme systems in snails (Narang and Narang, 1974b). The isoelectric point of each protein or enzyme is unique and is specified by the pH value, at which it carries a net zero charge, and hence is electrically neutral. Under ideal experimentation conditions, molecules with a difference of isoelectric points as low as 0.01 pH unit can be separated and characterized easily.

Gel-isoelectric focusing is essentially a type of electrophoresis, in which the supporting gel medium has a pH gradient as against a constant-pH gel in electrophoresis. It can be called a pH gradient electrophoresis. Separation of the molecules is based on the differences of the net charges without any sieving effect which is characteristic of gel electrophoresis. Because of the steady state end of the electrofocusing, it possesses a far superior fractionation power than electrophoresis. Although the technique of isoelectric focusing has been known for a long time, it has become widely used as a tool of protein separation and characterization only after the development of ampholine chemicals. These chemicals are mixtures of low molecular weight aliphatic poly-amino-polycarboxylic acids which, on exposure to electric field in a convection-free medium, create a pH gradient. The high molecular weight ampholytes, the proteins and enzymes migrate along the pH gradient until they reach a value of pH where they are electrically neutral. There they stop. The value of pH at which a molecule is electrically neutral is called its "isoelectric point," pI. The protein and enzyme molecules carry different charges which determine their pI's. More details of the basic principles of isoelectric focusing can be found in LKB 8100 Ampholine Instruction Manual, I-8100-EO3, LKB-Produkter AB, Bromma, Sweden.

MATERIALS AND METHODS

Ampholine-carrier ampholytes covering the pH range of 3.5–10 and fractions thereof, of 2 and 3 pH units were purchased from LKB-Produkter, Bromma, Sweden. All other reagents, substrates and coenzymes were obtained from Sigma Chemical Company, St. Louis, MO., U.S.A. *Anopheles strodei* Root and *Culex pipiens quinquefasciatus* Say were from the local collections (Brasilia, D.F.)

PREPARATION OF ISOELECTRIC GEL TUBES. Pyrex tubing (8mm O.D., 6mm I.D.) is cut to 125 mm length and filled to within 105 mm of one end with 3.5 to 5 percent gel solution, containing 0.5–0.75% ampholine of the desired pH range. The gel solution is prepared according to Table 1 and gelled under water. The sample is layered on the gel. Alternatively, the crude extract may be mixed with the gel solution before pouring into the tube to obtain a uniform distribution of the sample in the gel. Another possibility is to layer the sample at any chosen level and to follow this with a second layer of the gel. Precipitation of the sample by the anodic buffer is avoided by a layer of 10 percent sucrose separating the sample from the anodic buffer.

TABLE 1. Stock solutions: These should be prepared fresh every 15 days and stored at 4° C.

Stock solutions		Gel solution parts
*A	20% cyanogum-41 (acrylamide:Bis) 95 : 5	2 parts
B	0.84% TEMED	1 part
**C	Riboflavin (0.002%) or Ribo-persulfate solution (0.002% riboflavin:0.06% persulfate) 1 : 1	4 parts
D	40% Ampholine (desired pH range)	0.16 part
	Water	0.84 part

* If lower than 5% gel is desired, stock solution A can be diluted accordingly.

** For gels of lower concentration (3.5–4.0% gels) ribo-persulfate solution is better than riboflavin solution for photo-polymerization to yield mechanically stable gels.

PREPARATION OF SAMPLE.—About 10–15 μl of the crude extract or a partially purified enzyme is mixed with an equal volume of 40 percent sucrose solution to make it denser. Isoelectric focusing can be used to separate isoenzymes that are indistinguishable by electrophoresis. The 2 samples in question are first separated by electrophoresis. A representative gel of each sample is developed for the desired enzyme to localize the sites of activity. The gel portions containing apparently similar isoenzymes are cut from the unstained gel. These gel slices are washed in cold distilled water for 5–10 minutes to remove the electrophoretic buffer and then immersed in 40 percent sucrose solution. The slices may be placed on the isoelectric focusing gel or they may be macerated in a minimum amount of water and the resulting paste may be placed on the gel, or finally the paste may be centrifuged and the supernatant then layered on the gel.

BUFFERS AND ELECTROFOCUSING PARAMETERS. The anodic buffer consists of 0.05M phosphoric acid. One percent sulfuric acid can also be used. The cathodic buffer is made up of 0.05M triethanolamine and 0.05M sodium hydroxide. Alternatively 2 percent ethylenediamine or 8 percent sodium hydroxide may be used. An initial current of 1.0 mA per tube is applied and is regulated until a maximum of 350 volts (35 volts per cm. of gel) is reached. Then the voltage is regulated and the current is allowed to drop to a stable minimum value. The isoelectric focusing is considered to be complete, if the current is stable at the minimum value for at least half an hour. Under the described experimental conditions, it takes about 4 hours to reach a steady state equlibrium in the isoelectrofocusing gel.

A blank gel without sample or stain is cut into 0.5 cm. segments. Each segment is macerated in 1.5 ml of distilled water, pH 7.0 in small vials covered with saran wrap to avoid acidification by atmospheric carbon dioxide. These 0.5 cm. segment elutes are then used to determine

the relation of pH to gel length. The gels with the samples are developed for the desired enzyme. The isoelectric point of the isoenzyme in question is then read from the pH profile of the blank.

Choice of Ampholine Chemicals. When electrofocusing an unknown sample for the first time, a pre-run with the ampholine of 3.5–10 or 3.0–10 pH range is necessary to find the approximate pI values of the proteins of interest. Then a shallow-range ampholine is used to separate the proteins that have small differences in their isoelectric points.

RESULTS

The isoelectric technique has been used to distinguish and characterize the apparently electrophoretically similar esterase isoenzymes. Figure 1 shows zymograms of esterases of larvae of *A. strodei* and *C. p. quinquefasciatus* by the disc electrophoretic method of Clark (1964). The gel slices from unstained gels of each species containing the respective isoenzymes (indicated by arrow in Fig. 1) were subjected to electrofocusing. These apparently similar esterase forms in the two species of mosquitoes were found to be different molecules with distinct isoelectric points (Fig. 2). The most cathodic esterase forms of each species (marked by dots in Fig. 1), when analysed by isoelectric focusing proved to be similar molecules.

DISCUSSION

The main advantages of the gel method, described in this paper, over the more conventional method of Vesterberg and Svensson (1966), are that smaller samples of protein may be used, and that the proteins reach their isoelectric point in a shorter period of time. In addition, the great operational simplicity makes the technique an ideal tool for protein separation and characterization in routine research.

Low concentration gels, which can be supported by a dialysis membrane tied at the bottom of the gel tube help to minimize the time required for the proteins to reach the isoelectric end point. This in turn avoids the possibility of progressive flattening of the pH to distance curve with time. A minimum concentration of the ampholine, enough to give the desired buffering capacity to overcome that of the sample protein, is desirable. Higher concentrations cause a delay in reaching the steady state end, in addition to binding with the protein molecules and hence interfering with the staining of the proteins

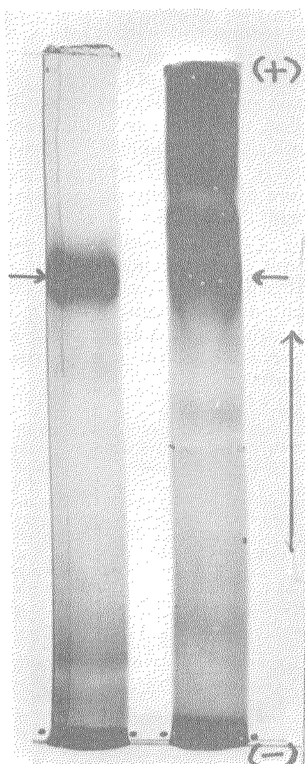

Fig. 1. Photograph of larval esterase zymograms of *Anopheles strodei* (left) and *Culex p. quinquefasciatus* (right). The apparently electrophoretically similar esterase bands in the two species are marked with arrows and dots.

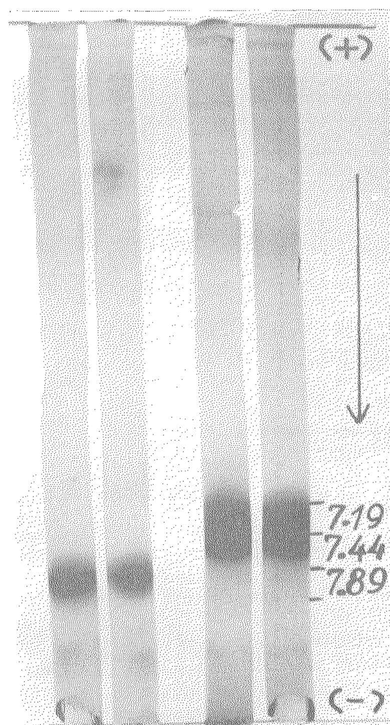

FIG. 2. Photograph of electrofocusing gels showing isoelectric positions of electrophoretically similar bands of *A. strodei* (left) and *C. p. quinquefasciatus* (right). The numbers represent the isoelectric points of the respective regions of the gels.

with the usual protein dyes. Treatment of the gel with 12.5% TCA for about 1.5–2.0 hours, both fixes the protein as well as removes the ampholine. Such a treatment is not possible in isoenzyme studies. The authors have found it useful to treat the gel with 0.3–0.5M Tris-HCl buffer for half an hour with agitation before developing the gel for the isoenzymes in question.

The distribution of pH gradient in the gel depends on various factors, such as the concentrations of ampholine, time taken to reach isoelectrofocusing end point, the minimum value of the current reached at which no further conductance is noted, the length of the gel in each run and many other minor variations from run to run. Hence there is no direct relationship between the position of a band on a given run and a pH vs gel length profile from another run. The pI should, therefore, be read from its position in the gel against the "blank" reference gel of the same run. The pI value characteristic of each protein may vary a little along the gel with various variables as mentioned above. The isoelectric focusing end-point should be found by systematic variation of the time of the run (after regulating the voltage) until pI of any given protein remains unchanged.

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VIABILITY OF PUERTO RICAN *AEDES AEGYPTI* EGGS AFTER LONG PERIODS OF STORAGE[1]

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ABSTRACT. Some eggs of the Arecibo, Puerto Rico laboratory strain of *Aedes aegypti* hatched after 21 months and 27 days storage, most of the time at 50% relative humidity. They became virile adults which eventually begot thousands of eggs. This is a new record for the duration of viability of *Aedes aegypti* eggs; the previous record was only 15 months.

Many authors have studied the longevity of *Aedes aegypti* eggs and the consensus seems to be that the eggs stay alive for about one year (Christophers, 1960; Gerberg, 1970). Bacot (1918) recorded 15 months. My purpose is to report on studies of eggs stored for much longer periods.

MATERIALS AND METHODS. To obtain eggs I placed finger bowls containing water and lined with paper towel strips in cages of adults of the Arecibo, Puerto Rico laboratory strain. These adults had been under insecticidal pressure for 10 or more generations, and at the time they were resistant to malathion by a factor of seven or more (Fox, 1973). They fed on guinea pigs. When many eggs had accumulated on the strips, I wrapped them in paper towels, put them in plastic bags, and stored them for 5 months (January through June, 1972) in a room which was not air-conditioned, the temperature varying from 72° F. to 86° F. (23° C. to 30° C.) and the relative humidity from 67% to 83%. But after this period I moved the bags to another building which was air-conditioned with the temperature from 68° F. to 78° F. (21° C. to 26° C.) and the relative humidity varying from 45% to 60%, but usually about 50%. There they stayed for almost 18 months (July, 1972 to November, 1973). There were 24 lots of eggs aged 7 to 22 months and each lot had 1,000 to 50,000 eggs. I hatched the eggs in tap water and reared the larvae on rabbit food pellets.

RESULTS. Of the 24 lots of eggs 10 did not hatch and 14 yielded some larvae. Table 1 gives the number of larvae obtained from eggs aged more than 15 months. Eleven larvae emerged and were reared to adults from 37,000 eggs more than 21 months old. These adults produced sufficient eggs to yield about 175 larvae, which became vigorous adults, and produced thousands of eggs.

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