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Acrylamide Gel Electrophoresis in a pH Gradient. Isoelectric Focusing

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General

In pH gradient electrophoresis (isoelectric focusing) (3,7,8) each protein migrates to a point in the pH gradient that is its isoelectric point. Isoelectric focusing was made possible by the invention of stabilized pH gradients obtained with histidine peptides from the partial hydrolysis of hemoglobin (7). The "Ampholine Carrier Ampholytes,"* produced by the LKB Produkter AB in Sweden, are low molecular weight synthetic ampholytes with a wide variety of isoelectric points. They are available as mixtures of such ampholytes whose isoelectric points are restricted to given ranges of pH. When electrophoresis is conducted in such a mixture, a pH gradient, covering the chosen pH range, is formed. The gel medium stabilizes the gradient, since it prevents convective mixing.

Longer times are required for isoelectric focusing than for disc gel electrophoresis since current heating interferes with the formation of the pH gradient, and the motion of the protein slows as it approaches its isoelectric point. Only one running gel is required for pH gradient electrophoresis, and by slicing the gels, making a water extract and measuring its pH, the isoelectric point of the protein band can be approximated. Resolution is comparable to standard disc electrophoresis (see Chapter by Groves), based on the number of components resolved for whole casein.

The method of Awdeh et al. (1) has advantages of economy since smaller amounts of the expensive ampholytes are required. Our

initial results on milk proteins were reported in 1969 (4) with thin gel sheets. Since then we have tried to improve the method, and have come to the conclusion that isoelectric focusing is best accomplished using disc electrophoresis apparatus. Both methods are described below. Urea is included in many of the gels since the caseins will not be resolved without it. However, urea should be omitted for easily denatured proteins such as β -lactoglobulin.

A. Focusing in Thin Gel Sheets

Sample Preparation

The samples are prepared by dissolving 1 mg of protein in 8% (vol/vol) acetic acid containing 6 M urea (the use of acid-urea solution prevents artifacts due to carbamylation of the protein). Urea should be omitted for resolution of whey proteins.

Apparatus

Gel molds are made by taking a 1 mm thick sheet of Teflon, 24 cm by 17 cm, and cutting out the center to produce an open rectangle 22 cm by 14 cm. The mold sides are stuck to a glass plate, 24 cm by 17 cm with Dow-Corning Silicone Stopcock Grease. A similar glass plate is used for the cover.

The cell used is a simple rectangular box made of acrylic plastic with holes bored in the side to hold two porous carbon rods parallel at a distance of 15 cm.

Gel Composition and Casting Procedure

The method consists of casting a thin gel, 1 mm thick, between two glass plates to exclude air, which inhibits polymerization. Gel formation is catalyzed by riboflavin,

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which is preferable to persulfate because of the greater possibility of artifact production by the latter (5).

1) *Gel preparation.* 2.5 g acrylamide, .1 g MBA, 13.5 g urea, 2.5 ml Ampholine buffer (pH range 3 to 10), and .5 mg riboflavin. Stir with sufficient distilled water to make to 50 ml of solution. Any excess solution may be stored in the dark at 3 C.

2) *Casting procedure.* The lower half of the mold is placed on a flat, fluorescent light slide viewer, and the cover plate is placed diagonally on top of the mold. By pipetting the gel mixture with the pipette tip in the space between the cover and the bottom of the mold, the capillary effect between the cover and the bottom fills the mold without trapping any bubbles. A slight turn then seals the mold, and a weight is put on the cover. The fluorescent light is turned on, and polymerization is complete in about an hour. The mold is inverted and the upper glass plate is pried off. The Teflon spacer must be removed for good contact of the gel with the electrodes.

Sample Application

One centimeter squares of Whatman No. 1 filter paper are dipped in the protein solutions and laid on the gel near the anode. Prior to use the anode rod is soaked in 1% phosphoric acid and the cathode rod in 1% ethylenediamine. The supporting plate holding the gel is inverted placing the gel in contact with, and supported by, the electrode rods. The apparatus is placed in the cold room at 5 C, and the cell bottom is filled with water to reduce drying of the gels.

Power Setting and Time

No more than 5 mA current are used initially since gels dry out. After about 8 h, the voltage is increased to about 400 V, and the run completed in 16 h for pH 3 to 10 ampholyte buffers.

Because most proteins cannot be seen in gels, colored proteins such as myoglobin and hemoglobin are used as markers in electrofocusing. Conway-Jacobs and Lewin (2) recommend a number of dyes for following the progress of electrofocusing as follows: Patent Blue, pH 3.00; Evans Blue, pH 5.35; and Congo Red, pH 5.80.

Fixing and Staining

At the completion of the run, the gel is placed in 10% TCA and removed from the glass plate. The electrofocused proteins are visible as sharp white lines. However, the ampholyte buffers must be washed out with several changes of TCA solution for a day before the proteins can be stained with 1% Amido Black dissolved in 7% acetic acid. If this is not done, a very heavy background stain will persist on destaining with 7% acetic acid. The gels should be stained for at least 1 h.

Coomassie Blue may be preferred to stain the proteins (6) since the gel requires less washing with TCA to remove ampholyte; however, the color staining of the bands is less intense than that produced by Amido Black.

B. Focusing in Disc Gels

The easiest method for pH gradient gel electrophoresis is that which uses the disc electrophoresis apparatus. It is not necessary in this case to produce a uniform pH gradient over a wide area; and if reference compounds are run in each tube until the markers are at equilibrium, comparisons can be made by lining up the markers. The pH of the sections can be measured by cutting small slices with a razor blade or a slicer, placing each slice into a 5 ml beaker and adding 1 ml of water. After an hour the pH is measured with a microelectrode.

Apparatus

Either 65 or 110 mm tubes, 5 mm in diameter, can be used with a disc-gel apparatus such as the Canalco No. 12.

Gel Composition and Casting Procedure

1) A 7.5% acrylamide gel is prepared as follows: 3.75 g acrylamide, .125 g MBA, 13.5 g urea, 2.5 ml Ampholine buffer (pH range 3 to 10), and .25 mg riboflavin. Make to 50 ml with distilled water.

2) The tubes are stoppered at the lower end, mounted in the polymerization rack and are syringe filled with gel solution to within 8

mm of the top. Polymerization is complete after 30 min of exposure to fluorescent light. The tubes are then filled to the top with 1% Ampholine buffer in 10% sucrose solution. Tubes are mounted in the rubber mounts of the upper buffer vessel and a .073 M phosphoric acid solution is poured into the upper vessel taking care to avoid disturbing the more dense Ampholine buffer.

Sample Preparation and Application

The samples, 1 mg of protein dissolved in .5 ml of 8% (vol/vol) acetic acid-6 M urea, are more dense than the Ampholine-sucrose solution. They are layered (10 μ l) under this solution with a microsyringe and lie on the gel surface. A trace of dye may be added so that the layering process can be observed.

The lower buffer vessel is filled with .5% ethylenediamine, and the negative terminal of the power supply is connected. The anode is then placed in the upper vessel.

Power Setting and Time

One mA of current per tube is applied from a constant current power supply. By the time the voltage has risen to 350V, the pH gradient is nearly formed and the power supply may be switched to constant voltage.

After about 3 h, the position of visible markers, such as hemoglobin, will have stabilized, and the run is terminated.

Location of the Protein

If the proteins are visible as opaque zones, their positions can be marked with small glass capillaries and the gel sectioned to determine isoelectric points. If they are not visible, dyeing of one duplicate set may be necessary to locate the proteins(s). The cylindrical gels are removed and washed with several changes of 10% TCA and stained with Amido Black. The sharpness of the bands is superior to those obtained with the thin gel sheet method.

Typical Results

To illustrate the detail obtained by disc electrophoresis in a pH gradient, the results for casein components in a pH 3 to 10

gradient are shown in Fig. 23. The five β -caseins, A¹, A², A³, B, and C, have been resolved by this technique. β -Lactoglobulin, because of urea, exhibits several bands, similar to the results for standard disc-gels.

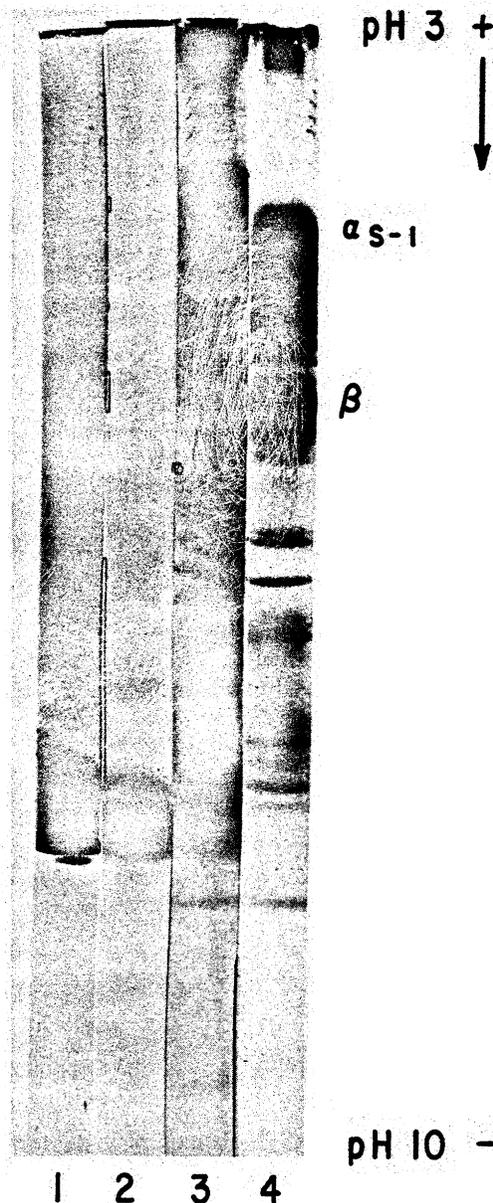


FIG. 23. Electrophoresis of milk proteins in a pH 3-10 gradient. pH 3 at top of photograph, pH 10 at bottom. Proteins: (1) reduced α -lactalbumin, (2) reduced β -lactoglobulin, (3) and (4) whole casein from different cows. The α_{S1} -caseins are nearest the top; the β -caseins are the strong band about one-third of the length of the gel from the top.

References

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