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N. Catsimpoolas[a]
[a] LABORATORY OF PROTEIN CHEMISTRY CENTRAL SOYA RESEARCH CENTER CHICAGO, ILLINOIS

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Scanning Density Gradient Isoelectric Separation of Proteins on a Microscale

N. CATSIMPOOLAS

LABORATORY OF PROTEIN CHEMISTRY
CENTRAL SOYA RESEARCH CENTER
CHICAGO, ILLINOIS 60639

Summary

An attachment for the Linear Transport System of the Gilford 2000 recording spectrophotometer is described which allows *in situ* continuous scanning of protein zones subjected to isoelectric focusing in sucrose gradients. Microgram quantities of protein samples can be analyzed in a short time (1.5 to 3 hr) in a quartz microcolumn. Resolution of separated components is excellent. Application of the method to the isoelectric separation of the commercially available proteins ovalbumin, beta lactoglobulin, myoglobin, Bowman-Birk soybean protease inhibitor, and rabbit gamma globulins is reported. The method provides for scanning at different wavelengths for differentiation of colored proteins, or proteins lacking tryptophan. The described procedure offers an opportunity for the studying of the kinetics of isoelectrofocusing in sucrose gradients.

INTRODUCTION

Isoelectric separation in density gradients as described by Svensson (*1*) and Vesterberg and Svensson (*2*) is a method of high resolution for the fractionation of proteins. The electrolysis columns (*1*, *2*) supplied commercially (LKB Produkter AB, Stockholm, Sweden) have a relatively large capacity (110 and 440 ml) and are not suitable for the analytical isoelectric separation of proteins in microgram quantities. Recently, Weller et al. (*3*), Koch and Backx (*4*), and Godson (*5*) designed electrofocusing columns of small capacity (7 to 11 ml) which can accommodate little amounts (0.5 to 5 mg) of sample. Detection of the separated components is performed either

by forcing the ampholine gradient upward and recording their UV absorbance (*3*, *5*) or by visual inspection using colored proteins (*4*). However, Weller et al. (*3*) and Godson (*5*) noticed that resolution following fractionation was not as good as resolution visible in the column itself. The problem of detection of the components was probably the major obstacle in developing true microcolumns which have volumes of 0.3 to 0.5 ml similar to those used for disc electrofocusing (*6*).

The present report describes an *in situ* scanning device and procedure for performing density gradient isoelectric separation of proteins in microquartz columns requiring only microgram quantities of sample. The advantage of the *in situ* scanning method includes the elimination of diffusion, increased resolution and sensitivity of detection, and following the isoelectrofocusing process continuously.

EXPERIMENTAL

The Scanning Isoelectrofocusing Device

The device shown in Fig. 1 consists of the following parts:

1. A Plexiglas block ($0.6 \times 2.5 \times 10.5$ cm) bearing two holes for attachment to the cuvet holder-supporting-base of the Linear Transport System (Model 2410) of the Gilford 2000 recording spectrophotometer operating with a Beckman DU monochromator. For identification purposes this piece is called supporting back plate (A).

2. An electrolyte reservoir (B) made of Plexiglas ($0.9 \times 1.3 \times 1.8$ cm) which bears two wells ($\frac{7}{32}$ in. diam) connected by a channel ($\frac{1}{8}$ in. diam) drilled in the block. The electrolyte reservoir is glued on the supporting back plate. One of the wells serves as a base for the quartz tube (described below), and the other well, filled with electrolyte, carries a platinum wire electrode.

3. An upper column-supporting Plexiglas block ($0.9 \times 1.0 \times 1.3$ cm) (C) which is glued on the supporting back plate (A) and serves for holding the upper part of the quartz tube (D).

4. A precision bore quartz tube (D) (Ace Glass, Inc.) 10.0 cm length and i.d. $\frac{3}{32}$ in., wrapped at one end (lower) with dialysis tubing—held in position with an O-ring—which is inserted into one of the wells of the electrolyte reservoir (B) described above. The upper end of the quartz tube accepts the other platinum wire electrode which is attached to the block C.

5. The electrode cables which pass through the Linear Transport

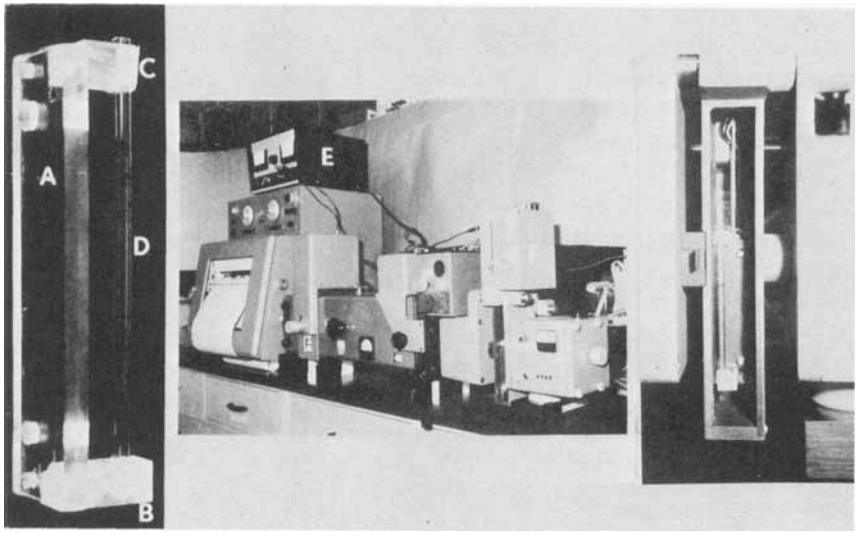

FIG. 1. Photograph of the equipment used for performing scanning density gradient isoelectric focusing. (Left) Scanning isoelectrofocusing device: A, supporting back plate; B, lower electrolyte reservoir; C, upper column-support; D, quartz tube. (Center) Over-all view of the equipment setup. Note that the monochromator, dual lamp housing, and photometer are resting on their "side" whereas the Linear Transport System (F) is positioned vertically. The electrode cables connected to the constant voltage power supply (E) pass through the synchronous motor box (F). (Right) Positioning of the scanning device inside the Linear Transport chamber.

System by means of two holes available on the back of the box (F) holding the motor (the cables are inserted by removing the appropriate screws and opening the box containing the synchronous motor). The cables are supported by two holes drilled in the supporting back plate (A) and are soldered to the platinum wire electrodes. It should be mentioned that the electrode cables should be thin and flexible so that they do not offer resistance to the movement of the scanning system.

The Linear Transport System with the attached device is mounted to the spectrophotometer in a vertical fashion. The monochromator, lamp housing, and photometer are placed on their "side" as shown in Fig. 1. This is done in order to position the light beam from the monochromator perpendicular to the length of the quartz tube and parallel to the slit opening supplied with the Linear Transport System.

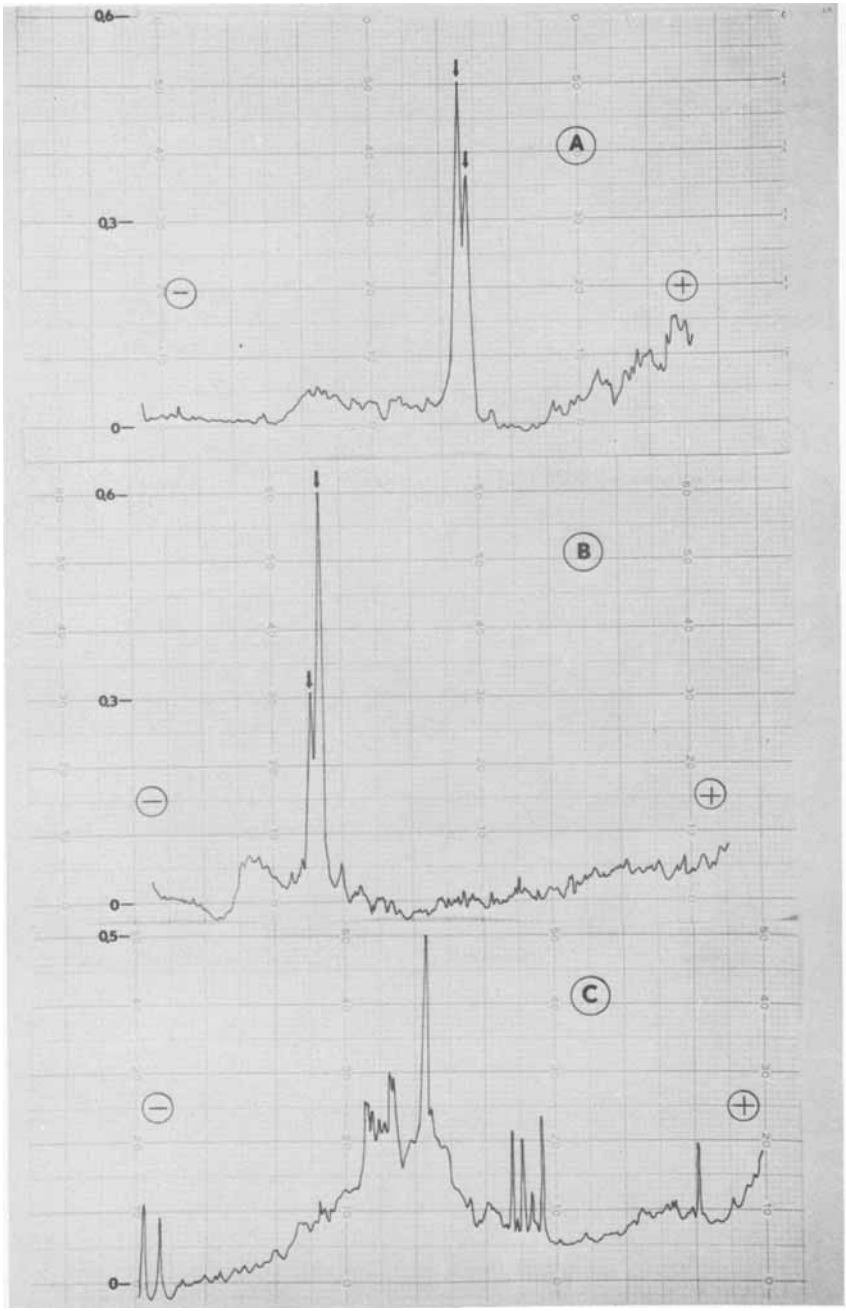

FIG. 2. Scanning density gradient isoelectrofocusing patterns of the fol-

This trick is necessary in order to achieve high resolution in scanning the electrofocusing column. The electrode cables are connected to a constant voltage power supply (E).

Procedure

The quartz tube is coated with methyl cellulose as described by Hjertén (*7*) in order to avoid electroendosmosis. The lower end is wrapped with a single square sheet of dialysis tubing fastened around the tube with an O-ring. The dialysis tubing seal makes unnecessary the use of a dummy arm (*3–5*). The quartz tube is held vertically with a clamp for filling with the ampholine gradient. This is prepared from a "dense" and a "light" solution by mixing calculated portions of the two solutions in a test tube (*2*). The "dense" ampholine solution contained 48 g sucrose and 2.5 ml of the 40% stock solution of Ampholine (LKB Produkter, AB), made to 100 ml with water. The "light" solution was prepared by dilution of 2.5 ml of the 40% stock Ampholine solution to 100 ml with water. Twenty-four fractions of progressively decreasing density were prepared by mixing 4.8 ml of the "dense" solution (d) and 0.2 ml of the light solution (l) in Tube No. 1, 4.6 ml of (d) and 0.4 ml of (l) in Tube No. 2, 4.4 ml of (d) and 0.6 ml of (l) in Tube No. 3, etc. until the 24th tube contained 0.2 ml of (d) and 4.8 ml of (l). The Ampholine gradient was formed by layering the contents of Tubes No. 1 through 24 with a 20-μl micropipet (Drummond Scientific Co.) in the quartz tube. The protein sample was usually dissolved in a portion of the mixture of Tubes No. 13 and 14. The top of the column was filled with the upper electrolyte which was made by dissolving 0.2 ml of ethylenediamine in 10 ml of water. The lower electrolyte was composed of 12 g sucrose, 0.2 ml of phosphoric acid, and 14 ml of water (*4*). The lower electrolyte reservoir (B) is then filled with this solution and the quartz tube is carefully inserted in the appropriate hole, avoiding any bubble formation between the dialysis tubing seal and the electrolyte solution. The device is placed in the Linear Transport chamber and the platinum wires are connected with the upper and lower electrolytes. The chamber is closed with the lid and the current is turned on with the

lowing proteins: A, Bowman-Birk soybean protease inhibitor (100 μg) in the pH 3–6 range scanned at 80 min; B, ovalbumin (50 μg) in the pH 3–6 range scanned at 125 min; and C, rabbit gamma globulins (100 μg) in the pH 3–10 range scanned at 120 min. Electrofocusing was performed at 500 V with 1.2–1.5 mA initial current.

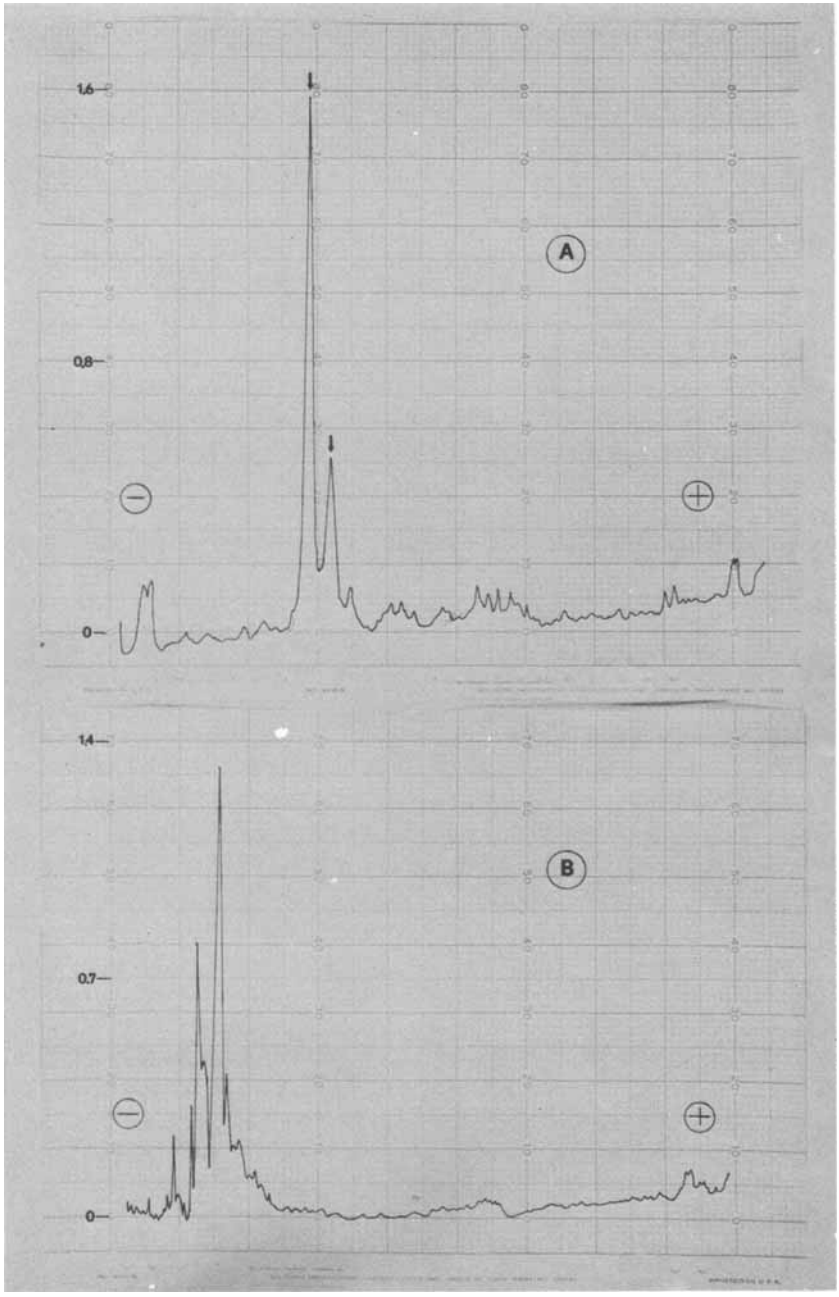

FIG. 3. Scanning density gradient isoelectrofocusing patterns: A, horse

simultaneous actuation of an electric timer. Scans (at 280 nm) are made continuously at a rate of 2.5 cm/min using the 0.20×2.36 aperture plate.

Test Materials

Ovalbumin (2X cryst) were obtained from Worthington, Freehold, New Jersey. Horse heart myoglobin (cryst) and beta lactoglobulin (cryst) were purchased from Pentex, Kankakee, Illinois. Rabbit gamma globulins (>98% pure by electrophoresis) was a product of Mann Research Laboratories, New York. Bowman-Birk soybean protease inhibitor was obtained from Miles Laboratories, Elkhart, Indiana.

APPLICATION AND DISCUSSION

Typical examples of separations achieved with commercially available proteins using the scanning isoelectrofocusing method are shown in Figs. 2 and 3. Excellent resolution of components has been observed which is not possible when the column is drained for detection of the focused zones. For example, the two components of the Bowman-Birk inhibitor resolved by the present method (Fig. 2A) appear as one only, when the 440 ml LKB column is used and the contents are drained (*8*). It is also evident that the fine isoelectric spectra of beta lactoglobulin (Fig. 3B) and gamma globulin (Fig. 2C) will be disturbed by fluid swirling during emptying of the column. The short time required for focusing is also an advantage offered by this technique.

The major obstacle in realizing the full sensitivity of the method in the detection of microgram quantities of separated proteins is the baseline noise which is mainly due to the presence of ampholytes and optical imperfections of the quartz column. Amounts of proteins in the 10 to 100 μg range can be easily distinguished from the background noise. Smaller amounts can be detected if the extinction coefficient of the protein at 280 nm is relatively high. With colored proteins baseline noise is considerably improved since scanning is performed in the visible region of the spectrum, and consequently

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heart myoglobin (80 μg) in the pH 3–10 range scanned at 180 min; and B, beta lactoglobulin (100 μg) in the pH 3–6 range scanned at 155 min. Electrofocusing was performed at 500 V with 1.2–1.5 mA initial current.

the sensitivity of detection is increased. The present method also offers the additional advantage of scanning the same electrofocused pattern of colored proteins at different wavelengths. Thus, full spectra of separated zones of interest can be compared. By using this technique in the UV region proteins that do not contain tryptophan, which usually absorb maximally at 274 nm, can be distinguished from protein zones containing both tryptophan and tyrosine. This was ascertained by scanning the major band of the Bowman-Birk inhibitor (Fig. 2A) (which does not contain tryptophan), the absorbance of which was found to be higher at 274 nm than at 280 nm.

The presently described scanning technique is mainly an analytical separation method and does not provide for direct determination of the isoelectric points of the separated components. The latter can be approximately estimated with the present instrument by construction of a "calibration curve" with marker proteins of known isoelectric points focused simultaneously with the test sample. An isoelectrofocusing scanner is currently under construction which will provide temperature control, pH monitoring, and a digital data acquisition system.

The continuous scanning of samples during focusing, which enables one to follow both the intensity of the focused zones and the resolution achieved, may considerably facilitate the study of the kinetics of the isoelectric focusing of proteins in sucrose gradients.

A horizontal scanning device and experimental procedure for polyacrylamide gel isoelectrofocusing were described recently (*9*).

REFERENCES

1. H. Svensson, *Arch. Biochem. Biophys., Suppl.* **1**, 132 (1962).
2. O. Vesterberg and H. Svensson, *Acta Chem. Scand.*, **20**, 820 (1966).
3. D. L. Weller, A. Heaney, and R. E. Sjogren, *Biochim. Biophys. Acta*, **168**, 576 (1968).
4. H. J. A. Koch and J. Backx, *Sci. Tools*, **16**, 44 (1969).
5. G. N. Godson, *Anal. Biochem.*, **35**, 66 (1970).
6. N. Catsimpoolas, *Anal. Biochem.*, **26**, 480 (1968).
7. S. Hjertén, in *Free Zone Electrophoresis*, Almquist and Wiksells, Uppsala, 1967.
8. N. Catsimpoolas, *Separ. Sci.*, **4**, 483 (1969).
9. N. Catsimpoolas and J. Wang, *Anal. Biochem.*, **39**, 141 (1971).

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