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Ultraviolet scanning of proteins on ordinary glass plates in thin-layer isoelectric focusing

The detection of proteins following isoelectric focusing in gel-stabilized systems is now usually accomplished by staining. With the majority of the dyes in current use, difficulties are experienced due to the carrier ampholytes, which in most instances must be washed out in a time-consuming step prior to staining. Direct scanning in the UV region would thus simplify the procedures. In view of the quantitative limitations of staining methods, UV scanning would also improve the quantitative evaluation of proteins.

Direct scanning of polyacrylamide gels in UV light at 280 nm has recently been described for electrophoresis[1,2] and isoelectric focusing[3,4]. Quartz tubes have been employed both for the separation and for scanning, thus eliminating transfer which could possibly cause damage to the gels. The use of quartz plates in thin-layer isoelectric focusing appears to be impractical because of their high cost. An attempt was made, therefore, to use ordinary glass plates which, in contrast to UV-transparent glass, will transmit only a small proportion of light at 280 nm. The quantity and subsequent detection of the transmitted light will depend on glass thickness, glass quality, intensity of the light source and sensitivity of the photomultiplier. Commercially available ampholytes have a low absorption at 280 nm (ref. 5) and do not interfere with the detection of proteins at this wavelength. Preliminary experiments described in this communication demonstrate that UV scanning of Sephadex layers on ordinary glass plates can be achieved in transmission, using the Schoeffel spectrodensitometer.

Thin-layer isoelectric focusing was performed as described previously[6]. Glass plates, 20 × 20 cm in size and 1 mm thick (precoated and thoroughly cleaned plates from Merck, Darmstadt, G.F.R.), were coated with a 0.75-mm layer of a Sephadex G-75 Superfine suspension containing 1% of pH 3–10 carrier ampholytes. In some experiments, the separation distance was reduced from 20 to 10 cm (ref. 7). The preparations used where horse cytochrome C and horseradish peroxidase (RZ = 0.6) from Boehringer, Mannheim, G.F.R., horse myoglobin and horse ferritin from Koch & Light, Colnbrook, Great Britain, and human serum albumin from Behringwerke, Marburg, G.F.R. Focusing with a potential gradient of 10 V/cm was complete after 5–7 h with the 20-cm separation distance and after 1–2 h with the 10-cm separation distance. After removal from the focusing apparatus, the plates were immediately scanned at 280 nm and at the Soret band (410 nm for myoglobin and 403 nm for peroxidase) in a Schoeffel SD 3000 spectrodensitometer equipped with a 200-W Xe–Hg lamp. The instrument was operated in transmission with single-beam illumination at 0.4 and 1.0 O.D. output ranges. The slit was 3–4 × 0.3 mm in most experiments. The recorder speed was twice that of the plate.

Scanning of uncoated glass plates at different output ranges (0.1, 0.2, 0.4 and 1.0 O.D.) demonstrated a sufficient homogeneity of the glass material. A uniform background was obtained, especially at the higher output ranges tested. Scanning of glass plates coated with a 0.75-mm Sephadex layer containing 1% of carrier ampholytes also gave a uniform background before and after focusing (without added

proteins) under standard conditions. Although the double-beam system gave slightly improved results, the use of single-beam illumination allowed focusing without any modification of the technique previously used. On the basis of these preliminary results, the single-beam system was used in further experiments. Fig. 1 shows the pattern of the protein mixture containing cytochrome C, myoglobin, human serum albumin and ferritin after focusing on a 20-cm plate. Separation of cytochrome C and myoglobin into a number of subcomponents is clearly visible. Both proteins exhibit patterns that consist of a main component with the highest isoelectric point and a number of subcomponents of decreasing quantities and isoelectric points. The distribution of cytochrome C is similar to that found in density-gradient isoelectric focusing in ampholytes with a narrow pH range[8]. Human serum albumin yields a rather broad peak, indicative of heterogeneity already previously found by staining following

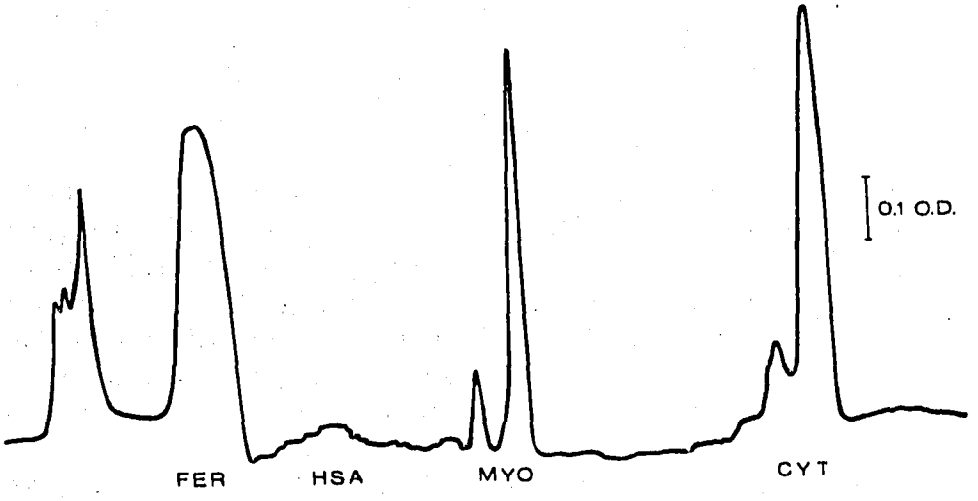

Fig. 1. Scanning at 280 nm of cytochrome C, myoglobin, serum albumin and ferritin (from right to left) focused in pH 3–10 ampholytes on a 20-cm plate. Forty μl of the mixture containing 5% of each protein in 1% glycine adjusted to pH 7.0 were applied at a distance of 5 cm from the anode. The peak furthest to the left corresponds to the starting zone.

thin-layer isoelectric focusing[6]. Although all the proteins were applied in equal amounts, a comparison of the peak areas discloses great differences due to the different extinction coefficients of these four proteins. The extinction coefficients of proteins at 280 nm reflect their aromatic amino acid compositions and varies strongly for different proteins; this presents a limitation to direct scanning at 280 nm.

The pattern obtained by scanning at 410 nm (Soret band) for myoglobin[9] and cytochrome C was very similar to that obtained at 280 nm, thus proving that both proteins are essentially free of non-haeme impurities. Scanning of the short-focusing plates (10 cm) gave essentially the same pattern as that on the long (20 cm) plates (Fig. 2). The advantage of short-focusing runs is the reduced focusing time.

Horseradish peroxidase has been previously focused in thin-layers and shown[10] to consist of about twenty isoenzymes with isoelectric points distributed in the pH range 3.5–9.0. The distribution of enzyme activity was paralleled by the curve obtained by direct scanning of the gel layer at 403 nm[11]. The pattern for horseradish peroxidase focused on a short plate and scanned at 280 nm is shown in Fig. 3. The pattern at 403 nm closely corresponded to that previously described for the 20-cm

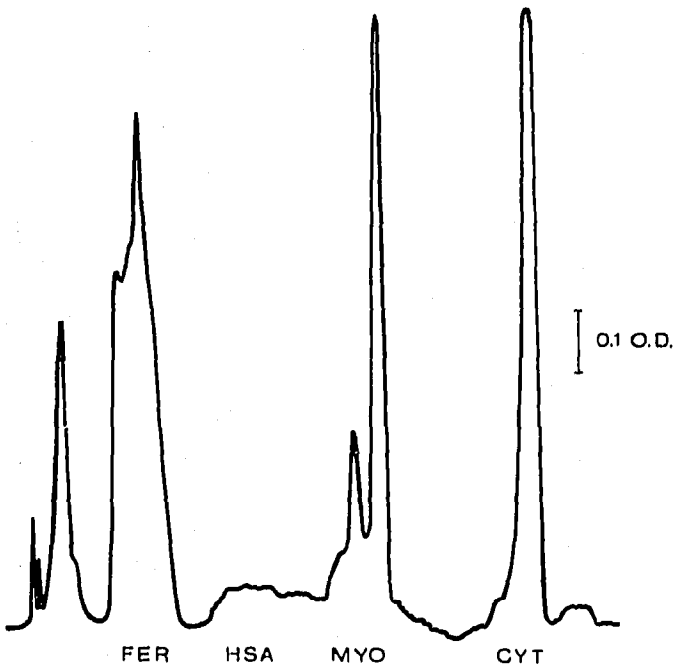

Fig. 2. Scanning at 280 nm of the protein mixture as in Fig. 1, focused on a 10-cm plate. Twenty μl of the mixture were applied at a distance of 2 cm from the anode.

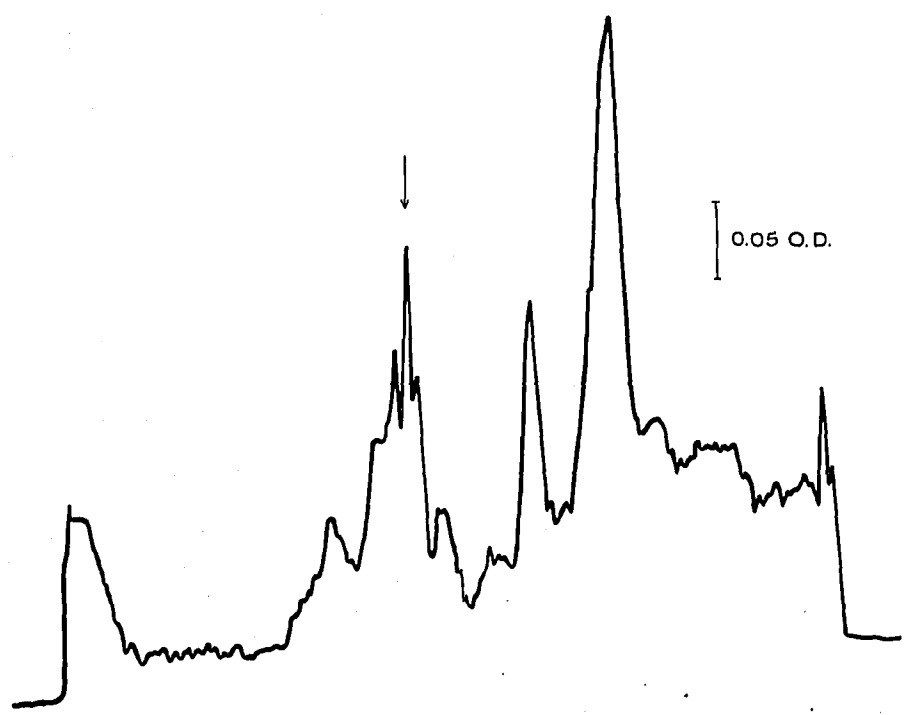

Fig. 3. Scanning at 280 nm of horseradish peroxidase focused on a 10-cm plate. Fifty μl of a 10% enzyme solution were applied in the middle of the plate. Anode to the left. The arrow indicates the application line.

plate. The 280-nm curve follows that of the 403-nm curve for some of the major components. In the region where the substance was applied, however, substantial amounts of non-haeme proteins appear. This finding is expected in view of the low absorbance ratio of *ca.* 0.6 and confirms previous results[11] obtained by staining the focusing prints with Coomassie Blue.

Direct scanning of gel layers at 280 nm in isoelectric focusing offers obvious advantages. Scanning can be performed without distortion of the gel layer and repeated scanning of the gel layer on a single track thus becomes possible during a focusing experiment. The proteins can be localized in the gel layer and the plate can subsequently be used for determination of the isoelectric points of selected peaks. Enzyme activity detected by the use of substrate-impregnated paper and other specific staining methods can be correlated easily with protein absorbance on the same track. The sensitivity of scanning gel layers at 280 nm is lower than that obtained on staining with some of the more sensitive dyes, *e.g.* Coomassie Blue, but is comparable with that claimed for the scanning of polyacrylamide gels in quartz tubes. The lower sensitivity and the varying response for different proteins due to variation of the extinction coefficients are compensated by the simplicity and speed of direct scanning. Although only the scanning of Sephadex gel layers following isoelectric focusing is described in this paper, application of the method to other thin-layer separation techniques for proteins, *e.g.* gel filtration and electrophoresis, appears to be feasible. Scanning of thin layers on ordinary glass plates in transmission will not be limited to proteins and will probably be applicable also to other substances with absorption maxima in the range 250–300 nm.

While the results of our experiments prove the suitability of ordinary glass plates for UV scanning, further work will be needed to improve the method. Screening of different glass materials, varying glass thickness, increasing intensity of the light source and a search for more sensitive photomultipliers are some possible approaches in this direction. Replacement of glass plates by UV-transparent plastic sheets could also offer an alternative, provided that their homogeneity and electrostatic properties are suitable.

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