Paper Electrophoresis of Enzymes

Amylase and Trypsin

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A large number of biologically active proteins have been studied by the classical Tiselius electrophoresis technique. Unfortunately this method only gives information on the individual protein components regardless of their biological activity unless isolation of the fractions is carried out, which is difficult in preparations which are not quite simple.

Wieland and Fischer¹ developed an electrophoretic method using filter paper, which has recently been used for the fractionation of the serum proteins by several workers (Durrum², Turba and Enenkel³, Cremer and Tiselius⁴). Paper electrophoresis offers better facilities for the fractionation of biologically active proteins than does the classical procedure, because the activity-mobility can be determined directly by a method specific for the substance of interest without showing simultaneously the inactive fractions.

The present authors have studied the practical application of the paper electrophoresis technique on amylase and trypsin, and it is the intention to extend the investigation to cover other enzymes and active proteins.

METHODS

Electrophoresis. An attempt was made to use the simplest possible electrophoresis system as such a system was likely to be the quickest and cheapest. Thus no refrigeration was used (*cf.* Cremer and Tiselius⁴) because experience showed that it was not indispensable.

The paper used was Swedish Munktell 20 filter paper cut into strips 32 cm × 4.5 cm, and the buffer was barbital, with a pH of 8.6 and an ionic strength of 0.05. At the concentrations used, this buffer has no effect on the enzyme activity. Electrophoresis was at first attempted with the paper pressed between two glass plates by a simple method which has been routinely used in this laboratory for serum proteins and the like. However it was found that the enzyme left behind a long tail towards the starting point, presumably due to adsorption on the glass and this prevented accurate measurements. Subse-

Fig. 1. The principle of the apparatus. Fig. 2. Standard curve for the determination of amylase.

quently the open pressing device, illustrated in Fig. 1, consisting of a glass lattice closed with glass plates was employed. This arrangement held up to 4 strips at once. After the paper in the lattice had become wet with buffer absorbed from the electrode bowls, 0.02 ml of enzyme solution was pipetted to a point 4 cm from the cathode pressing point. The time of electrophoresis ranged from 3 to 5 hours using a voltage of 250–300 V and a current of 2–4 mA per strip.

Demonstration of enzyme activity in the paper. 1. *Amylase.* Amylase was determined by a slight modification of the Smith and Roe⁵ method based on the photometric measurement of the blue colour of the iodine-starch reaction.

When electrophoresis was completed, the part of paper lying between the points of pressure (18 cm in length) was cut, while wet, into pieces 1 cm wide. These were placed in test tubes each containing 1 ml of 0.05 per cent starch solution and 5 ml of phosphate buffer-NaCl solution (1/50 M phosphate buffer of pH 7.2 to which NaCl was added to make it 1/20 M) at 37° C. The tubes were shaken to detach the enzyme from the paper and placed in a thermostat at 37° C. One hour later 5 ml aliquots were pipetted into a second series of tubes each containing 1 ml of 1 N HCl solution to arrest the enzyme activity. Finally, 1 ml of iodine potassium iodide solution (from a stock solution containing 30 g of potassium iodide and 3 g of iodine in 1 000 ml of distilled water, diluted 1:50) was added and the absorption at 620 μ was measured immediately with a Beckman spectrophotometer. This method was checked by a standard series made from starch solutions of varying concentrations (Fig. 2). It should be borne in mind that the concentrations of both the substrate solution and the iodine solution must be appropriate for the activity of the enzyme solution to be studied.

2. *Trypsin.* The proteolytic power of this enzyme was determined by Krijgsman's micro method⁶, in which casein, the substrate, is precipitated by quinidine, and the resulting turbidity is measured.

Fig. 3. Standard curve for the determination of trypsin.

In the manner described above, the electrophoresis paper was cut into pieces 1 cm wide, which were placed in test tubes containing 2 ml of 0.125 per cent casein solution (prepared by dissolving 2.5 g of casein in 12 ml of 1 *N* NaOH and adding distilled water to make 2000 ml), 2.5 ml of *M*/15 phosphate buffer of pH 8.04, 0.5 cc of physiological saline, and 1 ml of distilled water. The tubes were kept at 37° C for three hours, then 2 ml aliquots were transferred into clean tubes, and 2 ml of *M*/15 phosphate buffer of pH 7.73, 2 ml of distilled water and 2 ml of quinidine precipitant (hot water saturated with quinidine hydrochloride rendered a little acid to litmus with hydrochloric acid) were added. The resulting turbidity was measured after 30 minutes using a stufen-photometer with a S 61 filter. Fig. 3 shows the relation between the turbidity and the enzyme content, and demonstrates that a stufen-photometer can be used for these measurements in place of a nephelometer.

RESULTS AND DISCUSSION

Amylase. The study was carried out on Merck's non-crystalline pancreatic amylase. Tiselius electrophoresis showed that this was a heterogeneous protein mixture with at least three components (Fig. 4). After paper electrophoresis, however, only one of the fractions (the middle one) was found to have amylase activity. On filter paper its mobility was found to be 2.9×10^{-5} cm² · sec⁻¹ · volt⁻¹ (small fluctuations from this value were obtained in dif-

Fig. 4. Tiselius-electrophoresis at pH 8.6 of amylase preparation used. Descending limb.

Fig. 5. Electrophoresis of amylase activity on filter paper. pH 8.6, ionic strength 0.05.

ferent experiments) (Fig. 5), a value approximately the same as that obtained by Meyer, Fischer, and Bernfeld⁷, who prepared a crystalline amylase from the pancreas of hog, which on Tiselius electrophoresis proved to be uniform, with a mobility of $3.1 \times 10^{-5} \text{ cm}^2 \cdot \text{sec}^{-1} \cdot \text{volt}^{-1}$ at pH 7.9 (ionic strength 0.10) and isoelectric point at pH 5.2–5.6. Bernfeld, Duckert, and Fischer⁸ isolated an amylase from human pancreas which was also electrophoretically uniform, with a mobility of $3.75 \times 10^{-5} \text{ cm}^2 \cdot \text{sec}^{-1} \cdot \text{volt}^{-1}$ at pH 10.14.

Trypsin. The preparation used was Merck's non-crystalline pancreatic trypsin. Tiselius electrophoresis revealed two components (Fig. 6). The diagram of activity-mobility obtained by paper electrophoresis was obviously identical with that yielded by Tiselius electrophoresis, and showed that both fractions possessed some proteolytic activity (Fig. 7). The larger (and apparently also most active) fraction was almost iso-electric at pH 8.6, while the smaller had a mobility of $7.0 \times 10^{-5} \text{ cm}^2 \cdot \text{sec}^{-1} \cdot \text{volt}^{-1}$ at this pH.

It is interesting to compare these results with earlier experience on the electrophoresis of proteolytic pancreatic factors. Anderson and Alberty⁹ found that the iso-electric points of chymotrypsin were 8.6 and 8.1 at ionic strengths of 0.01 and 0.1 respectively. Approximately the same result was obtained a little later by Kubacki, Brown and Laskowski¹⁰, who found that

Fig. 6. Tiselius-electrophoresis at pH 8.6 of trypsin preparation used. Descending limb.

Fig. 7. Electrophoresis of tryptic activity on filter paper. pH 8.6, ionic strength 0.05.

the iso-electric point of chymotrypsin α prepared according to Kunitz and Northrop, was 8.3. Brown, Shupe and Laskowski¹¹ isolated an active protein from bovine pancreas and called it chymotrypsin B because it clearly differed from chymotrypsin α in physico-chemical properties. Electrophoretically, the iso-electric point of chymotrypsin B has been determined to be 4.7 (Kubacki, Brown and Laskowski¹⁰). It is possible that in the preparation examined by the present authors the larger fraction was chymotrypsin α and the smaller one chymotrypsin B.

SUMMARY

A technique for the study of enzymes by paper electrophoresis has been described and applied to amylase and trypsin. The amylase activity was found to be electrophoretically uniform, with a mobility of $2.9 \times 10^{-5} \text{ cm}^2 \cdot \text{sec}^{-1} \cdot \text{volt}^{-1}$ at pH 8.6 and ionic strength 0.05. On the other hand, the trypsin activity was, under the same conditions, split up into two components, the larger with a mobility of 0 and the smaller with a mobility of $7.0 \times 10^{-5} \text{ cm}^2 \cdot \text{sec}^{-1} \cdot \text{volt}^{-1}$.

After this investigation was completed, the writers learnt that Wallenfels and Pechmann¹² had used paper electrophoresis for the differentiation of enzymes (amylase, proteinase, lipase, phosphatase) of mould extract. They demonstrated the position of the proteinase using undeveloped cinematographic film as substrate, and the amylase by starch-iodine staining. At 120 V, the proteinase travelled, in 12 hours, at pH 6.8 about 23 mm towards the cathode, and the amylase about 10 mm towards the anode. It seems that the results obtained by the present authors are roughly the same as far as mobility is concerned.

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