

# Technical methods

## A direct staining technique for amylase isoenzyme demonstration

SIDNEY B. ROSALKI *From St Mary's Hospital, London*

Remazolbrilliant Blue covalently linked to amylose (Amylose Azure) has been used for the determination of  $\alpha$ -amylase (Rinderknecht, Wilding, and Haverback, 1967). It forms an insoluble suspension in aqueous media, but liberates a soluble highly coloured blue dye as a result of amylase activity. This communication describes the use of this compound for the demonstration of amylase isoenzymes separated on cellulose acetate membranes.

### Materials and Methods

Pancreatic homogenates, saliva, urine, and sera were separated on cellulose acetate membrane. Pancreatic extracts and saliva were diluted with serum of very low amylase activity (less than 60 Somogyi units) to yield activities of approximately 1,000 Somogyi units.

Cellulose acetate separations were carried out using Oxoid cellulose acetate membranes or the Beckman microzone system. For the former, cellulose acetate membranes, 12 × 5 cm, were used and the sample applied 2 cm to the anode side of the midline using an 8 cm bridge gap. Separations were carried out at 4°C using barbital buffer, pH 8.6,  $\mu$  0.07, at a constant voltage of 200 V for two hours.

Using the Beckman microzone system, samples were applied at the slots nearest to the anode side of the midline. For tissue extracts, one application (0.25  $\mu$ l) of sample was used. For urine and serum, four applications were applied. Separation was carried out at 4°C using buffer as above, and a constant current of 250 V for one hour.

Enzyme substrate was prepared by adding Amylose Azure<sup>1</sup> at a concentration of 100 mg per ml to 0.05 M phosphate buffer, pH 7.4, containing 0.05 M sodium chloride. The resulting suspension was warmed to 37°C, well mixed, added to an equal volume of melted 2% agar which had

Received for publication 28 August 1969.

<sup>1</sup>The gift of Calbiochem, USA

been allowed to cool to 56°C, and rapidly poured into a shallow plastic tray. Following electrophoretic separation the cellulose acetate strip was layered on to the surface of the Amylose Azure substrate gel. Incubation was then carried out in a moist chamber at 37°C, the time of incubation being judged by visual inspection. For tissue extracts five to 15 minutes was satisfactory; however, for sera or urine with activities of 200 Somogyi units (the lower limit of sensitivity of the method) one hour's incubation was required. Overincubation should be avoided because of diffusion of stained bands. The amylase isoenzymes appear on the cellulose acetate strip as blue staining bands on a relatively unstained background. After staining, the cellulose acetate was removed from the substrate gel and air dried. It is important that the cellulose acetate is not washed or immersed in fixative, as this disperses the staining. The stained bands are stable for many months on the dried membrane.

### Results

Amylase isoenzymes could be detected in samples with activities of 200 Somogyi units or above. Isoamylase activity was demonstrated only in the gamma globulin region. Clear separations of salivary and pancreatic isoamylases were obtained (Fig. 1), the pancreatic isoenzyme having a greater mobility towards the cathode. Mixtures of pancreatic and salivary isoamylases could be clearly resolved into two separate bands. Examination of normal urines (not concentrated) showed the presence of two isoamylases (Fig. 2), the most prominent of which occupied a position corresponding to the pancreatic isoamylase. In sera showing raised amylase activity from patients with mumps parotitis, isoamylase corresponding in mobility to that of the salivary enzyme could be demonstrated (Fig. 2).

These results were in accord with findings obtained by other separation and isoenzyme demonstration methods (Kamaryt and Laxova, 1965; Hobbs and Aw, 1968); subcomponents of the isoamylases were not resolved.

### Comment

Electrophoretically separated isoamylases may be demonstrated by a variety of methods. However, elution and saccharogenic assay is somewhat tedious; staining techniques utilizing the starch-iodine reaction are subject to interference by protein, and a coupled-enzyme staining technique used for demonstrating maltose formed from starch (Joseph, Olivero, and Ressler, 1966) is somewhat non-specific (Hobbs and Aw, 1968).

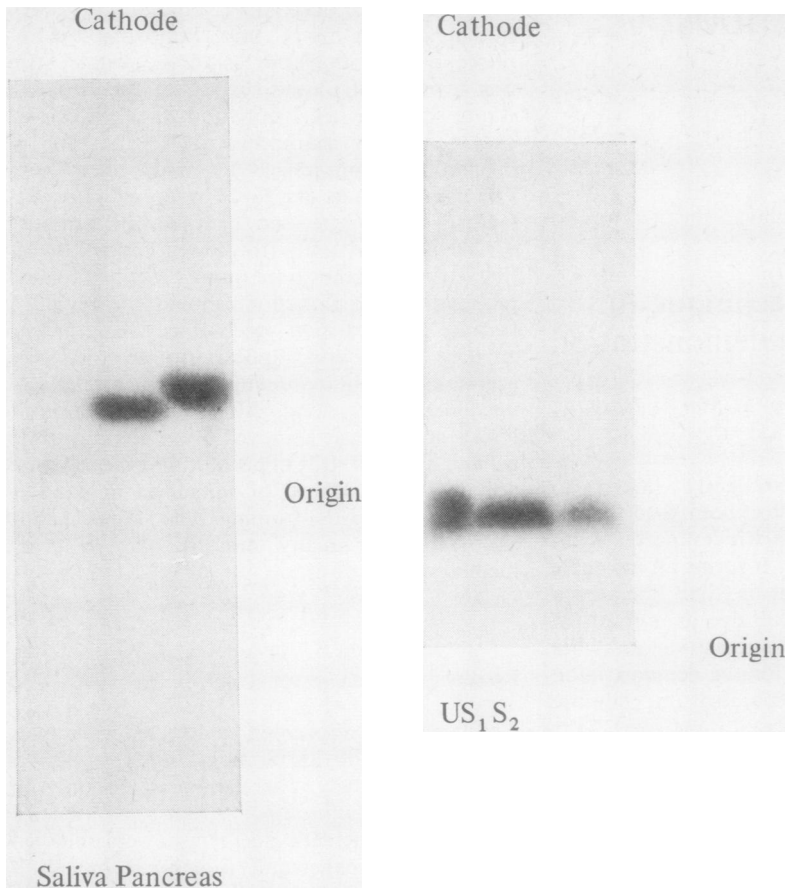


Fig. 1 Amylases of saliva and pancreatic extract (diluted to yield activities of approximately 1,000 Somogyi units), separated on Beckman microzone<sup>1</sup> cellulose acetate membrane. The pancreatic isoenzyme has a greater mobility towards the cathode.

Fig. 2 Amylases of normal urine, U (activity 320 Somogyi units) and sera, S1 and S2 from children with mumps parotitis (activities 980 and 205 Somogyi units respectively) separated on Beckman microzone cellulose acetate membrane. Two isoamylases are visible in the urine sample. The sera each show a single isoamylase of mobility identical to that of simultaneously separated diluted saliva.

For these reasons, and because of its simplicity, the staining method described above is preferred.

<sup>1</sup>The gift of Dade Reagents, USA

#### References

- Hobbs, J. R., and Aw, S. E. (1968). *Enzymes in Urine and Kidney*, p. 281. Huber, Berne and Stuttgart.
- Joseph, R. R., Olivero, E., and Ressler, N. (1966). Electrophoretic study of human isoamylases. A new saccharogenic staining method and preliminary results. *Gastroenterology*, 51, 377-382.
- Kamarýt, J., and Laxová, R. (1965). Amylase heterogeneity. Some genetic and clinical aspects. *Humangenetik*, 1, 579-586.
- Rinderknecht, H., Wilding, P., and Haverback, B. J. (1967). A new method for the determination of alpha-amylase. *Experientia (Basel)*, 23, 805.

## Letters to the Editor

### Modification of Plasma Fibrinogen Method

The article by Burmester, Aulton, and Horsfield (*J. clin. Path.*, 23, 43-46) published in your recent issue describes a modification of the plasma fibrinogen method originally published by Miss A. Stransky and myself. I wish to point out an error in their article which seems to have been missed by them.

They recommend that venous blood is anticoagulated with one tenth its volume of 3.8% sodium citrate. In their eventual calculation of the fibrinogen result they include a correction factor of 10/9 for anticoagulant dilution. This of course is wrong because, first, the plasma dilution by anticoagulant is much more, and secondly their calculation ignores variations in haematocrit. The significance of these criticisms may be illustrated by the following examples:

Let (a) 4.5 ml blood be anticoagulated with 0.5 ml 3.8% sodium citrate and, (b) the plasma (+citrate) fibrinogen as measured by the method be 250 mg%. Using their factor 10/9 this would give a corrected fibrinogen concentration of 278 mg%.

The following calculations show the results in three examples where the packed cell volume is 40%, 50%, and 60% respectively, assuming, of course, that citrate dilutes the plasma only.

#### 1 PCV 40%

Volume of plasma in 4.5 ml blood	= 2.70 ml
Citrate dilution	= 0.5 in 3.2
	= 1 in 6.4
Plasma fibrinogen =	
$\frac{250 \times 6.4}{5.4}$	= 296 mg%

Their corrected result is too low by 18 mg%, ie, an error of 6%.

#### 2 PCV 50%

Volume of plasma in 4.5 ml blood	= 2.25 ml
Citrate dilution	= 0.5 in 2.75
	= 1 in 5.5
Plasma fibrinogen =	
$\frac{250 \times 5.5}{4.5}$	= 305 mg%

Their corrected result is too low by 27 mg%, ie, an error of 9%.

#### 3 PCV 60%

Volume of plasma in 4.5 ml blood	= 1.80 ml
Citrate dilution	= 0.5 in 2.30
	= 1 in 4.6