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A Technique for the Identification and Separation of Enzymes by Paper Chromatography

BY K. V. GIRI, A. L. N. PRASAD, S. GOWRI DEVI AND J. SRI RAM
Department of Biochemistry, Indian Institute of Science, Bangalore

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Paper partition chromatography, first described by Consden, Gordon & Martin (1944), has proved an effective method for separating the simpler chemical compounds from complex mixtures such as protein hydrolysates and for identifying them on a micro-scale. So far, paper partition chromatography has been used chiefly in the analysis of amino-acids, carbohydrates, purines, nucleic acids, organic acids, vitamins and other substances of biological importance. Very little is recorded in the literature on the application of this technique or of adsorption chromatography on paper to the study of enzymes, except the recently published reports by Franklin & Quastel (1949), Mitchell, Gordon & Haskins (1949) and Reid (1950). Franklin & Quastel (1949) have reported their preliminary investigations on the enzyme urease. They studied the movement of this enzyme on paper, using a cysteine-glycine solution as the developing solvent and determined the activity of the enzyme on paper, manometrically. They showed that the movement of the enzyme on paper can be followed by this technique, without the enzyme losing its activity during the experiment. However, they did not attempt to separate enzymes by this technique. Mitchell *et al.* (1949) made use of the 'chromatopile' (a pile of filter-paper disks) for the separation of the constituents of takadiastase preparations, by placing the enzyme mixture near the top of the filter-paper pile and fractionating by a process involving solubility in a concentration gradient. They report that some separation of adenosine deaminase from amylase was obtained by using aqueous ammonium sulphate as the solvent. In these methods the enzymes are located by carrying out a series of determinations of the activity of the enzymes

present in different parts of the paper. This procedure is very cumbersome and time-consuming for carrying out preliminary investigations on the movement of enzymes on paper. As a consequence, we have employed a simpler technique for locating the enzymes on paper, using agar plate containing the substrate on which the enzyme acts and suitable reagents for the detection of the hydrolysed products (cf. Goodall & Levi, 1947). The ease with which the enzymes on paper can be located by means of this technique prompted us to investigate the movement of enzymes on paper, with a view to separating enzymes by paper chromatography. Reid (1950) has given a preliminary account of similar studies, chiefly with fungal enzymes. The essential feature is the use of precipitating solvents such as aqueous acetone or alcohol or salt solutions as the moving phase (for references see Swingle & Tiselius, 1951). A preliminary account of this technique has already been given (Giri & Prasad, 1951).

It is intended in the present paper to demonstrate the usefulness and potentialities of this technique in the study of the chromatographic behaviour of enzymes and to describe some examples of the separation of enzymes, which have so far been achieved in preliminary investigations of some important enzyme systems. The study of other enzymes is in progress.

MATERIAL

The various enzyme preparations used in the present investigations were either isolated as dry powder by precipitation or obtained as aqueous extracts from natural sources.

Amylases

Amylase from sweet potato. This enzyme was prepared from aqueous extract of the dried powder of sweet potatoes by precipitation with 4 vol. of 95% (w/v) aqueous ethanol as described by Giri (1934). 0.10 g. of the preparation was dissolved in 1 ml. of water and centrifuged to remove the suspended impurities, and the clear aqueous extract was used.

Amylase from saliva. The enzyme was prepared from saliva according to the method of Hanes & Cattle (1938). 0.30 g. of the enzyme preparation was dissolved in 10 ml. of water.

Amylase from *Aspergillus niger*. The amylase solution was prepared by extraction of 1 part of dry mycelium with 10 parts of water for 4 hr. and subsequent filtration. The clear filtrate was used, without further treatment, as source of the enzyme.

Amylase from rice. Germinated and dried rice powder was extracted with 8 times its volume of water and the enzyme mixture was precipitated from the extract by the addition of three volumes of acetone. The precipitate obtained from 15 ml. of original extract was dissolved in 0.25 ml. of water.

Phosphatases

Kidney and liver phosphatases. The enzyme preparations were obtained from acetone powders which were prepared from the kidney and liver (sheep and rat) in the conventional manner. The tissues were washed free from blood with cold water and minced well. The minced tissue was ground with acetone and filtered. It was treated with a further quantity of acetone followed by ether and filtered. The preparation thus obtained was first dried in a current of air and then in a vacuum desiccator over H_2SO_4 . The dry material was ground finely and used for extraction of the enzymes.

For extraction, 1.0 g. of the powder was triturated with 20 ml. of cold water for 2–3 hr. The insoluble material was removed by centrifugation. The supernatant liquid was kept in a refrigerator under toluene. This solution was used directly.

Serum phosphatase. Human, rat and chick sera were used as such as source of the enzyme.

Phosphorylase

Green gram (*Phaseolus radiatus*), both resting and germinated, was used as source of the enzyme. The resting seeds were powdered, sieved (100 mesh) and extracted with 5 vol. of water for 3–4 hr. at 0°. The clear solution obtained on centrifugation was used as a source of the enzyme. For the preparation of enzyme extract from the germinated seeds, the same proportion of water to the weight of the seeds was used for extraction.

METHODS

With a micropipette (7–12 μ l.) known volumes of the enzyme solutions were placed on a filter paper (Whatman no. 1; 45–50 cm. long and 14–18 cm. wide) at intervals of about 2.5 cm. on a line drawn about 10 cm. from one end of the filter paper. Care was taken to confine the enzyme to a spot of as small an area as possible (about 1 cm. in diameter). A total of 50 μ l. of the enzyme solution could be deposited on the paper within such an area by applying it in 7–10 μ l. portions and allowing to dry before the next application. After drying, the paper was hung from a glass trough fitted near the top of a rectangular glass chamber. The whole assembly was kept in a refrigerator at 0–5°.

The bottom of the glass chamber was covered with the aqueous solution to maintain suitable conditions of humidity. The solvent was then poured into the glass trough and allowed to spread down the paper sufficiently far beyond the starting line. Usually the chromatogram was allowed to run until the solvent had advanced about 20 cm. from the starting line. Approximately 4–8 hr. run was sufficient in most cases. With *n*-butanol as solvent, however, about 16–20 hr. were necessary for the solvent front to travel the same distance as the other solvents. After the solvent had travelled a convenient distance, the paper strip was removed and the limit of excursion of the solvent marked. The paper was allowed to dry at room temperature. The position of the enzymes on the paper was located as follows.

Identification of enzymes on the paper

The agar-substrate media used for the detection of the various enzymes consisted of 2 g. agar-agar, with the following additions, made to final volume 100 ml. with water.

Amylases. 'Soluble starch,' 1 g. (British Drug Houses Ltd.) and 30 ml. of 0.2M-sodium acetate buffer (pH 4.6) for sweet-potato amylase or 30 ml. of 0.067M-phosphate buffer ($KH_2PO_4 + Na_2HPO_4$; pH 7.0) for salivary and *Aspergillus niger* amylases.

Phosphatases. Sodium phenolphthalein phosphate (0.1 g.) and 30 ml. of 0.02M-sodium acetate buffer (pH 5.2) for acid phosphatase or 30 ml. of 0.1M-sodium glycine buffer (pH 9.2) containing also 0.58% (w/v) NaCl for alkaline phosphatase.

Phosphorylases. Glucose-1-phosphate, 0.2 g. (K_2 salt) and 30 ml. of 0.2M-sodium citrate buffer (pH 6.0).

The mixtures were usually made after autoclaving the agar solution. The agar-substrate medium, while still hot, was poured on a glass plate 16 by 8 in. This was allowed to cool. The dried paper-strip chromatogram was laid gently on the agar plate and allowed to remain on it for 4–12 hr. at room temp. (20–30°), depending on the activity of the enzyme. The paper was then removed gently without disturbing the surface of the agar layer and the surface was flooded with the appropriate reagent. Iodine solution (0.01 N) was used for the detection of amylases and phosphorylases and 0.1 N-NaOH for the detection of phosphatases. We could also locate the position of phosphatases by locating the inorganic P formed by their action on sodium glycerophosphate. It was, however, found that clear spots were obtained using phenolphthalein-phosphate as substrate. The positions of the enzymes on paper were clearly indicated by the formation of coloured or colourless spots on the agar plate. The presence of β -amylase or α -amylase was indicated by the formation of violet or colourless spots respectively against a blue background. Phosphorylase produced a blue spot while phosphatases produced pink spots against a colourless background. The position of the enzymes on the paper was also indicated by spraying the above reagents on the paper itself. It was, however, found that well-defined and clear spots were best obtained on the surface of the agar plate.

For locating on the same chromatogram enzymes which hydrolyse the same substrate at different pH's (such as the alkaline and acid phosphatases) or act on different substrates (like phosphorylase and amylase) the paper was cut into strips longitudinally, each carrying the enzyme concerned. The strips were then placed on the agar plate containing suitable substrate and adjusted to suitable pH.

Developing solutions

The choice of developing solution for the chromatography of enzymes is rather limited on account of the labile nature of enzymes. It appeared probable that the movement of enzymes on paper might be obtained by using the ordinary solvents employed for precipitating enzymes. Aqueous acetone, aqueous ethanol and saline solution were, therefore, chosen for trial. *n*-Butanol saturated with water was also tried, as it was found to have no deleterious effect on the enzymes. After a series of trials with all these solvents mixed in various proportions with water, it was found that aqueous acetone (20–50%, v/v) and sodium chloride (2–20%, w/v) proved the most useful developing agents for the separation of some of the enzymes investigated. The enzymes did not travel at all when *n*-butanol saturated with water was used as solvent.

RESULTS

Movement of enzymes in various developing solutions

The technique described was first applied to the study of the movement of various important enzymes and later extended to the resolution of the individual enzymes in mixtures and in extracts of plant and animal tissues. Table 1 gives the position on the chromatograms of the enzymes from plant and animal tissues and blood sera in various developing solutions. The R_F values (Consden *et al.* 1944) represent the average of several experiments. This table is intended to serve as a guide to the relative positions of the enzymes on paper, under the experimental conditions. The movement of the enzymes is influenced by the nature of the accompanying substances present in the enzyme preparations employed, the concentration of the enzyme, the nature of the developing solution and other unknown factors.

It will be apparent from the data given that some of the enzymes investigated have high R_F values while others do

not show any movement at all. Aqueous ethanol (50%, v/v), acetone (50%, v/v) and NaCl solution (2%, w/v) bring about the movement of many of the enzymes investigated. β -Amylase (from sweet potato), amylase from *A. niger*, phosphorylase from green gram and amylase from germinated rice show considerable movement, while α -amylase (salivary) does not move at all. One of the alkaline phosphatases of kidney and the alkaline phosphatases of rat and human sera also do not show any movement.

Solvents such as *n*-butanol and acetone containing very little water do not bring about the movement of the enzymes.

Chromatography of mixtures of enzymes

The observation that enzymes move on paper without losing their activity and that the rate of movement differs from one enzyme to another depending on their degree of aggregation and the strength of association with other proteins and accompanying substances indicates the basis for a chromatographic separation of enzymes. The technique described above may, in fact, be applied to the separation of enzymes from one another, and examples of the separation of enzymes which has so far been achieved in preliminary investigations are given below.

Separation of α -amylase (salivary) and amylase from A. niger. We have succeeded in separating the components of such a mixture, using aqueous acetone (50%, v/v) as the developing solvent. A typical chromatogram of a mixture of these two amylases is shown in Fig. 1. It can be seen from the figure that the separation of the two amylases is readily achieved. The salivary amylase remains at the starting point, while the amylase of *A. niger* travels down the paper. Thus it is possible by means of this technique to separate from one another even closely related enzymes like the two α -amylases. Although a mixture of salivary amylase and the amylase of *A. niger* can be separated, the complicated shape of the chromatogram obtained in the case of a mixture of salivary amylase and β -amylase

Table 1. Behaviour of enzymes on paper chromatograms

Enzyme	Source	Solvent used	R_F
α -Amylase	<i>Aspergillus niger</i>	Aqueous acetone (50%, v/v)	0.75
β -Amylase	Sweet potato	Aqueous acetone (50%, v/v)	0.50
α -Amylase	Saliva	Aqueous acetone (50%, v/v)	0.00
Amylases	Germinated rice	0.33 M-NaCl	0, 0.67*
Amylases	Germinated rice	Acetone-water 75/25 (v/v)	0
Phosphorylase	Green gram (14 μ l.)	0.33 M-NaCl	0.77
	(21 μ l.)	0.33 M-NaCl	0.74
	(28 μ l.)	0.33 M-NaCl	0.65
	(42 μ l.)	0.33 M-NaCl	0.55
	(28 μ l.)	3 M-NaCl	0.68
Acid phosphatase	Rat kidney	0.33 M-NaCl	0.61
Alkaline phosphatase	Rat kidney	0.33 M-NaCl	0.00, 0.67*
Acid phosphatase	Sheep kidney	0.33 M-NaCl	0.53
Alkaline phosphatase	Sheep kidney	0.33 M-NaCl	0.00, 0.61*
Acid phosphatase	Sheep kidney	Aqueous acetone (30%, v/v)	0.53
Alkaline phosphatase	Sheep kidney	Aqueous acetone (30%, v/v)	0.00, 0.60*
Acid phosphatase	Rat liver	0.33 M-NaCl	0.63
Alkaline phosphatase	Rat liver	0.33 M-NaCl	0.67
Alkaline phosphatase	Chick serum (22 μ l.)	0.33 M-NaCl	0.65
Alkaline phosphatase	Human serum (88 μ l.)	0.33 M-NaCl	0.00
Alkaline phosphatase	Rat serum (55 μ l.)	0.33 M-NaCl	0.00

* Two components.

from sweet potato renders the separation of these two enzymes from one another somewhat difficult at the present stage.

Amylases from germinated rice. Similarly, clear separation of amylases occurring in aqueous extracts of germinated rice into two enzymes, one having no movement at all and the other having an R_f value of 0.67 was achieved using NaCl solution (Fig. 2). We have not as yet been able to characterize these two amylases.

Fig. 1. Chromatograms of a mixture of α -amylase (salivary) and amylase from *A. niger* 50% (v/v) aqueous acetone: O, starting line; S, solvent front; 1, α -amylase (salivary); 2, α -amylase (spot at the starting line) and amylase from *A. niger* (spot at the bottom); 3, amylase from *A. niger*.

Kidney phosphatases. Clear separation of the phosphatases present in rat, guinea pig and sheep-kidney extracts into two distinct alkaline phosphatases (one of them having an R_f value of 0.62–0.68 and the other showing no movement) and one acid phosphatase with an R_f value of 0.53–0.61 which moves slightly more slowly than the corresponding alkaline phosphatase was achieved by this method (Fig. 3). Further investigation is in progress on the characterization of the two alkaline phosphatases which have been chromatographically shown to be quite distinct from one another. Pending further knowledge of the nature of these phosphatases it is proposed to name them as stationary and mobile phosphatases.

Liver phosphatases. The examination of the chromatograms (Fig. 3) reveals the presence of mobile acid and alkaline phosphatases.

Serum phosphatases. The chromatograms of the phosphatases of human and rat sera showed the existence of alkaline and acid phosphatases both of which were stationary, while

the chromatogram of chick serum phosphatase showed the existence of a mobile phosphatase. The difference in the movement of the phosphatases of rat and human sera on the one hand and that of chick serum on the other is rather striking. This characteristic difference in the chromato-

Fig. 2. Chromatogram of amylases of aqueous extract of germinated rice showing the presence of two distinct amylases. One remains at the origin while the other moves down. 2% (w/v) aqueous NaCl; O, starting line; S, solvent front.

Fig. 3. Chromatograms of phosphatases from kidney and liver of rat. 2% (w/v) aqueous NaCl; O, starting line; S, solvent front; A1, alkaline phosphatase of liver; A2, alkaline phosphatase of kidney; B1, acid phosphatase of liver; B2, acid phosphatase of kidney.

graphic behaviours of the sera of various animals in both normal and pathological conditions is being investigated.

Amylase and phosphorylase of green gram. Our attempts to separate the amylase and phosphorylase of germinated green gram using various solvents did not prove fruitful due to the fact that both enzymes moved together. Fig. 4 shows the interesting fact that on developing the agar plate with iodine solution there is a small break in the middle

lase, alkaline phosphatase of human serum and one of the alkaline phosphatases of kidney do not show any movement at all, and they are found at the starting point, irrespective of the solvent used. The other enzymes investigated, namely, amylase from *A. niger*, β -amylase from sweet potato, phosphorylase from green gram, the acid phosphatase and one

Fig. 4. Chromatograms of the amylase and phosphorylase of aqueous extract of germinated green gram (*Phaseolus radiatus*). 2% (w/v) aqueous NaCl; O, starting line; S, solvent front. 1, amylase; 2, phosphorylase (showing the position of the amylase by the absence of phosphorylase activity at the centre of the spot).

of the long blue spot. This is clearly the position of the amylase which either prevents the phosphorylase from acting on the Cori ester present in the agar or hydrolyses the starch synthesized from it.

DISCUSSION

The present data offer an idea of the chromatographic behaviour of enzymes on paper. Most of the enzymes investigated travel on paper in solvents with high water content, such as aqueous acetone or alcohol and salt solutions. From the above results of preliminary investigations certain generalizations on the movement of enzymes on paper may be made. (1) Enzymes can be made to move on paper by selecting suitable solvents. The rates of movement of the enzymes investigated differ from one another. Some enzymes such as salivary amy-

Fig. 5. Chromatograms of phosphorylase from green gram (*Phaseolus radiatus*) showing the influence of concentration of the enzyme on the rate of movement. 2% (w/v) aqueous NaCl; O, starting line; S, solvent front. 1, 14 μ l.; 2, 28 μ l.; 3, 42 μ l. enzyme solution applied to paper.

of the alkaline phosphatases of kidney, acid phosphatase of liver and one of the amylases of germinated rice grain show considerable movement. (2) Increase in the concentration of the enzyme decreases its rate of movement. This has been observed particularly in the case of phosphorylase with 2% (w/v) sodium chloride as the solvent (Fig. 5). (3) The R_f values of the enzymes are also influenced by the distance to which the solvent front has advanced. This is clearly shown in the case of the amylase from *A. niger* with aqueous acetone as solvent. The R_f values decrease slightly with increase in distance travelled by the solvent front (Giri & Prasad, 1951). (4) The rate of movement of the enzymes is slower with aqueous acetone than with sodium chloride as solvent. (5) Proteins and other non-enzymic impurities associated with them

tend to alter the movement of the enzyme or suppress it altogether. Separation of enzymes under such conditions can still be achieved by choosing a suitable solvent. It may be that in some instances the lack of movement of enzymes on paper is due to protein interaction with other impurities present in the extract or adsorption of the enzyme on to other substances which are absorbed on paper. Too little is known about these factors. For this reason it is clearly important that the material used should be as free as possible from impurities, and exact conditions which give complete separation must be determined for each of the investigated enzymes. (6) The observation that there are two distinct and well-defined spots in the chromatogram of the alkaline phosphatase (Fig. 3) of the acetone-treated kidney extract would indicate the existence of at least two distinct alkaline phosphatases in kidney. The chemical basis for the differences between the alkaline phosphatase is not understood. In crude extracts the presence of substances acting as adsorbents or protective agents might contribute to the observed differences in the movement of the two enzymes. However, whatever effects such substances may have, the two phosphatases retain their individuality irrespective of the solvent used. But this evidence should be confirmed by the isolation of the enzymes by chromatography using cellulose columns, and study of their characteristics before their non-identity is finally accepted. The difference in the mobility of the enzymes may lie in the enzymes themselves, or be due to something which remains firmly associated with the enzyme. In addition to the existing numerous histochemical and biochemical methods for distinguishing the phosphatases from one another, the paper-chromatographic method may provide a further basis for distinguishing these enzymes present in various tissues. (7) The results are easily reproducible as

long as the same experimental conditions are maintained. When duplicates are run simultaneously on the same paper, the differences in R_f values are negligible. Under the experimental conditions it was found possible to reproduce with reasonable exactness the characteristic positions occupied by the enzymes.

The paper-chromatographic method is undoubtedly a useful addition to the methods used in enzyme chemistry for determining the identity or diversity of enzymes and for separating individual enzymes from a mixture. It is obvious that the chromatographic behaviour of enzymes may provide a basis for the differentiation of enzymes of overlapping or identical substrate specificity. The very small amounts of the enzyme preparations used in these methods make preparative work difficult. For preparative work based on the same principle columns made from powdered paper may, however, prove satisfactory.

SUMMARY

1. A technique for the identification and separation of enzymes by paper chromatography is described.
2. The application of this technique to the study of the movement of amylases, phosphorylases and phosphatases on paper has been examined and R_f values tabulated.
3. Aqueous acetone, aqueous ethanol and sodium chloride solutions have proved useful as solvents for the study of the movement of the enzymes on paper.
4. Some examples of the separation of enzymes by means of this technique are given.
5. The usefulness and potentialities of this technique in the study of the chromatographic behaviour of enzymes are discussed.

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