THE ASSAY OF ENZYME ACTIVITY BY THE PLATE-DIFFUSION TECHNIQUE

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The technique described provides a convenient means for estimating various enzymes quantitatively. Only a few examples are quoted, but provided suitable indicator substrates can be found the range can be extended. Having established the procedure for one enzyme, it is applicable to others, subject only to the variation in substrate required and the different dilutions of the enzyme preparation necessary to obtain satisfactory responses. Thus, the method is more convenient and easier to manipulate than are the existing methods, including those official in the B.P. and U.S.P.; moreover, as already stated, the assay is quantitative, the limits of error being only about 8 per cent ($P = 0{\cdot}95$).

Enzymes will diffuse through an agar gel and digest a suitable substrate in the agar in the same way in which antibacterial substances diffuse to inhibit bacterial growth. Provided, therefore, that the extent of the digestion can be measured by some visible means, the principle of the cup-plate technique can be applied, and we have developed methods based on this principle for the assay of pepsin, trypsin and amylase but not lipase (in pancreatin), diastase and papain. The idea is not new; it was used, for example, by Dingle, Reid and Solomons (1953) mainly for determining saccharolytic enzyme activity during fungal metabolism. It also formed the basis of methods described by Castrèn (1956), but our methods differ from hers in several respects, notably in the enzyme concentrations needed to obtain satisfactory responses, and sometimes in the substrate used.

In contrast to the existing methods, the diffusion method allows a direct quantitative assessment of activity—the present methods are little more than qualitative, being simply "minimum performance" tests at given levels of dilution.

Experimental and results

The basis for the procedures described is the well-known four-point assay, that is, two dilution levels of the standard preparation are compared with two equivalent dilutions of the test material, the stronger dilution in both instances being four times that of the weaker. Large, flat-bottomed plates or trays measuring about 10 in. × 10 in. are used with an 8 × 8 Latin square design, thus allowing three samples to be examined simultaneously.

The diffusion medium in the plates is a gel of washed agar (1·2 per cent of Davis New Zealand agar has been found to be satisfactory) to which has been added a suitable concentration of a substrate appropriate for the enzyme being assayed. For storage purposes, the more concentrated gel from which this final dilution is made (see below) must be sterilised, but asepsis in the assay is not necessary.

The depth of agar-substrate mixture in the plates is about 1/10 in. (2·5 mm.), 180 ml. of agar-substrate being required for each dish, and the cups, cut with a No. 4 cork borer, are about 7 mm. diameter. The same volume of solution must be filled in each cup and for this a platinum-tipped dropping pipette is recommended. After filling, the plates are incubated at 37° for 16–18 hr.—neither the temperature nor the time of incubation is critical—and the diameters of the zones of digestion read, preferably with an optical projection device giving a magnification of about 5 diameters.

Calculations of the regression of zone diameter on log concentration of the enzymes show a linear relation. As an example, data and the

TABLE I

ASSAY OF PEPSIN BY THE DIFFUSION METHOD: SUBSTRATE 0·25 PER CENT CASEIN AT PH 3·5: RELATION BETWEEN LOG DILUTION AND ZONE DIAMETER

Replicate zone diameters at dilutions of:							
1:200		1:600		1:1,800		1:5,400	
23·0	23·0	19·0	19·5	17·0	17·0	15·0	15·0
21·2	22·0	19·5	21·0	18·0	17·2	15·0	15·2
22·0	21·5	20·2	20·0	17·7	18·0	15·0	15·2
22·0	21·7	20·0	20·2	17·2	17·2	15·7	15·0
22·0	22·5	19·0	19·7	18·0	17·0	15·0	15·0
21·2	21·0	20·5	19·2	17·5	17·5	15·5	15·0
22·8	22·0	19·5	19·5	17·0	17·2	15·0	15·2
22·0	21·0	19·5	19·2	17·0	17·7	15·2	15·7

Analysis of Variance

Source of variance	Sum of squares	Degrees of freedom	Mean squares
Between concentrations	409·329	3	
Regression	409·286	1	409·286***
Deviation from regression	0·043	2	0·022
Within concentrations (residual) ..	14·291	60	0·238 N.S.
Total	423·620	63	

*** = highly significant (P = < 0·001) N.S. = not significant (P = 0·1)

analysis of variance for pepsin are given in Table I. The limits of error are about 8 per cent (P = 0·95).

Pepsin. Casein, gelatin, haemoglobin and egg-white were tried as substrates in the assay of pepsin and casein proved the most suitable. Although the optimum activity of pepsin is at about pH 1·6, the best and most clearly defined zones occur at pH 3·5, the value chosen for all subsequent assays; at pH 5, the level used by Castrèn, the zones are much smaller and badly defined.

At pH 3·5, the casein is present as a suspension in the agar medium and the plates need to be prepared carefully. The method is:

To 90 ml. of sterile 2·4 per cent agar gel in water, previously melted and cooled not below about 75°, add 45 ml. of a 1 per cent solution of light soluble casein and then 45 ml. of double strength McIlvaine buffer at pH 3·5. By this means the casein is obtained as a finely dispersed suspension in the agar base. Pour immediately on a levelled plate and

allow to cool and solidify. Cut out the cups and fill them with the standard and test preparations diluted to the required concentrations with half-strength McIlvaine buffer at pH 3·5. The standard preparation should be prepared from pure pepsin, and suitable dilutions are given in Table II. (A 1 per cent solution of pepsin in acidified chloroform water is a convenient stock supply.)

Trypsin. The procedure is the same as for pepsin, except that a 4 per cent casein solution is used and the assay is carried out at pH 6, using McIlvaine buffer at this value. The zones of digestion show themselves as white haloes with a sharp outer edge on a clear background.

TABLE II

ASSAY OF ENZYMES BY THE DIFFUSION METHOD: RECOMMENDED DILUTIONS AND SUBSTRATES

Enzyme	Substrate and pH value	Recommended dilutions for:	
		Pure enzyme	Commercial preparations
Pepsin	Casein pH 3·5	1:1,600 and 1:6,400	B.P. "2,500" 1:50 and 1:200
			"10,000" 1:200 and 1:800
Trypsin	Casein pH 6	1:10,000 and 1:40,000	Pancreatin 1:200 and 1:800
Amylase	Starch pH 6	1: 2,000 and 1: 8,000	Pancreatin 1:200 and 1:800
Lipase		No reliable	assay
Diastase	Starch pH 6	Not available	B.P.C. '34 1:200 and 1:800
Papain	Casein pH 6	Not available	1:100 and 1:400

Using this method at the most suitable dilution levels (see Table II) the pure trypsin content of pancreatin was found to be between 1 and 2 per cent. An anomaly arose, however, in attempting to confirm this by the B.P. assay for trypsin. The pancreatin gave the expected results (an amino-acid titration equivalent to at least 3 ml of 0·1N NaOH) but trypsin, even with ten times the theoretical equivalent, gave only very low amino-acid titrations (0·5–1·0 ml.). When pure trypsin was added to pancreatin in sufficient quantity to double the activity in the diffusion assay, the B.P. method gave increases of only 25–50 per cent, and reducing the pH value and temperature of the B.P. assay did not effect these results. This indicated the presence of some controlling factor other than the trypsin.

Amylase. Amylase is most easily determined with starch as the substrate in an agar medium at pH 6. To prepare the plates, add 45 ml. of a 2 per cent starch gel in water, 45 ml. of McIlvaine buffer at pH 6 and about 1·8 g. sodium chloride (this is necessary as an activator for the enzyme) to 90 ml. of a 2·4 per cent agar gel in water, previously melted and cooled not below 75°, mix and pour on a levelled plate. After incubation, the zones of digestion appear only faintly, but they are

developed by flooding the plate with a 0·1 per cent solution of iodine in water, when they stand out as colourless zones on a blue background.

The recommended dilutions for the assay of pure α-amylase and amylase in pancreatin are given in Table II. It is essential that the amylase used as the standard be derived from pancreatic extracts since we have found that other α-amylases give different slopes in the assay.

Trypsin and amylase in pancreatin. Trypsin and amylase can easily be assayed simultaneously on one plate by combining the two methods just described. To prepare the plates, melt and cool not below 75° 90 ml. of agar gel as before and add to it 22 ml. of an 8 per cent solution of light soluble casein, 22 ml. of a 4 per cent starch gel in water, 45 ml. of double-strength McIlvaine buffer at pH 6 and about 1·8 g. sodium chloride, and pour on a levelled plate. After incubating, measure the diameters of the outer edges of the white haloes to estimate the trypsin content then flood the plate with 0·1 per cent iodine solution and measure the clear zone diameters on the blue background to estimate the amylase content.

Lipase. For the detection and estimation of lipase activity several substrates have been suggested: Castrèn (1957) used Tween 20, Dingle, Reid and Solomons (1953) used polyethylene glycol monolaurate and Jones and Richards (1952) olive oil, butter fat or triacetin, each with a suitable indicator or developing agent. We have tried several of these, and other substances, but so far only triacetin, with bromocresol purple as the indicator, has shown any promise. The zones appear rapidly in 2–4 hr. but they are not clearly defined and more work in this direction is needed.

Diastase. The procedure for the assay of diastase is the same as that described for amylase and the definition of the zones is equally clear. The recommended concentrations with commercial diastase (Diastase B.P.C. '34) are given in Table II.

Papain. The same assay procedure is followed for papain as for trypsin, again with an optimum zone definition at pH 6. Above this value the zones become progressively less well defined and the characteristic halo disappears. Suitable concentrations of commercial papain to use in the assay are given in Table II.

Discussion

Although 37° is not necessarily the optimum for several of the enzymes examined, this temperature has been found most suitable in the diffusion assays described. At the higher temperature of 55°, for example, as specified in the B.P. assays for pepsin and trypsin, and even with shorter incubation periods, the zones are less regular, their edges are less well defined and the agar is liable to dry out during incubation; moreover it is generally more convenient to choose one temperature for all such assays rather than to have a different one for each enzyme. It is also desirable to limit the number of substrates, and for proteolytic enzymes casein has proved most suitable giving clear zones or haloes under the correct conditions and being readily available as a reasonably pure

substrate. Gelatin is probably the next most satisfactory substrate; it is more sensitive than casein, but is more variable in composition and involves a development stage in the assay with sulphosalicylic acid (Dingle and others, 1953) with the resulting zones less clearly defined. Egg-white, the substrate used in the B.P. assay for pepsin, is too variable and insufficiently sensitive in its response and haemoglobin, although giving reasonable zones, is not easy to use.

Where possible, comparative assays were made using the B.P. and the diffusion methods. Thus, taking into account the much greater precision of the latter, we have confirmed that Pepsin B.P. has only about one-twentieth of the enzymic activity of pure pepsin, and that pancreatin contains about 12 per cent of α-amylase. With trypsin, however, a similar comparison was not possible in that pure trypsin by the B.P. test appears to be almost inactive. This is because trypsin alone does not release the necessary amino-acids from casein as does trypsin in pancreatin. From this and the experimental evidence quoted earlier it is evident that the B.P. test is not a specific assay for trypsin but a composite one involving other enzymes which can further break down the tryptic degradation products to the amino-acids on which the B.P. test depends.

In the trypsin assay the white halos produced are of particular interest in that they only occur if the initial pH value is at 6·0. Even if the pH value is raised to only 6·5 no halos are produced and the digestion zones must be developed with trichloroacetic acid; similarly at pH 5·5 the casein is beginning to be precipitated and this interferes with the zone definition. At pH 6·0 the casein and its final digestion product is in solution, hence the insoluble material forming the halo appears to be an intermediate product.

In all assays by the diffusion method it is desirable to use the pure enzyme as the standard. Such material may not always be available, however, and for routine work standardised samples of commercial products can equally well be used.

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References

Castrèn, E. (1956). *Svensk. farm. Tidsk.*, **60**, 871–878.
Dingle, J., Reid, W. W. and Solomons, G. L. (1953). *J. Sci. Food Agric.*, **4**, 149–155.
Jones, Audrey and Richards, T. (1952). *Proc. Soc. appl. Bact.*, **15**, 82–93.

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