PEPTIDE MAPPING ON CELLULOSE THIN LAYERS

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Fingerprinting techniques, originally developed by INGRAM[1] in studies with haemoglobins, are becoming increasingly important in the characterisation of proteins. For example, peptide mapping has been used for a comparison of immunoglobulins[2], TCA precipitable products of bacteriophage RNA-directed protein synthesizing systems[3,4] and cytochrome *c* from normal and mutant strains of *N. crassa*[5].

Most studies have used separation methods on paper. The low sensitivity of the ninhydrin reagent for peptides has required the application of at least 1 mg of digest at the origin. In contrast, thin layer methods on silica[6] use only 0.05–0.5 mg per plate. The procedure developed by BIELESKI AND TURNER[7] using a cellulose–silica mixed layer[8] has, for amino acid separation, advantages of speed, increased sensitivity, and a high degree of resolution. In this paper we report the extension of similar thin-layer methods to peptide mapping on cellulose.

MATERIALS

Pierce Co. ninhydrin was used. TPCK* was purchased from Calbiochem (Lot 53227) and trypsin (2 times recrystallised) from Worthington Biochemical Co. Kodak royal blue medical X-ray film was used for autoradiography. Micro-capillaries were obtained from Drummond Scientific Co. and Miracloth from Calbiochem. All chemical reagents used were "Analar" grade.

Four proteins were used for digestion.

(i) Carboxymethylated protein subunits from the B1 component[9] of TYMV prepared by R. E. F. MATTHEWS.

(ii) Globin prepared from canine haemoglobin (Mann Research Laboratories)[10].

(iii) ^{14}C-lysine and arginine labelled R17 bacteriophage coat protein, prepared according to CAPECCHI[4].

(iv) Ribonuclease B. (Worthington Biochemical Co., 5 times recrystallised).

A tryptic digest of performic acid oxidised yeast glyceraldehyde-3-phosphate dehydrogenase was supplied by J. I. HARRIS.

METHODS

The protein is subjected to either alkylation with iodoacetic acid [11] or performic acid oxidation[12] to render it susceptible to enzymatic digestion. The treated protein is

* Abbreviations: TPCK = L-1-tosylamido-2-phenylethyl chloromethyl ketone; TYMV = turnip yellow mosaic virus; GPDH = glyceraldehyde-3-phosphate dehydrogenase.

dissolved in $0.05 M$ ammonium bicarbonate buffer, pH 8.4, to a concentration of 2 mg/ml and TPCK-treated trypsin[13] is added to give a final enzyme to substrate ratio of 1/75. The treated trypsin is used to eliminate the possibility of chymotryptic activity. The digest is incubated for 5 h at 37°, then freeze dried and redissolved in 10 % isopropanol for application to the plates.

Standard 20 × 20 cm plates are spread (using Shandon equipment) with a 250 μ layer of cellulose. The glass plates are soaked overnight in an alcoholic potassium hydroxide bath to remove all traces of grease before spreading. Washed surgical gloves are worn at all stages during handling of the plates to reduce contamination from the hands. The cellulose layers are washed by two successive upward elutions, the first in 1 % acetic acid and the second in the buffer to be used for electrophoresis. This treatment moves heavily staining, ninhydrin-positive material originating in the cellulose to the top of the plate. It also serves to reduce the possibility of blistering during subsequent buffer application. The top 1 cm edge of layer now containing the interfering material is scraped off and the plates stored in an ammonia-free atmosphere before use.

Volumes up to 80 μl containing 120 μg of digest are applied by 5 μl micro-capillary to a band origin 2.5–3.5 cm long.

Separation in the first dimension is achieved electrophoretically. A volatile buffer (glacial acetic acid–98 % formic acid–water, 170:50:2800, pH 2) which can be used in a Varsol cooled electrophoresis tank[14] is sprayed on the plate, blotted and wicks applied[7]. Miracloth strips which have been prewashed in the buffer are substituted for paper in the composite paper-dialysis tubing wicks[14]. The plate is immersed in the electrophoresis tank maintained at 15° and run for 25 min at 50 V/cm. The plate is then removed and dried at 30°.

Prior to ascending chromatography in the second dimension, the plate is dipped into 1 % acetic acid and elution allowed to compress the series of separated bands resulting from electrophoresis into a fine line of spots[7]. The plate is dried again at 30°.

Several chromatographic solvents have been employed for separation in the second dimension. The most suitable solvent for a particular protein digest is decided by a preliminary trial. Most runs take 4–5 h at 20°.

Peptides are detected by spraying with a sensitive Cd^{2+}-containing ninhydrin reagent[15]. The plate is then dried for 15 min at 30° and left overnight at room temperature in an ammonia-free atmosphere for the colour to develop fully.

COMMENTS ON METHODS AND RESULTS

Preparation of protein digests

Purity of the starting protein is an important factor in the production of reproducible peptide maps. Small amounts of extraneous amino acids cannot be swamped out by the loading factor because of their far greater sensitivity to ninhydrin, and thus may provide significant background contamination.

Alkylation was preferred to performic acid oxidation as a method of breaking the disulfide bonds prior to tryptic digestion. Performic acid causes total destruction of tryptophan and converts methionine to its sulfone.

Thin layer plates

Pure cellulose layers proved superior to several other types of layer tested.

Silica layers proved unsuitable because of the general fragility and lower sensitivity of the layer. It was difficult to spot aqueous digests without disrupting the layer of silica. The standard of results obtained on silica layers[6] could not be adequately reproduced with the solvent systems used.

Mixed cellulose–silica layers[8] gave fair separations though tailing of several spots occurred.

A mixed cellulose layer was tested using a medium made up of varying proportions of Macherey-Nagel MN 300 cellulose and Whatman CF 11 fibrous column cellulose. Plates spread with a 50/50 composition by weight proved useful for extracts where streaking occurred on standard cellulose plates. The streaking was eliminated but the spots were more diffuse.

Pure cellulose layers 500 μ thick could take much higher loadings of digest but resolution and colour development were decreased.

Only Macherey-Nagel MN 300 provided a firmly-bound layer which could withstand the conditions of separation without lifting. Layers of Whatman CC 41 cellulose, Camag D-O cellulose and microcrystalline "Avicel" cellulose lacked the necessary robustness. All brands of cellulose contained the ninhydrin-positive impurities which necessitated the double washing technique in 1% acetic acid and buffer.

Origin

Band origins gave consistently better results than spot origins. The latter were found to suffer from two disadvantages: (i) much less liquid could be applied without disturbing the layer; and (ii) peptides tended to diffuse during separation.

Electrophoresis

Electrophoresis in the first dimension followed by second dimensional chromatography gave the most satisfactory results. Electrophoresis was carried out at right angles to the direction in which the layer had been spread. The electrophoretic separation could be increased by using a larger plate if required.

Peptides have been reported in chromatography paper[16]. In the early stages paper wicks were used and found to be a troublesome source of ninhydrin-positive material which banded across the width of the plate. A quantity of this material which had been eluted off and hydrolysed, was shown to be composed of amino acids. Miracloth wicks have been employed because they have considerable wet strength and can easily be washed free of any impurities in buffer.

Drying

Plates were dried in a 30° forced air oven against the direction of solvent elution[17]. It was found advisable to avoid heating the plate above 30° because higher temperatures increased adsorption of the larger peptides. No dissolution of spot compactness was evident so the use of freeze drying techniques for plates[18] was unnecessary.

Chromatography

Several solvents were generally useful for separation of protein digests following electrophoresis.

n-Butanol–acetic acid–pyridine–water (15:3:12:12)
Isobutanol–pyridine–water (35:35:30)
Isoamyl alcohol–pyridine–water (35:35:30)
Butanol–acetic acid–water (5:1:4; top phase used)

Slight alterations in the constitution of these "standard" solvents sometimes gave appreciably better separation. A second run in the same solvent was occasionally employed to get a better separation, or two successive solvent systems could be used in the same dimension.

Autoradiography

Thin-layer methods lend themselves particularly well to autoradiography allowing detection of labelled peptides at low levels of radioactivity within 2 weeks (see Fig. 3).

Peptide maps of proteins

The peptide maps for all five proteins studied showed a clear separation of most of the spots, which were compact with very little streaking. Speed, and in particular, much reduced loading requirements appear to be major advantages of the method. The application of 50–100 μg of digest is adequate to give good colour development with ninhydrin. Paper methods require milligram quantities of material to give comparable development.

A two-dimensional ionophoretic separation on paper of 1 mg quantities of a

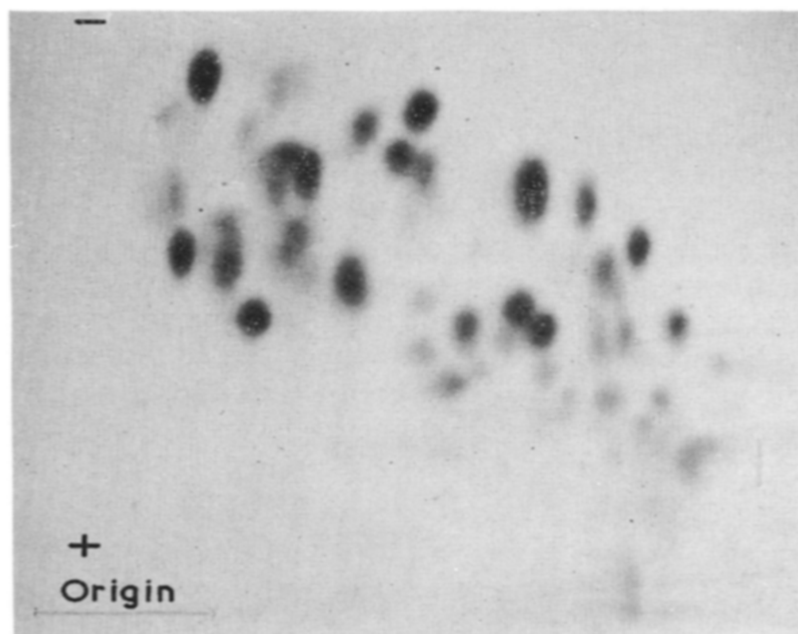

Fig. 1. Map of tryptic digest of performic acid oxidised glyceraldehyde-3-phosphate dehydrogenase (supplied by Dr. J. I. HARRIS) separated by thin-layer electrophoresis (pH 2.0, 25 min, 1000 V, 20 mA, 15°) followed by ascending chromatography in isoamyl alcohol–pyridine–water–ethanol–glacial acetic acid, 35:35:30:10:2.5 for 4 h at 25°.

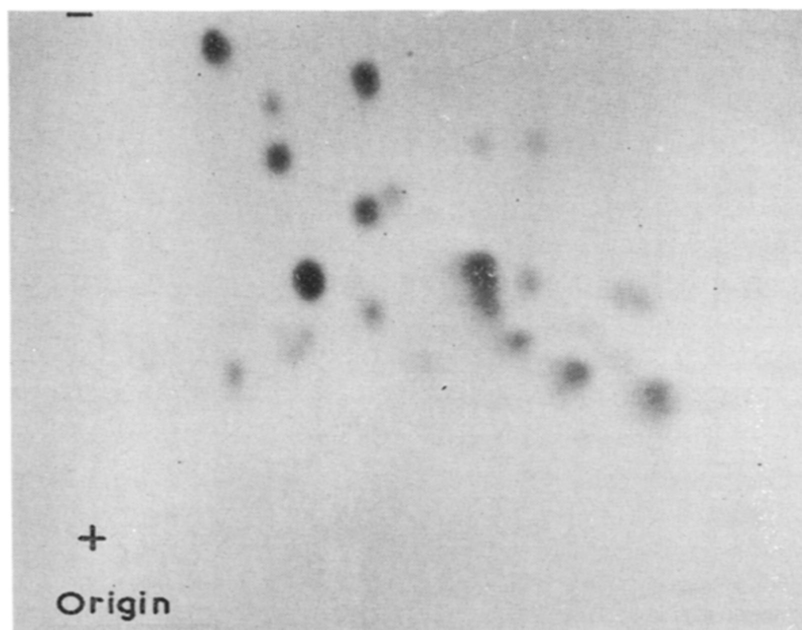

Fig. 2. Map of tryptic digest of carboxymethylated TYMV coat protein separated by thin-layer electrophoresis (as in Fig. 1) followed by ascending chromatography in *n*-butanol–acetic acid–pyridine–water, 15:3:12:12 for 5 h at 20°.

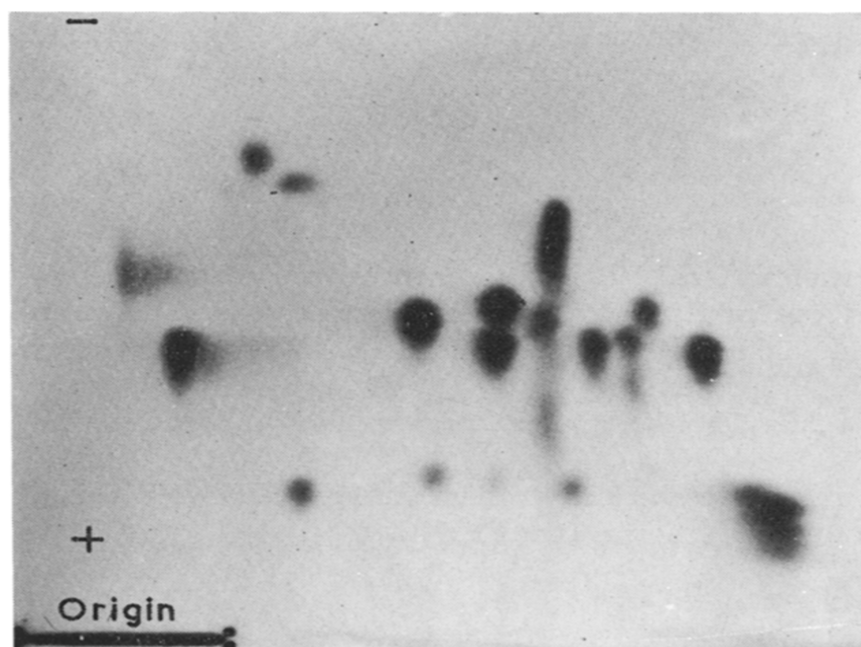

Fig. 3. Autoradiograph of tryptic digest of carboxymethylated ^{14}C-labelled (lysine, arginine) R17 bacteriophage coat protein. Same system as for Fig. 2. 600 c.p.m. (measured with G.M. detector) were spotted on the origin. Exposed for 3 weeks.

tryptic digest of pig GDPH has been shown to give rise to about 30 spots[19]. If the electrophoretically neutral peptides are run in a third dimension a total of 35–40 spots is obtained. A 75 μg sample of a tryptic digest of yeast GDPH which was kindly supplied to us by Dr. J. I. HARRIS gave rise to 43 distinct spots with 7 more uncertain when mapped by the thin-layer system (Fig. 1). This number is greater than expected

from the lysine and arginine residues in the protein (about 38) although in this case the trypsin had not been pre-treated with TPCK. With the exception of haemoglobin, an even greater proportion of extra spots was found in all the digests of the other proteins. The spots are reproducible in number and pattern for different preparations of the same protein. The purity of either the enzyme or the protein may be suspect; alternatively the increased sensitivity of thin-layer methods may enable the detection of peptides which are not present in sufficient quantity to be noticed on paper. The extra spots are presumably not due to autolytic tryptic digestion because a buffer blank containing trypsin which was incubated under the standard conditions gave no spots on the plates. The purity of the enzyme was tested by doing a time course of digestion for TYMV coat protein. Reproducibility of peptide maps from samples removed at 5, 17 and 45 h was found. A 2 h sample gave a comparable map containing one additional spot.

The inherent uncertainty in the number of tryptic peptides arising from a protein, and consequently in the value placed on peptide maps has been discussed by HARRIS AND HINDLEY[11].

Other workers using an improved procedure for separation of tryptic peptides by column chromatography on an ion exchange resin[20] have recently reported many minor peptides in excess of the theoretical number anticipated for digests of ribonuclease and egg white lysozyme. Both these results and ours obtained with completely independent separation systems and with different proteins raise doubts as to the absolute specificity of trypsin for peptide bond cleavage at lysine and arginine residues in a polypeptide chain. The possibility that trypsin has an inherent chymotrypsin activity has been suggested[21].

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SUMMARY

A method is described which uses electrophoresis followed by chromatography on cellulose thin layers to achieve separation of peptides resulting from tryptic digestion of protein. Tryptic digests of 5 proteins were tested: glyceraldehyde-3-phosphate dehydrogenase, TYMV coat protein, canine haemoglobin, R17 bacteriophage coat protein and ribonuclease. The application of 50–100 μg of digest to a band origin is sufficient to give good colour development of peptide spots with ninhydrin. More spots were found in all digests than was anticipated from the number of lysine and arginine residues in the proteins.

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