THIN-LAYER GEL FILTRATION OF PROTEINS AND MUCOPOLYSACCHARIDES

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Since its introduction a few years ago the chromatographic technique of gel filtration has become an important fractionation procedure and has been the subject of many recent papers. GRANATH AND FLODIN[1] showed that fractionation of dextrans occurred according to molecular weight and this was extended by ANDREWS[2] to provide a method of molecular weight determination by correlating the elution volumes with the logarithms of the molecular weights of the proteins examined. A similar approach was used in this laboratory[3] for the investigation of the relative molecular sizes of fragments obtained by degradation of epithelial mucopolysaccharides, the agar columns used being first calibrated with dextrans of known molecular weight ranges. Although this procedure has proved extremely valuable in degradative studies on proteins and mucopolysaccharides, the necessity for prior calibration of the columns has tended to make the method time consuming. It appeared feasible that the technique of thin-layer chromatography might be used to advantage in this problem, the standards and material under investigation being run at the same time, hence eliminating any errors caused by differences in column performances during different runs.

The application of Sephadex G-25 and G-75 to thin-layer chromatography has already been reported from two laboratories[4,5] and whilst this paper was in preparation other papers[6–8] appeared describing the thin-layer gel filtration of proteins on Sephadex G-100 and G-200. The present paper describes an improved detection procedure which can be used to detect proteins, mucopolysaccharides, peptides and amino sugars.

EXPERIMENTAL

Materials

The gel filtration media used were the commercially available Sephadex G-25 fine and G-50 fine. Samples of Sephadex G-100 and G-200 $<$ 400 mesh size and blue dextran 2,000 were generously supplies by Pharmacia, Uppsala, Sweden. The proteins used were samples kindly supplied by Dr. P. ANDREWS and their source has been described by him[2]. The mucopolysaccharides and mucoproteins were as used by GIBBONS AND ROBERTS[3].

Preparation and development of thin-layer chromatograms

The Sephadexes used were allowed to soak in an excess of 0.05 *M* sodium

phosphate buffer (pH 7.0) containing 0.15 *M* potassium chloride and saturated with chloroform for at least 72 h before use. Thin-layer chromatograms were prepared by pouring off the excess buffer from the gels and spreading the resulting free flowing suspension on degreased plates (20 × 10 × 0.4 cm) using a spreader manufactured by C. Desaga G.m.b.H., Heidelberg, with a slit width of 0.25 mm. The plates were allowed to dry in air for a few minutes until the excess moisture had drained off leaving a moist gel surface. As a development tank a plastic bowl covered with a sheet of polythene was found quite adequate. A glass histological dish (11 × 11 × 4 cm) served to hold the buffer and the thin-layer plate was inclined against this at an angle of approximately 10°, contact with the buffer being made by means of a filter paper strip. The plate was allowed to equilibrate overnight with 0.05 *M* sodium phosphate, pH 7, containing 0.15 *M* potassium chloride before use. As a spotting guide a strip of paper marked with an origin line was placed underneath the glass plate at a distance of about 2 cm from the upper end of the plate. Along this origin line 1 % (w/v) protein solution (1 or 2 μl) together with a guide spot of india ink were spotted using calibrated capillary tubes and the plate developed until the india ink spot had reached within a few cm of the bottom of the plate. The time required for development varied with the height of buffer in the reservoir and with the Sephadex used. Under the conditions used it was approximately 2–3 h for G-25 and G-50, 4 h for G-100 and about 12 h for G-200. India ink was not entirely satisfactory as a marker because irreversible adsorption occurred occasionally and in later work it was replaced by blue dextran 2,000 which did not suffer from this disadvantage.

Detection and recording procedure

The procedure used to stain proteins and mucopolysaccharides was a modification of the chlorination technique first described by RYDON AND SMITH[9]. After excess moisture had been removed from the developed plates by allowing them to stand at 37° for 5–10 min they were placed in an atmosphere of chlorine for 10–15 min then allowed to stand in a stream of air for 15 min and sprayed with a solution containing 20 % (w/v) ammonium sulphate and 5 % (w/v) sodium bicarbonate. This treatment, which was the main modification to the original method of RYDON AND SMITH[9], served to destroy all the excess chlorine leaving the N-chloro derivatives intact. After allowing the plate to stand for a further 15 min, the N-chloro derivatives were located as blue spots on a white background by spraying with a solution containing 1 % (w/v) starch and 1 % (w/v) potassium iodide.

A permanent record of the plate was obtained by placing it on a sheet of photographic contact paper and illuminating it with a photocopying lamp held at a distance of 4 cm above the plate for about 5 sec. The correct exposure time depended on the intensities of the spots and was found by trial and error. The photographic paper was then developed and fixed in the usual manner and in this way the proteins were shown up as a series of white spots against a black background. The distance of the centres of these spots from the origin could then be measured with some accuracy.

RESULTS AND DISCUSSION

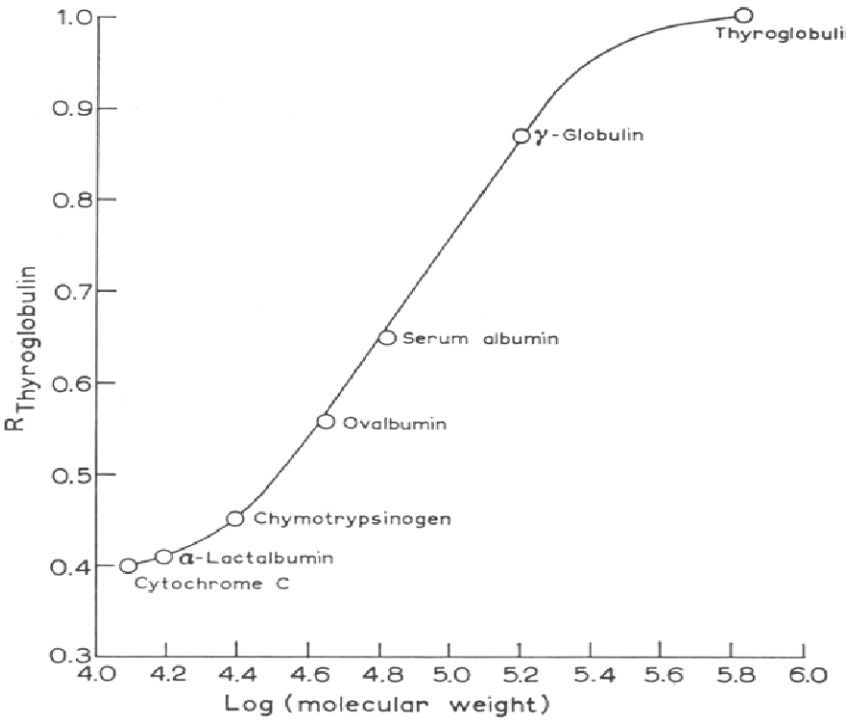
In the previous papers published on thin-layer gel filtration the detection of peptides and proteins was accomplished by staining with ninhydrin or amido black.

These techniques were found inconvenient for the detection of large proteins on the more open Sephadexes G-100 and G-200 because of the large amount of shrinking of the gel which occurred on drying. They are also very insensitive for carbohydrate-rich glycoproteins. As an alternative method for detecting proteins, staining in an atmosphere of iodine was first used in this laboratory[10] but was found to be too insensitive. The chlorination staining technique was found to be much superior and by using this method less than 1 μg of protein could be detected and a permanent record of the plate produced. This staining technique has also been used satisfactorily with mucopolysaccharides although the sensitivity is slightly less here because of their lower nitrogen content. It has also proved extremely useful for detecting oligosaccharides containing hexosamines and other nitrogenous compounds on paper chromatograms but in this case it was necessary to wash the chromatographic paper with 2 N acetic acid and water before use. This modified staining procedure possesses the advantages of taking less time and being more reproducible than RYDON AND SMITH's[9] original chlorination method but still retains the wide specificity of the original method so that it can be applied for the detection of small molecules as well as macromolecules. This wide specificity is a great advantage when carrying out degradative studies since the usual protein stains would not detect any smaller fragments which might be released.

Other workers[2,11] have shown that with gel filtration columns a linear relationship exists between elution volume of proteins and the logarithm of their molecular weights within certain limits of molecular weights determined by the gel filtration media used. When using thin-layer chromatograms, $R_{thyroglobulin}$ defined as:

$$\frac{\text{distance travelled by protein}}{\text{distance travelled by thyroglobulin}}$$

was found to be a convenient index of the rate of travel of a protein. Fig. 1 shows a plot of $R_{thyroglobulin}$ against log (molecular weight) for a number of proteins separated on a Sephadex G-200 thin-layer chromatogram. This is seen to be linear within certain limits of molecular weight but as might be expected it deviates from linearity at the lower and higher ends of the molecular weight range. This linear relation can only be expected to exist for molecules which are of similar shape; since most of the proteins examined are compact folded structures this is true but would not hold if the more asymmetric polysaccharides and mucopolysaccharide molecules were examined on the same scale. A comparison of the behaviour of proteins on the different Sephadexes is shown in the form of $R_{thyroglobulin}$ values in Table I. These cannot be taken as absolute values since they show slight variation from plate to plate, this possibly being due to small changes in the equilibrium of the gel media with the aqueous phase. Approximate molecular weights of unknown proteins can be obtained using thin-layer gel filtration provided the standards and unknown proteins are run on the same plate. For this purpose when working with G-200 it was found convenient to have a standard mixture of thyroglobulin, human γ-globulin, bovine serum albumin, ovalbumin, and α-lactalbumin. A 1.0 % (w/v) mixture of these proteins was completely resolved into discrete spots on Sephadex G-200 and a typical thin-layer chromatogram is shown in Fig. 2. Occasionally diglycylglycine was added to this mixture in order to show the complete inclusion limit but as shown in Fig. 2, this material although separated



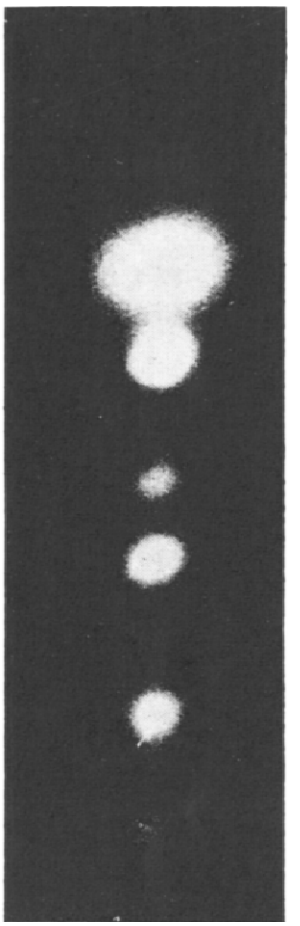


Fig. 1. Plot of $R_{thyroglobulin}$ against logarithm (molecular weight) for thin-layer gel filtration of proteins on Sephadex G-200.

Fig. 2. Thin-layer chromatogram on Sephadex G-200. Mixture containing from top to bottom respectively, diglycylglycine, α-lactalbumin, ovalbumin, bovine serum albumin, human γ-globulin and thyroglobulin.

TABLE I

COMPARISON OF $R_{thyroglobulin}$ VALUES OF PROTEINS ON THIN-LAYER CHROMATOGRAMS USING DIFFERENT SEPHADEXES

Protein	*Molecular weight*	$R_{thyroglobulin}$		
		G-50	*G-100*	*G-200*
Diglycylglycine	189	0.53	0.45	0.30
Cytochrome c	12,400	0.82	0.52	0.40
α-Lactalbumin	15,500	0.85	0.55	0.41
Chymotrypsinogen	25,000	0.89	0.63	0.45
Ovalbumin	45,000	1	0.74	0.56
Bovine serum albumin	67,000	1	0.82	0.65
Human γ-globulin	160,000	1	0.97	0.87
Thyroglobulin	670,000	1	1	1

from α-lactalbumin tended to give a larger spot due to the greater amount of diffusion with the smaller molecule. Although more precise values for molecular weights may be obtained by gel filtration on columns, 10–20% accuracy is obtainable by thin-layer gel filtration and for many purposes this is sufficient. This is especially true of degradative studies when approximate molecular sizes of the degraded molecule are required for comparison with the intact molecule which can be run on the same plate. As with the column method there is always the possibility of interaction of the protein with the gel media by adsorption or ionic interaction thus producing an erroneous result. In the case of such an anomalous result occurring thin-layer gel filtration possesses an advantage over the column method since it is possible to see exactly what is happening on the thin-layer plate and in this case the wastage of material is negligible if the gel has to be discarded on account of protein adsorption.

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SUMMARY

A method of detecting proteins and mucopolysaccharides on thin-layer gel filtration chromatograms which is sensitive to less than 1 μg of protein has been developed and a procedure for making a permanent record of the chromatogram is described. A correlation between the rate of travel of proteins on Sephadex G-200 and the logarithm of their molecular weights has been obtained.

REFERENCES

1 K. A. GRANATH AND P. FLODIN, *Makromol. Chem.*, 48 (1961) 160.
2 P. ANDREWS, *Nature*, 196 (1962) 35.
3 R. A. GIBBONS AND G. P. ROBERTS, *Ann. N.Y. Acad. Sci.*, 106 (1963) 218.
4 B. G. JOHANSSON AND L. RYMO, *Acta Chem. Scand.*, 16 (1962) 2067.
5 H. DETERMANN, *Experientia*, 18 (1962) 430.
6 B. G. JOHANSSON AND L. RYMO, *Acta Chem. Scand.*, 18 (1964) 217.
7 P. ANDREWS, *Biochem. J.*, 91 (1964) 222.
8 C. J. O. R. MORRIS, *J. Chromatog.*, 16 (1964) 167.
9 H. M. RYDON AND P. W. G. SMITH, *Nature*, 169 (1952) 922.
10 G. P. ROBERTS, unpublished work.
11 J. R. WHITAKER, *Anal. Chem.*, 35 (1963) 1950.