# POLYNUCLEOTIDE-ACRYLAMIDE GEL ELECTROPHORESIS OF SOLUBLE NUCLEASES FROM TOBACCO LEAVES

L. C. van LOON

*Department of Plant Physiology and Laboratory of Virology, Agricultural University, Wageningen, The Netherlands*

Received 30 December 1974

## 1. Introduction

To detect zones of hydrolytic activity after electrophoresis in polyacrylamide gels, advantage can be taken of the inclusion of high molecular weight substrate in the gel during polymerization [1]. This technique obviates the need for diffusion of either enzyme into a substrate-containing medium, or low molecular weight substrate and, if necessary, coupling factors into the gel, thus allowing longer incubation times and producing sharper bands. After electrophoresis under conditions in which the enzyme is not active, gels are incubated in a suitable medium for hydrolysis to take place. Subsequently, staining of residual substrate in the gel leaves bands of activity as colourless zones. Methods based upon this principle have been described for both deoxyribonuclease [2] and ribonuclease [3], but found only limited application, probably due to the complicated procedures involved (cf. [4]) and because the nucleic acids used are not necessarily uniformly degraded, leaving blurred zones on the gels instead of sharp, colourless bands.

In the soluble protein fraction of tobacco leaves, at least four types of nuclease activity have been characterized on the basis of their substrate specificity [5,6]. Although cultured tobacco cells upon electrophoresis yielded four bands of RNase activity as detected by diffusion of low molecular weight RNA into the gel [4], in our hands similar methods led to a poorly defined, remarkably complex pattern when applied to soluble proteins from tobacco leaves. Therefore, a variant of the substrate–gel method was developed which, by making use of simple high molecular weight polynucleotides, offered increased resolution and versatility and allowed us to define multiple bands of ribonuclease activity. The method has general applicability and may also be used to evaluate substrate specificity of individual enzyme forms.

## 2. Materials and methods

Leaves from 10–13 week old tobacco plants (*Nicotiana tabacum* L. cv. Samsun NN) were harvested and soluble proteins were extracted and purified as described before [7]. Aliquots of centrifuged or purified extracts containing 100–300 $\mu$g soluble protein, were subjected to electrophoresis in 7.5% polyacrylamide gels, using the discontinuous system of Davis [8] with minor modifications [7].

To be able to visualize zones of ribonuclease (EC. 3.1.4.23) activity, during preparation of the gels polyuridylic acid (poly-U; Calbiochem, K-salt, A grade was included in the gel matrix at a final concentration of 0.05%. After electrophoresis, gels were incubated for 2 hr in 7% acetic acid. During this time interval, the pH in the gel falls from 9.5 to about 2, and activity of enzymes with different pH optima was manifested before inactivation and fixation of the proteins took place. Subsequently, gels were stained overnight in a solution of 1% pyronin Y in 7% acetic acid, destained by washing with several changes of 7% acetic acid, and stored in the same solvent. Most active bands were even better resolved by immediately dropping the gels in the staining solution after electrophoresis. As a control, gels were heated to 100°C for 10 min in 7% acetic acid immediately after electrophoresis.

Deoxyribonuclease activity was located on gels



*North-Holland Publishing Company – Amsterdam*

containing 0.05% poly-d(A-T) (Sigma). After electrophoresis gels were incubated for a total of 60 min in two changes of 0.2 M Na-acetate buffer pH 5.0, stained overnight with 0.25% methyl green [9] in 0.02 M Na-acetate buffer pH 4.1 containing 0.001 M EDTA, and subsequently destained by washing with 0.1% acetic acid. Active bands were also resolved by immediately dropping the gels in the staining solution after electrophoresis. In an adaptation of the method of Boyd and Mitchell [2], in which 0.067% calf thymus DNA was included in the gel matrix, reversed electrophoresis of the proteins out of the gel after incubation was omitted, because tobacco leaf proteins irreversibly precipitate at the pH necessary for incubation[7].

Phosphodiesterases were visualized after Lerch [10] and polynucleotide phosphorylase was detected by an adaptation of the methods of Thang et al. [11] and Fitt et al. [12]. Gels were preincubated for a total of 20 min in two changes of 0.1 M Tris–HCl pH 9.0 containing 4 mM $MgCl_2$ and 0.25 mM EDTA at 0°C, then incubated overnight at room temperature in the presence of 0.01 M ADP. After incubation, gels were stained with 1% methylene blue, 1% lanthanum acetate in 0.4 M Na-acetate buffer pH 4.7 [13,14] and subsequently destained by washing in 0.1% acetic acid.

Enzyme patterns on the gels were recorded by densitometry, using a Photovolt Model 520-A densitometer with reduced slit width, equipped with a Varicord 43 linear/log recorder.

## 3. Results

Tobacco leaf ribonuclease, revealed by diffusion of low molecular weight yeast-RNA into the gel after Wolf [14] exhibited a very complex pattern, consisting of several poorly defined zones of activity over the entire gel length, as evidenced in fig.1a. The complexity of the pattern was confirmed by the poly-U method, as shown in fig.1b, by which the presence of seventeen bands of enzymatic activity was established. In strong contrast to the rather poor differentiation of the pattern shown in fig.1a, the latter method yielded very sharply bounded, colourless bands on a bright red background. Minor components could be clearly distinguished by visual inspection of the gels but were not always resolved well by densitometry.

Although several common features are apparent in the patterns obtained on gels incubated in yeast-RNA (fig.1a), or containing poly-U (fig.1b), they showed a quantitatively different distribution of RNase activity. This may be a consequence of differences in substrate preference and of the incubating conditions. In poly-U gels, the relative activity of individual enzyme bands was also affected by gel width, most likely as a

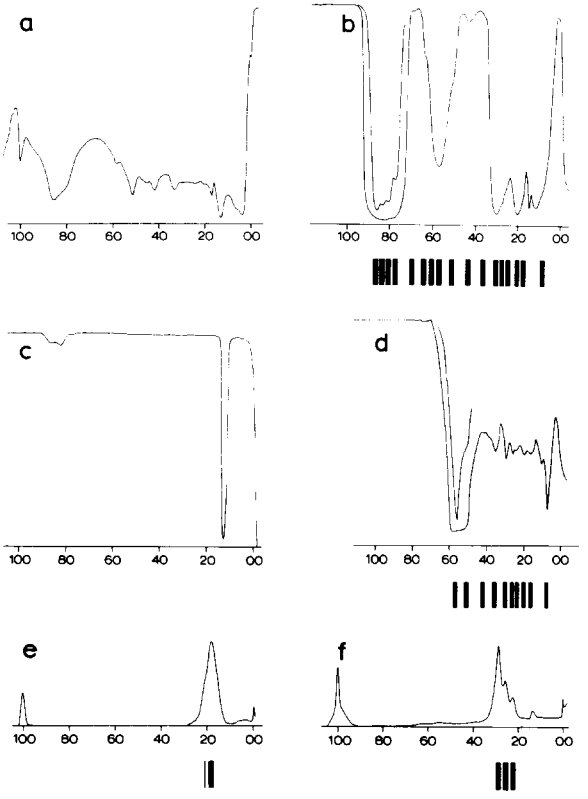


Fig.1. Densitometer tracings and diagrammatic representations of electrophoretic patterns of tobacco soluble leaf enzymes after electrophoresis in 7.5% polyacrylamide gels. (a) RNases, developed by incubation in 1% low molecular weight yeast-RNA at pH 5.0 and staining with methylene blue after Wolf [14]; (b) RNases, developed in gels containing 0.05% poly-U by incubation in 7% acetic acid and staining with pyronin Y (insert: incubated in staining solution only); (c) as (b), but heated to 100°C for 10 min in 7% acetic acid immediately after electrophoresis; (d) DNases, developed in gels containing 0.05% poly-d(A-T) by incubation in 0.2 M acetate buffer pH 5.0 and staining with methyl green (insert: incubation in staining solution only); (e) polynucleotide phosphorylase and (f) phosphodiesterases. $R_f$ values × 100 indicate band positions relative to the Bromophenol Blue tracking dye.

result of differences in the rate with which the pH in the gel was lowered, thus leading to differing effects on individual enzyme forms with different pH-ranges of activity. Apart from giving rise to deeper troughs in the densitometric patterns, higher activity appeared to manifest itself especially in broader bands in the gels, a phenomenon commonly encountered [3,15] and leading to decreased resolution. This could be overcome by dropping the gels immediately after electrophoresis into staining solution (fig.1b, insert).

In control gels, a single strong band was present around $R_f$ 0.13 (fig.1c). This band is aspecific and results from the presence of 'Fraction I protein' [7], which contains a very high amount of protein and impedes the penetration of stain in the corresponding area of the gel. Otherwise, only traces of the most active bands were apparent.

Except for these four most active, rapidly migrating enzyme bands, and probably one or more of the three relatively poorly active bands in the area 0.60-0.72, all further bands of RNase activity also degraded poly-d (A-T) (fig.1d). Although the relative activities of these enzyme forms on poly-U- and poly-d(A-T)-gels were different (cf. band 0.57), their ability to degrade both substrates classify them as aspecific nucleases. While gradual lowering of the pH by incubation in acetic acid also clearly revealed these nucleases on poly-d (A-T) gels, gels containing calf thymus DNA showed the two strong bands and diffuse staining in the upper part in the gel only after incubation for at least 30 min at an optimal pH of 5.5. As the presence of EDTA did not alter the pattern, $Mg^{2+}$ is not required for activity of these enzymes.

Three of these enzymes forms possessed phosphodiesterase activity when assayed at pH 7.0 (fig.1e). Staining for polynucleotide phosphorylase revealed one prominent band with a slight shoulder (fig.1f).

To elucidate the relationship of the four major bands possessing exclusively RNase activity, electrophoresis was conducted at a series of gel concentrations from 7.5% to 12.5% polyacrylamide. Plotting the log of their relative mobilities against the gel concentration yielded a set of parallel lines (fig.2), demonstrating that these enzyme forms are charge isomers [16].

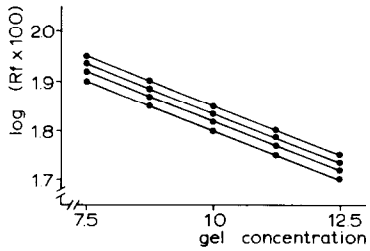


Fig.2. The effect of gel concentrations on relative mobilities of the four major RNase bands. After electrophoresis in gels containing 0.05% poly-U, RNases were visualized directly by incubation in staining solution.

## 4. Discussion

Inclusion of simple, synthetic polynucleotides in polyacrylamide gels for the detection of RNase or DNase isoenzymes, coupled with incubation over a wide pH range, yielded patterns which are characterized by high sensitivity and excellent resolution. If the base specificity of a particular enzyme is such that it will hydrolyze the homopolynucleotide present, exclusively mononucleotides can be produced, which easily diffuse out and do not leave material in the gel that would result in background staining. By using different polynucleotides, different types of nuclease activity can be distinguished. It may also be envisaged that comparison of the patterns obtained in gels with different homopolymers gives information about the base specificity or prefence of individual enzyme forms. In the case of soluble leaf proteins from tobacco the enzyme patterns were produced to a full extent upon gradually lowering the pH to about 2. Incubation for a given time at pH 5.0 led to excessive band broadening, while subjecting smaller quantities of protein to electrophoresis and incubation under these conditions did not clearly reveal minor enzyme components. The same method has also been succesfully applied to the study of RNase isoenzymes in barley leaves [17].

Although both the $R_f$ values of individual bands and the general shape of the patterns give indication that ten enzyme bands show both RNase and DNase activity, at present their presumed identity can only be regarded as tentative. However, the results obtained corroborate and extend the findings of Wyen et al. [5] about the presence of different types of nuclease activity in tobacco leaves. Four major and probably three minor enzyme bands fall into the class of (endo) ribonuclease; two major and eight minor bands show

nuclease activity; three of these can be classified as acid phosphodiesterases. Alkaline phosphodiesterase was not detected. This enzyme has an pH optimum of 9.3 [5] and, if present, should be represented in both poly-U-, poly-d(A-T)- and control gels. As this type is eluted from Sephadex columns before the acid phosphodiesterase and separation of tobacco leaf proteins in polyacrylamide gels is due mainly to differences in size [7], this enzyme may have been responsible for the slight activity seen in fig.1c in the area 0.00–0.10 and the stronger activity observed in this area in poly-d(A-T) control gels. The four major ribonuclease isoenzymes were found to be charge isomers, as was expected from the determination of a single molecular weight for this enzyme in tobacco leaves [5,6].

Although the detection method used for polynucleotide phosphorylase was based on the synthetic capacity of the enzyme, which appeared to be primer-independent, the enzyme is considered to have a function in the degradation of RNA in vivo. As bands of RNase activity in this area also showed DNase activity, it seems unlikely that this enzyme is represented among the RNases, unless overlapping bands were present.

## References

[1] Stegemann, H. (1968) Z. Anal. Chem. 243, 573–578.
[2] Boyd, J. B. and Mitchell, H. K. (1965) Anal. Biochem. 13, 28–42.
[3] Randles, J. W. (1968) Virology 36, 556–563.
[4] Wilson, C. M. (1971) Plant Physiol. 48, 64–68.
[5] Wyen, N. V., Udvardy, J., Erdei, S. and Farkas, G. L. (1972) Virology 48, 337–341.
[6] Jervis, L. (1974) Phytochemistry 13, 709–714.
[7] Van Loon, L. C. and Van Kammen, A. (1968) Phytochemistry 7, 1727–1735.
[8] Davis, B. J. (1964) Ann. N.Y. Acad. Sci. 121, 404–427.
[9] Kurnick, N. B. (1950) Exp. Cell Res. 1, 151–158.
[10] Lerch, B. (1968) Experientia 24, 889–890.
[11] Thang, M. -N., Thang D. -C. and Leautey, J. (1967) C. R. Acad. Sci. Paris D265, 1823–1826.
[12] Fitt, P. S., Fitt, E. A. and Wille, H. (1968) Biochem. J. 110, 475–479.
[13] Peacock, A. C. and Dingman, C. W. (1967) Biochemistry 6, 1818–1827.
[14] Wolf, G. (1968) Experientia 24, 890–891.
[15] Wilson, C. M. (1969) Anal. Biochem. 31, 506–511.
[16] Hedrick, J. L. and Smith, A. J. (1968) Arch. Biochem. Biophys. 126, 155–164.
[17] Lontai, I., Van Loon, L. C. and Bruinsma, J. (1972) Z. Pflanzenphysiol. 67, 146–154.