Specific Soluble Leaf Proteins in Virus-infected Tobacco Plants are not Normal Constituents

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SUMMARY

The conclusion by Barker (1975) that the four proteins previously thought to occur only in virus-infected tobacco leaves are probably normal constituents is based on an erroneous interpretation of the banding pattern obtained by polyacrylamide gel electrophoresis. Patterns shown by Barker for non-infected and water-injected tobacco leaves are similar to those for non-inoculated or water-inoculated plants from our laboratory. ‘New’ protein components induced by virus infection relate to bands not observed under the above-mentioned conditions. This is illustrated by a comparison of protein patterns from water-inoculated and TMV-inoculated tobacco plants, obtained under various conditions and after staining with either amido black or Coomassie blue. Preferential extraction of the new protein components at low pH further supports the view that these components do not occur in non-infected plants.

The occurrence of four new protein components, designated I to IV, in Nicotiana tabacum cv. Samsun NN reacting hypersensitively to tobacco mosaic virus (TMV), was first described by van Loon & van Kammen (1970). Subsequently, Gianinazzi, Martin & Vallée (1970) observed induction of similar, and in fact identical, components in the tobacco variety Xanthi-nc after infection with TMV, and later Kassanis, Gianinazzi & White (1974), and Gianinazzi & Kassanis (1974) found at least three of the four proteins (II to IV) in tobacco leaves either systemically infected with potato virus Y, cucumber mosaic virus, potato virus X, potato aucuba mosaic virus or alfalfa mosaic virus, or injected with polyacrylic acid. The specificity of the induction of the components I to IV by different viruses in different tobacco varieties, some properties of these components and, in particular, their relationship to acquired resistance, have been further analysed by van Loon (1972, 1973, 1975a, b).

Van Loon & van Kammen (1970) concluded that the occurrence of these specific proteins represented qualitative changes because no bands migrating at these positions were observed after electrophoresis of proteins from water-inoculated plants. This view has been challenged recently by Barker (1975) who stated that three of the four proteins previously thought to occur only in virus-infected or polyacrylic acid-injected tobacco leaves are probably normal constituents. As will be shown, this conclusion is based on an erroneous interpretation of the banding patterns obtained by polyacrylamide gel electrophoresis. Protein components I to IV induced by virus infection are not detectable in non-infected plants. Neither, in our laboratory, have conditions other than virus infection been found to induce new protein components (cf. van Loon, 1972, 1975b).

Tobacco plants were grown and inoculated, and soluble proteins were extracted, centrifuged and separated by electrophoresis on polyacrylamide gels as described previously (van Loon & van Kammen, 1968, 1970; van Loon, 1972). These methods are essentially similar to those described by both Gianinazzi & Kassanis (1974) and Barker (1975), except
Fig. 1. Densitometer tracings of electrophoretic patterns of soluble protein extracts from tobacco cv. Samsun NN, 7 days after inoculation with distilled water (a, c and e) or 100 μg TMV-W U/ml (b, d and f) in 10% polyacrylamide gels. Extracts were subjected to electrophoresis immediately after preparation (a, b, e, and f) or after storage for 1 day at 0 to 4°C (c and d). Gels were stained with amido black (a to d) or Coomassie blue (e and f). Band positions are indicated by their $R_p$ values $\times 100$. Protein bands labelled ph and 0 to 4 refer to those described by Barker (1975); I to IV refer to protein components induced by virus infection.
that the extraction medium contained ascorbic acid and cysteine instead of mercaptoethanol. After electrophoresis, gels were stained either with a saturated solution of amido black in 5% (w/v) trichloroacetic acid (TCA), or with 0.05% (w/v) Coomassie blue in 12.5% TCA (van Loon, 1973). Protein patterns were recorded by densitometry, using a Chromoscan or a Photovolt Model 520-A densitometer with a reduced slit width. The position of the protein bands on the gels is expressed by the \( R_v \) value, taking the distance travelled by the bromophenol blue tracking dye as 1.00.

Fig. 1a, b shows densitometer tracings of protein patterns typical of centrifuged extracts from water-inoculated and 7 day TMV-infected Samsun NN tobacco leaves, respectively, applied to 10% (w/v) polyacrylamide gels immediately after preparation. The species specificity of these patterns (van Loon & van Kammen, 1970; van Loon, 1972) allows a comparison to be made with the photographic reproductions given by Barker (1975) and Gianinazzi & Kassanis (1974). In Fig. 1a only the area between \( R_v \) about 0.60 to 1.00 shows very low background staining, due to the limited number of protein bands migrating that far in 10% polyacrylamide. Only this area is clearly illustrated in the photographs presented by Barker (1975), the upper parts of his gels appearing overloaded due to the relatively long exposure times necessary to reveal the weakly stained bands in the lower parts. The difficulties encountered in the photographic representation of leaf protein patterns have been discussed (van Loon, 1973) and led us to prefer the more quantitative densitometric depiction.

Although it is difficult to calculate exactly the \( R_v \) values of the bands labelled 0 to 4 by Barker (1975), no other bands in this area of the gel can be distinguished in extracts from non-inoculated or water-inoculated plants. Both the relative positions of the bands and the general similarity of the protein pattern in this area of the gel thus point to the correspondence of bands 1, 2, 3, and 4 with bands 0.92, 0.83, 0.76, and 0.72, respectively, in Fig. 1a. ph marks the corresponding plant phenol and bromophenol blue band 1.00. A band corresponding to band 0 is not normally seen as a separate peak under our conditions but a similar band migrating immediately behind the front marker can be distinguished by visual inspection of the gels. Proteins corresponding to bands 1, 2, 3, and 4 are equally found in the patterns from TMV-infected plants (Fig. 1b). The components I to IV, however, occupy different positions at \( R_v \) 0.57, 0.60, 0.70 and 0.86, respectively. All these bands can be seen to be separate from each other by visual inspection of the gels. By densitometry not all components are resolved as single peaks (van Loon, 1973), so that, for instance, the presence of band 4(\( R_v \) 0.72) is indicated only by the asymmetry of the peak of component III (\( R_v \) 0.70).

Browning of the protein extracts as a result of polyphenol oxidation during preparation or storage affects the quality of the separation, as demonstrated in Fig. 1c, d (cf. van Loon & van Kammen, 1968; van Loon, 1972). These patterns, obtained from extracts stored for one day at 0 to 4°C, are less well resolved than those shown in Fig. 1a, b. Due to the migration, under these conditions, of a broader band of brown oxidation products together with and directly behind the bromophenol blue tracking dye, the protein band immediately behind the front marker is retarded, giving rise to a separate peak 0.97, apparently corresponding to Barker’s band 0. Bands 1 to 4 show \( R_v \) values slightly different from those in Fig. 1a, namely 0.90, 0.81, 0.77 and 0.71, respectively. Notwithstanding the poorer separation, again the same bands can be traced in infected plants, whereas the components III and IV are characterised by the dissimilar \( R_v \) values of 0.68 and 0.84, respectively. Thus, one has to conclude that the patterns shown by Barker (1975) for non-infected and water-injected tobacco leaves are similar to those of water-inoculated and non-inoculated leaves of plants from our laboratory. Bands labelled 1, 2 and 3 by Barker (1975) do not correspond to our components IV, III and II, which in turn are identical to the bands b1, b2 and b3.
described by Gianinazzi & Kassanis (1974). Only the components III and IV are present in the area of the gel referred to by Barker (1975), components I and II exhibiting lower $R_p$ values.

A broadening of band 2 and the appearance of a new band 5 with an $R_p$ value slightly lower than that of band 4 were noticed by Barker (1975) upon injection with polyacrylic acid or after infection with alfalfa mosaic virus. Unfortunately, Barker did not examine TMV-infected leaves. However, the occurrence of the components I to IV after infection with several different viruses, including alfalfa mosaic virus (van Loon, 1972, 1975b; Kassanis et al. 1974) can now be considered well established. Barker’s observations are therefore to be interpreted as the presence of the components IV and III, respectively. The reason why Barker was unable to resolve the broadened band into two components is unclear, but may be sought in the differences in the extraction medium and the staining conditions (cf. van Loon & van Kammen, 1968; van Loon, 1973). Both components III and IV thus appear to be newly induced upon virus infection.

As discussed by van Loon & van Kammen (1970), components I and II are also to be considered proteins appearing only after infection. This is particularly obvious after staining with Coomassie blue, as demonstrated in Fig. 1e, f, corresponding to the patterns stained with amido black shown in Fig. 3e, f from the paper by van Loon and van Kammen (1970). No bands at the positions of the components I and II are observable after electrophoresis of proteins from non-infected plants. Additional evidence that the components I to III do not correspond to proteins already present in non-infected plants has been obtained by varying the gel concentration (van Loon & van Kammen, 1970; van Loon, 1972).

Extraction of TMV-infected leaves with phosphate-citrate buffer, pH 3.0, containing 0.5 M-NaCl and 0.1% (v/v) mercaptoethanol quantitatively liberated the components I to IV, whereas only 30% of the amount of protein extracted at pH 8.0 was found to be soluble under these conditions. After dialysis against electrophoresis buffer and electrophoresis in 10% polyacrylamide gels, the patterns shown in Fig. 2 were obtained. All bands distinguishable in the area from $R_p$ 0.50 to 1.00 for non-infected plants are indicated by the short arrows in Fig. 2a. In addition, the positions of the components I to IV, as indicated in Fig. 2b for TMV-infected plants, are marked. With the exception of component III, no correspondence

Fig. 2. Densitometer tracings of electrophoretic patterns of the proteins extracted at pH 3.0 from tobacco cv. Samsun NN, 7 days after inoculation with (a) distilled water or (b) 100 µg TMV-W U1/ ml both in 10% polyacrylamide gels. Similar amounts of protein were applied to the gels as in Fig. 1. Gels were stained with amido black. The long arrows in (a) mark the corresponding positions of the components I to IV shown in (b).
between the components induced by virus infection and bands already present before infection could be established at this gel concentration. Moreover, by varying the gel concentration, the correspondence of component III with a band already present appeared fortuitous.

Hence, the components I to IV do not appear to be normal constituents but to be newly induced as a result of infection. Their preferential extraction at low pH is in agreement with our previous suggestion (van Loon, 1973) that these proteins may contain a high percentage of basic amino acids. In this respect, they differ from the combined soluble proteins in amino acid composition (L. C. van Loon, unpublished results), which points to a specific role in pathogenesis. Moreover, extraction of tissues at low pH will facilitate detection of the components in those virus-host plant combinations in which they are present only in trace amounts.

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REFERENCES


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