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POLYACRYLAMIDE DISC ELECTROPHORESIS OF THE SOLUBLE LEAF PROTEINS FROM *NICOTIANA TABACUM* VAR. "SAMSUN" AND "SAMSUN NN"—I.

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Abstract—Different conditions for the extraction of soluble proteins from tobacco leaves were evaluated. Best results were obtained by mixing and homogenizing freshly harvested leaf material with a Tris-HCl buffer containing 0·5 M sucrose, 0·1 per cent ascorbic acid and 0·1 per cent cysteine-HCl at pH 8·0. The extract was fractionated by centrifugation, further purified by gel filtration and concentrated by ammonium sulphate precipitation. The resulting solution showed an u.v. spectrum characteristic of protein, and was separated by electrophoresis on polyacrylamide gels of different concentrations into about thirty protein zones. No differences were detected in the electrophoretic patterns of purified extracts from the varieties Samsun and Samsun NN.

INTRODUCTION

WHEN a susceptible plant is infected by a virus, symptoms develop which are characteristic for the combination virus (e.g. virus strain)–host plant (e.g. plant variety). The formation of symptoms is the result of changes in the physiology of the host plant caused by a specific interaction of the virus and the host-plant genome. In the presence of different viruses, different metabolic pathways may be either activated or reduced within the same plant species. Conversely, the same virus may induce the formation of different symptoms in different plant species. The mechanism by which these symptoms are induced is poorly understood, since it is generally observed that the underlying metabolic changes are only detectable shortly before or at the same time as symptoms appear.[1–3]

The varieties Samsun and Samsun NN of tobacco (*Nicotiana tabacum* L.) differ only by a single gene, determining hypersensitivity with regard to infection by tobacco mosaic virus (TMV).[4] Yet, after infection with the common strain of TMV, the response of these varieties is strikingly different in several respects. In Samsun NN local lesions appear after a few days on the inoculated leaves and the virus remains localized within a narrow zone around these lesions. At the same time glucose catabolism via the pentose shunt pathway is activated,[5] and the activities of peroxidases and phenolases increase.[6] In Samsun, on the other hand, the virus becomes systemic, giving rise to a mosaic pattern in 10–14 days on the young developing leaves. No changes in glucose catabolism have been observed.[7] Peroxidase and phenolase activities are increased to a lesser extent than in the hypersensitive host.[3, 8]

[1] G. L. FARKAS and M. A. STAHMANN, *Phytopathology* **56**, 669 (1966).
[2] F. SOLYMOSY, J. SZIRMAI, L. BECZNER and G. L. FARKAS, *Virology* **32**, 117 (1967).
[3] H. SUSENO and R. E. HAMPTON, *Phytochem.* **5**, 819 (1966).
[4] F. O. HOLMES, *Phytopathology* **28**, 553 (1938).
[5] F. SOLYMOSY and G. L. FARKAS, *Virology* **21**, 210 (1963).
[6] A. VAN KAMMEN and D. BROUWER, *Virology* **22**, 9 (1964).
[7] J. R. BAUR, R. S. HALLIWELL and R. LANGSTON, *Virology* **32**, 406 (1967).
[8] C. MARTIN, *Compt. Rend.* **246**, 2026 (1958).

Since these changes may be associated with changes in protein constitution, it appeared interesting to investigate whether any differences in the soluble protein fraction, which could account for the specific reaction of the host plant after infection, could be detected in uninfected plants. Alternatively, such differences might only become detectable after infection as a consequence of the interaction of the virus and the host-plant genome. Changes in protein constitution connected with symptom formation may therefore be virus or host-plant specific, e.g. coded or by the virus RNA or the DNA of the host plant. After infection of bean plants by southern bean mosaic virus, changes in protein constitution due to the induction of host-specific enzymes have been demonstrated.[1] However, apart from the coat protein and a RNA-dependant RNA polymerase necessary for virus multiplication, no specific proteins of plant viruses themselves have been found which could be involved in symptom formation.

In this paper a method for preparing a purified soluble protein fraction from tobacco leaves is presented. Because of its great resolving power, polyacrylamide disc electrophoresis was used to separate the proteins present and the patterns obtained were used for comparisons between Samsun and Samsun NN.

RESULTS AND DISCUSSION

Extraction Solutions

Protein extractions in buffers based on those described by Steward, Lyndon and Barber (0·1 M Tris-glycine, pH 8·3),[9] Van Kammen (0·05 M Tris-HCl, 0·5 M sucrose, 0·01 M magnesium chloride, 0·006 M 2-mercaptoethanol, pH 7·2),[10] Staples and Stahmann (0·1 M Tris-HCl, 0·5 M sucrose, 0·1 per cent ascorbic acid, 0·1 per cent cysteine-HCl, pH 8·0),[11] and Stavely and Hanson (0·1 M Tris-HCl, 0·5 M sucrose, 0·075 M ascorbic acid, 0·0066 M cysteine-HCl, 0·0142 M 2-mercaptoethanol, pH 7·4),[12] and containing different amounts of particle stabilizers and antioxidants, were compared on the basis of the electrophoretic pattern after centrifugation of the extracts. A water extraction at pH 7·2, adjusted with 0·1 N sodium hydroxide,[13] was included as a control. The main criteria for the evaluation of the extraction procedures were the number and distinctness of stained protein zones on the gel and the absence of interfering background staining, a phenomenon commonly encountered in electrophoresis of proteins from higher plants.[14]

Water extracts turned brown rapidly and yielded extremely diffuse patterns upon electrophoresis, with only a few distinct bands. Extraction with buffers yielded solutions which varied from nearly colourless to bright yellow. These extracts gave essentially similar patterns, completely different from the water extract, in all cases, with distinct protein zones but varying degrees of background staining. Best results were obtained with a medium based on that described by Van Kammen,[10] pH 7·2, and the pH 8·0 extraction buffer described by Staples and Stahmann,[11] given above. Since the pH 7·2 buffer gave an extract which was nearly colourless and has been used successfully in our laboratory in *in vitro* amino acid incorporation studies, it seemed particularly suitable in extracting soluble proteins. However, it appeared that even when plants were grown under controlled conditions of light, temperature and humidity, a seasonal variation occurred in the content of phenolic compounds

[9] F. C. STEWARD, R. F. LYNDON and J. T. BARBER, *Am. J. Botany* **52**, 155 (1965).
[10] A. VAN KAMMEN, *Arch. Biochem. Biophys.* **118**, 517 (1967).
[11] R. C. STAPLES and M. A. STAHMANN, *Phytopathology* **54**, 760 (1964).
[12] J. R. STAVELY and E. W. HANSON, *Phytopathology* **57**, 482 (1967).
[13] J. W. HILTY and A. F. SCHMITTHENNER, *Phytopathology* **56**, 287 (1966).
[14] F. C. STEWARD and J. T. BARBER, *Ann. N.Y. Acad. Sci.* **121**, 525 (1964).

in tobacco leaves, leading to rapid browning of extracts in this buffer during the summer months. In this case it was not possible to separate the protein fraction from oxidation products by gel filtration, and after electrophoresis diffuse protein zones and increased background staining in the gels were observed, probably due to the binding of phenolic compounds with proteins.

Under these conditions no browning occurred in extracts prepared in the pH 8·0 buffer which contained ascorbic acid, and background staining in gels after electrophoresis was markedly reduced. Except for the presence of ascorbic acid, which is known to be one of the most effective quinone-reducing agents,[13, 15] raising the pH also has a beneficial effect in that phenolic compounds dissociate and no longer form hydrogen bonds with proteins. Results obtained by using this buffer as the extraction medium were superior to all others tested, and this extraction buffer was finally adopted in all further experiments.

Homogenization and Centrifugation

Instruments by which high pressures were applied to the leaf tissue during extraction were all found to be less effective than homogenizing for a short time in a Waring Blendor. In this way disruption of cell organelles was minimized, and the protein pattern was detectable upon electrophoresis of the initial extract after an initial centrifugation at low speed (Fig. 1A)

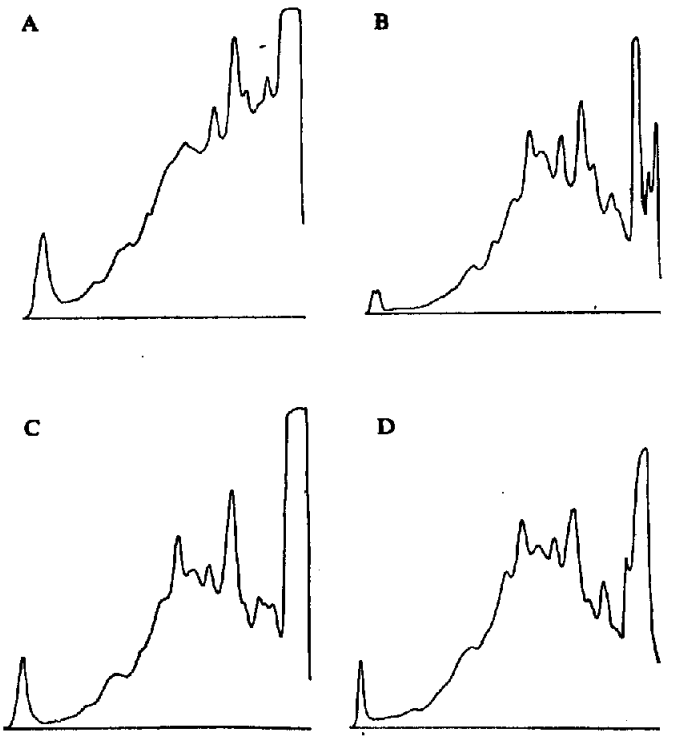

FIG. 1. DENSITOMETER TRACINGS OF ELECTROPHORETIC PATTERNS OF SOLUBLE PROTEIN EXTRACTS OF TOBACCO LEAVES (*Nicotiana tabacum* L.) var. Samsun NN AT DIFFERENT STAGES DURING THE PURIFICATION PROCEDURE.
(A) after extraction and centrifugation at 1000 *g* for 10 min; (B) after centrifugation at 105,000 *g* for 150 min; (C) after fractionation on Sephadex G50; (D) purified extract.

[15] W. D. LOOMIS and J. BATTAILE, *Phytochem.* 5, 423 (1966).

On the contrary, by applying high pressures during extraction darkly stained diffuse zones were obtained.

Cell particles were completely removed from the extract by centrifugation since no u.v.-absorbing material was detected in the eluate before protein appeared, when samples were separated on a Sephadex G200 column. The electrophoretic pattern obtained after centrifugation was highly reproducible and showed comparatively low background staining (Fig. 1B).

Effect of Storage of Leaf Material

Fresh tissues were superior to tissues stored at $-20°$ and thawed before extraction. In extracts from frozen material, protein zones were less distinct and background staining was increased considerably.

Gel Filtration

After centrifugation, the extract was applied on a Sephadex column to separate proteins from yellow pigments and low molecular weight components. Samples were assayed on columns of Sephadex G25, G50, G75, G100 and G200. In all cases, except on G25, a complete separation between proteins and coloured components was possible. Best separations were achieved on G200, but the protein was always eluted in a single peak which showed considerable tailing on the lower-crosslinked gels. In this case, it appeared advantageous to collect the protein rapidly and without excessive dilution. Therefore, filtration was carried out on columns containing G50, and the protein fraction was collected immediately after the void volume (Fig. 2; 1C). Yellow pigments absorbing strongly at 260 nm were eluted after the protein peak together with the low molecular weight fraction.

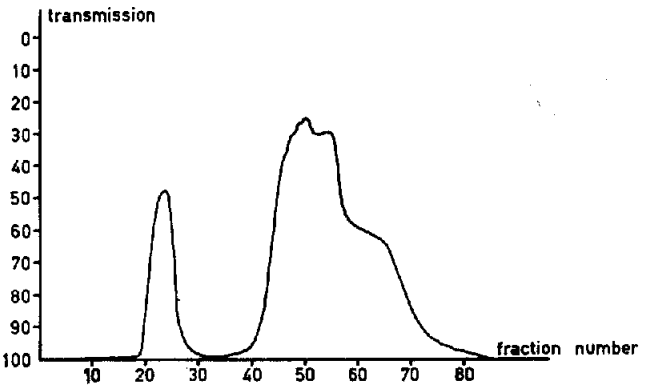

FIG. 2. ELUTION PATTERN OF CENTRIFUGED EXTRACT ON SEPHADEX G50.
Transmission was recorded using a LKB Uvicord II, operating at a wavelength of 280 nm with a 0·3 cm light path. Protein (absorptivity (A) 280 nm: A 260 nm > 1) was eluted in the first peak. Yellow pigments and low molecular weight substances were eluted in the following peaks in which A 260 nm exceeded strongly A 280 nm. Column dimensions: 48 × 3 cm dia. Load: 36 ml.

Concentration

Protein was precipitated by adding solid ammonium sulphate to 95 per cent saturation followed by centrifugation. The resulting precipitate was slightly yellow but turned greyish brown quickly after decanting. This phenomenon could be prevented completely by recrystallizing ammonium sulphate from a solution of 0·05 M EDTA (tetrasodiumsalt) in distilled water.

After precipitation the protein was dissolved in either 0·06 M Tris-phosphate buffer, pH 6·9[16] (spacer gel buffer in the electrophoretic procedure), or 0·005 M Tris-glycine buffer, pH 8·3[17] (reservoir buffer), containing 0·05 per cent 2-mercaptoethanol. Dialysis and subsequent centrifugation resulted in considerable loss of material in the pH 6·9 buffer, but not in the pH 8·3 buffer. Comparing the electrophoretic patterns did not reveal any qualitative differences between the two treatments, indicating that there was no denaturation of any specific protein in the pH 6·9 buffer. Upon storage, the protein solution at pH 6·9 turned rapidly turbid again, resulting ultimately in loss of some protein bands. The solution at pH 8·3 remained stable for about 1 week and even longer in the presence of 0·5 M sucrose.

Criteria for Purity; Yield

The protein fraction obtained was nearly colourless and showed an u.v. spectrum characteristic for protein (max. 279 nm; min. 251 nm; average 280:260 ratio: 1·50). As most of the RNA present had been removed by the ammonium sulphate treatment, the final protein solution contained less than 5 per cent RNA as judged by the orcinol method of Dische and Schwarz.[18] The average yield was about 2 mg protein per g leaf material.

Gel Electrophoresis and Protein Comparisons

The presence of 2-mercaptoethanol in the buffer containing the protein solution prevented polymerization of the sample gel. Moreover, exposure of the protein solutions to room temperature resulted in less discrete bands or even trapping of protein in the spacer gel. Therefore, after polymerization, gels were cooled to 4° and the sample was layered directly over the spacer gel in 0·5 M sucrose.

In this way, protein solutions consistently separated in up to twenty distinct zones in a 7·5 per cent standard acrylamide gel. The patterns obtained were essentially similar to those obtained from extracts immediately after centrifugation (Fig. 1B, D), indicating that no loss of components had occurred. More fast migrating zones were detected in purified extracts, but normally background staining was slightly increased. Near the top of the gels a thick band of protein was observed, being mostly considerably thinner upon electrophoresis immediately after centrifugation. This has been found by Macko *et al.*[19] to occur in different plant tissue extracts and is regarded as a nonspecific aggregate, probably containing fraction I protein.

More detail could be obtained when electrophoresis was conducted simultaneously in 5, 7·5 and 10 per cent acrylamide gels. Proteins which migrated slowly in 7·5 per cent gels were better resolved in 5 per cent gels; those which migrated rapidly in 7·5 per cent gels were better resolved in 10 per cent gels. The results obtained by both visual inspection of the gels and densitometry were compared in a great number of experiments. Patterns were found to be highly reproducible, and no significant differences could be detected in gels run in different experiments. Due to an improved method of staining the proteins in the gels,[20] a few more bands could be detected by visual inspection of the gels than in the densitometric tracings, as narrow bands lying side by side could not in all cases be resolved in densitometry. The relatively low background staining of the gels enabled us to detect minor bands consistently

[16] L. THELANDER, *J. Biol. Chem.* **242**, 852 (1967).
[17] B. J. DAVIS, *Ann. N.Y. Acad. Sci.* **121**, 404 (1964).
[18] Z. DISCHE and K. SCHWARZ, *Mikrochim. Acta* **2**, 13 (1937).
[19] V. MACKO, G. R. HONOLD and M. A. STAHMANN, *Phytochem.* **6**, 465 (1967).
[20] L. C. VAN LOON, to be published.

by both visual inspection and densitometry. When the same gel was scanned in different positions, only slight quantitative differences were observed.

Bands were numbered according to increasing mobility in the 7·5 per cent gel. It was found that soluble proteins from tobacco differ very little in negative charge at the pH used in electrophoresis, as only one protein zone with two slight shoulders was obtained by agar gel electrophoresis.[21] Therefore, in polyacrylamide gel electrophoresis separation is achieved mainly by the molecular sieving action of the gel. By changing the gel concentration between

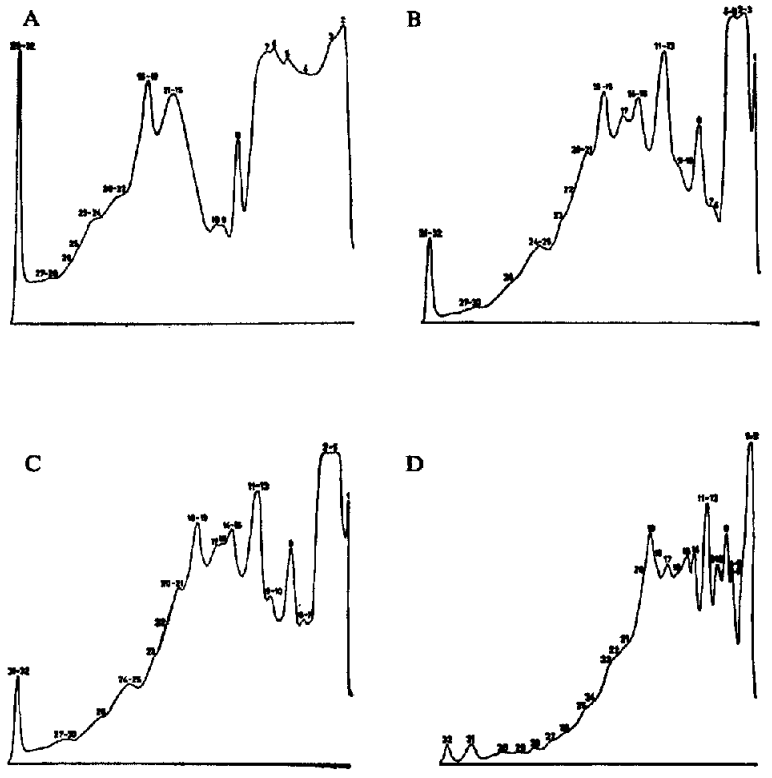

FIG. 3. DENSITOMETER TRACINGS OF ELECTROPHORETIC PATTERNS OF THE PURIFIED SOLUBLE PROTEIN FRACTION OF TOBACCO var. Samsun AFTER ELECTROPHORESIS IN:
(A) 5 per cent acrylamide gel, stained with amido black; (B) 7·5 per cent acrylamide gel, stained with amido black; (C) 7·5 per cent acrylamide gel, stained with light green; (D) 10 per cent acrylamide gel, stained with amido black.

5 and 10 per cent, the sequence of the proteins in the gels will not be affected.[22] Hence, using a dominant band with a R_f value of 0·31 in a 7·5 per cent gel (band nr. 11–13) as a reference, it was possible to compare the proteins in the three gels (Figs. 3, 4), and numbering of the bands was extended to those which were better resolved in the 5 and 10 per cent gels. In this way, up to thirty-two distinct zones were observed, indicating the presence of at least the same number of proteins in the soluble protein fraction.

[21] L. C. VAN LOON and A. DIELEMAN-VAN ZAALJEN, unpublished results.
[22] J. ZWAAN, *Anal. Biochem.* 21, 155 (1967).

No qualititative differences were evident between extracts of Samsun and Samsun NN, neither with amido black, nor with light green as a general protein stain. Slight quantitative differences were found between the two staining methods (Figs. 3, 4).

As no differences were demonstrable in protein constitution of the two varieties, it can be concluded that the difference in response to TMV infection is not the result of the action of a specific protein already present, but that these differences are induced as a result of host-pathogen interaction. However, the hypothesis that a special protein is present in very small amounts, cannot be excluded.

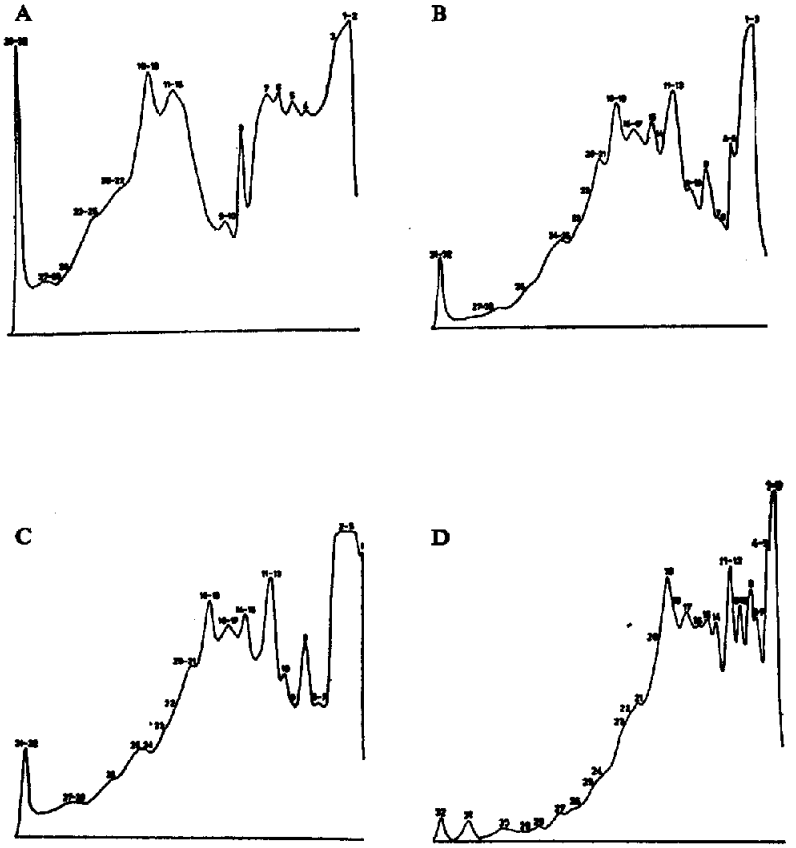

FIG. 4. DENSITOMETER TRACINGS OF ELECTROPHORETIC PATTERNS OF THE PURIFIED SOLUBLE PROTEIN FRACTION OF TOBACCO var. Samsun NN AFTER ELECTROPHORESIS IN:
(A) 5 per cent acrylamide gel, stained with amido black; (B) 7·5 per cent acrylamide gel, stained with amido black; (C) 7·5 per cent acrylamide gel, stained with light green; (D) 10 per cent acrylamide gel, stained with amido black.

GENERAL DISCUSSION

To obtain maximum resolution of plant protein patterns by electrophoresis in polyacrylamide gels, special attention should be given to the preparation of the protein solutions. During extraction and fractionation all treatments leading to minor alterations of the protein components will influence the separation obtained. Non-protein components present may interfere by binding to the proteins, influencing their electrophoretic behaviour to some extent or resulting in diffuse patterns. Therefore, the protein-containing extract

should be purified under mild conditions to maintain the proteins in their native form, but interfering substances should be removed completely. In the case of the soluble leaf proteins from tobacco, every step in the purification process was checked by electrophoresis. Although R_f values varied somewhat after different treatments, protein patterns were essentially similar indicating no alteration of the protein components had occurred during the purification procedure.

As the purified protein solution is free of interfering substances, protein concentrations can easily be determined. In this way, upon electrophoresis, accurate qualitative and quantitative comparisons will be possible.

Although the conditions of electrophoresis are not critical, the sample gel should be omitted since protein may be trapped or denatured during polymerization. On the other hand a spacer gel was found to be absolutely necessary to separate the complex mixture of proteins into thin zones. Omission of the spacer gel led to only a few broad bands.

MATERIALS AND METHODS

Tobacco plants (*Nicotiana tabacum* L. var. "Samsun" and "Samsun NN") were grown from seed in 12-cm pots in a growth chamber under controlled conditions (day 16 hr, light intensity about 25,000 erg sec^{-1} cm^{-2} at plant height; temp. 18–20°; relative humidity 65–80 per cent).

After 10–13 weeks the young and two fully expanded leaves were harvested, washed with distilled water, deribbed and blotted dry on filter paper.

All subsequent procedures were carried out in a cold room at 0–4°. Leaf material was precooled for 15 min. Small amounts (15–20 g) were minced with a razor blade and gently ground with a mortar and pestle in 1·25 vol (v/w) 0·1 M Tris-HCl, 0·5 M sucrose, 0·1 per cent ascorbic acid, 0·1 per cent cysteine-HCl buffer, pH 8·0.[11] Larger amounts (30–50 g) were cut in a vegetable slicer (Braun) and the expressed sap collected in part of the buffer. The residue was extracted in a Waring Blendor with the rest of the buffer (30 sec half speed, 15 sec top speed). The resulting slurry was squeezed through several layers of sterile gauze.

The extract was centrifuged for 15 min at 15,000 *g* and subsequently for 30 min at 30,000 *g*. The resulting supernatant was centrifuged for 2·5 hr at 40,000 rev/min in the R40 rotor of the Spinco L50. The yellow supernatant solution containing the soluble protein fraction was either used directly for electrophoresis or further purified in the following way: 12 ml of the protein-containing solution was passed through a Sephadex G50 column (32 × 2 cm dia.) equilibrated with 0·05 M Tris-HCl, pH 7·5. The column was eluted with the same buffer and absorptivity of the eluate was determined at 260 and 280 nm. The protein-containing fractions were pooled and the solution brought to 95 per cent saturation with solid ammonium sulphate under constant stirring.

After standing for at least 1 hr, protein was collected by centrifugation at 30,000 *g* for 10 min. The precipitate was dissolved in 1·5–3 ml of either 0·005 M Tris-glycine buffer, pH 8·3, or 0·06 M Tris-phosphate buffer, pH 6·9, and dialysed for 20 hr against 1000 vol. of buffer with one change.

After dialysis the protein solution was centrifuged at 30,000 *g* for 10 min and the supernatant solution used as purified soluble protein solution. This solution was brought to 0·5 M sucrose by adding solid sucrose or by diluting with a solution of 1 M sucrose in buffer.

Disc electrophoresis was performed essentially according to Ornstein[23] and Davis[17] with some minor modifications. The large pore spacer gel was polymerized over the small

[23] L. ORNSTEIN, *Ann. N.Y. Acad. Sci.* **121**, 321 (1964).

pore separation gel and did not contain sucrose. Instead of the Tris-HCl buffer, pH 6·7, in the spacer gel solution a buffer containing 0·47 M Tris-phosphate, pH 6·9, was used. This buffer facilitates polymerization of the gel and has a higher buffering capacity.[24] Samples in 0·5 M sucrose and containing 300 μg of protein were applied directly upon the spacer gel. The electrophoresis buffer contained 0·5 per cent 2-mercaptoethanol and was diluted 1:10 before use.

Electrophoresis was conducted in a commercial apparatus (Acrylophor, from Pleuger, Amstelveen, Holland) for 20 min at 2 mA per tube and subsequently for about 1 hr at 4·5 mA per tube (approximately 40 V per cm), until the tracking dye had migrated a few mm beyond the lower end of the gel.

After electrophoresis gels were stained at room temperature for at least 1 hr in a saturated solution of amido black 10 B in 5 per cent TCA or 0·25 per cent light green in 5 per cent TCA as described elsewhere.[20] By this method better resolution was obtained than when using the recommended staining solution. Excess dye was removed by electrophoretic destaining in 7 per cent acetic acid.

Densitometer tracings of gels were obtained by using a Photovolt model 520-A densitometer with reduced slit width, equipped with a Varicord 43 Linear/Log recorder. An orange filter with a maximum transmittance at 595 nm was used for scanning light-green patterns. No filter was used for scanning amido-black patterns.

Protein concentrations were determined by the method of Lowry *et al.*,[25] and by the absorption at 280 nm. Using purified dry frozen soluble leaf protein from tobacco as a standard, the A 280 nm for 1 mg/ml was calculated to be 1·2. RNA was determined according to the orcinol method of Dische and Schwartz.[18]

Acknowledgements—The authors are grateful to Professor J. P. H. van der Want for his stimulation and continuous interest in this work and to Mrs. A. Dieleman-Van Zaaijen for performing part of the initial experiments.

[24] L. Ornstein and B. J. Davis, *Preprint by Distillation Products Industries, Rochester, N.Y.* (1960).
[25] O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).