Plant and Aphid Protein Patterns as Influenced by Pea Enation Mosaic Virus

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ABSTRACT

Isoelectric focusing was used to study changes in protein patterns and some enzyme activities in pea plants (*Pisum sativum* 'Perfected Wales') infected with pea enation mosaic virus (PEMV) and in viruliferous and nonviruliferous aphids (*Myzus persicae* and *Acyrthosiphon pisum*). A peroxidase-active band with an isoelectric point at pH 7.2, which did not result from accelerated senescence, was observed in patterns developed from protein extracts of diseased peas, but not in patterns of noninfected pea tissue.

Viruliferous pea aphids (*Acyrthosiphon pisum*) contained a protein with an isoelectric point at pH 5.1 which was not present in extracts from nonviruliferous aphids and did not react with PEMV antiserum. A similar effect of the virus on *Myzus persicae*, green peach aphid, was not found.

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In the past there has been a considerable amount of discussion (15) as to whether or not pea enation mosaic virus (PEMV) multiplies in its insect vectors (1) [*Acyrthosiphon pisum* (Harris), pea aphid; *Myzus persicae* (Sulzer), green peach aphid; *Microsiphum euphorbiae* (Thomas), potato aphid]. In some of the leaf hopper-borne propagative plant viruses, metabolic changes have been shown to occur as a result of virus multiplication in leaf hopper tissues (20).

Assuming that the interaction of PEMV with its hosts at the biochemical level is similar in peas and aphids, when PEMV multiplication occurs one would expect to see similar metabolic changes. These may then be reflected in the occurrence of similar changes in protein patterns and enzyme activities in the two different cell systems.

Acrylamide disc electrophoresis has been used intensively in the study of virus plant host interactions

(6, 12, 16, 18). Changes in protein and enzyme patterns have been observed where viruses caused local lesions (18). However, in those instances where systemic viruses have been studied, no apparent changes in protein patterns except for the occurrence of virus protein were observed (12, 18). Mainly, quantitative differences in some enzymes were observed in these virus plant-host interactions (12), usually not different from those occurring during plant senescence.

This paper describes a change occurring in PEMV-infected plant tissues that does not occur in comparable healthy tissues as a result of normal senescence, and reports on the effect of PEMV on the protein patterns of two aphid species known to be PEMV vectors.

MATERIALS AND METHODS.—*Plants.*—*Pisum sativum* L. 'Perfected Wales' were grown from seed in a greenhouse where daytime temperatures were kept at ca. 75 F and night-time temperatures were 60 F. The plants were mechanically inoculated with PEMV (corundum, cotton swab) 13-18 days after planting. Buffer inoculated plants of the same age served as controls. Leaves from infected and healthy plants were harvested and processed at various times between 7-40 days after inoculation.

Aphids.—Green peach aphid populations were reared on Chinese cabbage [*Brassica pekinesis* (Lour.) Rupr] and then transferred in mass to healthy and PEMV-infected peas. Aphid populations were kept on healthy and diseased pea for 1 and 4 weeks, respectively, before harvesting and processing.

Pea aphids were reared on healthy pea plants and subsequently transferred to healthy and PEMV-infected peas of the same age and reared for 20 days before they were harvested and processed.

Protein extraction.—Harvested leaves were washed in distilled water and immediately ground in cold 0.2-M ammonium acetate buffer at pH 6.0 (1 ml/g tissue). The extract was filtered through four layers of cheesecloth, then centrifuged for 60 min at 10,000 rpm in a SS34 rotor in a Servall preparative centrifuge. Protein concentrations were determined by the method of Lowry et al. (10). Sucrose (10% v/v) was added to the leaf protein preparations which were then stored frozen. Except for the distilled water wash, soluble proteins were extracted from aphids in the same way as described for leaf material.

Isoelectric focusing (19) in 6% acrylamide gels was employed to develop the protein patterns from the leaf and aphid extracts. Two carrier ampholine preparations containing 40% ampholytes were used, with pH ranging from 3-10 and 7-9. The gels were prerun electrophoretically for 60 min to establish the pH gradients. The protein samples were applied in 10% sucrose under an ampholyte protective layer. A 1-mg sample of protein was used for the pH 3-10 gels; and 2-mg samples, for the pH 7-9 gels. Electrophoresis was conducted at 4 C for 4 hr with 1.5 ma/gel, raising the voltage gradually to 300-350 v. The pH gradient in the gels was determined by cutting a gel without a protein sample into 5-mm pieces. We macerated the cut pieces by squeezing them through a syringe into 1 ml of distilled water. The macerate was left for 1 hr; then the pH was determined.

Samples with or without 8 M urea incorporated were also run in gels containing 8 M urea.

After electrophoresis, soluble total proteins, peroxidase, acid-phosphatase, and esterase tests were made in the gels.

The peroxidase and esterase determination was adapted from Desborough & Peloquin (3, 4). A 0.5-M acetate buffer at pH 5 was used for the peroxidase determinations instead of the recommended aqueous solution. Acid phosphatase isoenzymes were detected according to Macko et al. (11). Total proteins were stained with Coomassie blue (2).

A peroxidase assay of crude extracts from healthy and diseased plant tissue was made according to Sequeira & Mineo (14), except for the use of 0.01 ml of extract and 0.05 M phosphate buffer at pH 6.

We recovered proteins from gels by cutting sections of unstained gels containing the desired band and macerating the sections with a syringe. Macerated sections were soaked overnight in a 0.2 M ammonium acetate buffer at pH 6. The solutions were then filtered and dialyzed overnight against 0.02 M ammonium acetate buffer at pH 6 and concentrated with Aquacide II.

Gels were scanned in a Gilford spectrophotometer at 550 nm. The acid and basic halves of the gels were often scanned at different slit widths because of different staining intensities of the bands in these areas.

Serology.—Whole virus was located in the gels as described by Van Loon & Van Kammen (18), using PEMV antiserum that was prepared from a mixture of whole and degraded PEMV.

RESULTS.—*Plant extracts.*—The technique of isoelectric focusing (19) was judged to be superior to the Ornstein method (13) because of the better resolution obtained. Therefore, it was used throughout this investigation to separate proteins.

The number of bands routinely resolved in gels from plant extracts using gradients of pH 3-10 were 24, 17, 10, and 40 for peroxidase, acid phosphatase, esterase, and total proteins, respectively. When urea was omitted from the gels, no differences in the isoenzyme patterns of these enzymes were found between healthy and diseased plants.

The stain intensity of peroxidase bands from infected tissues was strongest 7 to 14 days after inoculation (Fig. 2-A), and always much stronger than bands from comparable healthy tissue when equal amounts of protein were compared. Throughout the testing period, band A was stronger in the total protein patterns from diseased tissues than in those from healthy tissues (Fig. 2-B, I). The higher peroxidase activity in infected tissue was evident also from the total peroxidase activity (act/mg protein) determined in extracts (Fig. 1).

The patterns for total protein obtained in gels with a gradient from pH 3-10 showed many dark bands in the acid half of the gels with main bands at pH 6.7 and 6.1. The basic half of the gels showed fewer bands which were generally not as intensely stained (Fig.

2-B). A difference was found between healthy and infected plant material in the region of the gels between pH 7.2 and 8.3 (Fig. 2-B, 3). Band B was observed in the total protein patterns developed from diseased tissues and not in those from healthy tissues (Fig. 2-B, I and II) using equivalent amounts of protein in the samples.

These differences could be expanded when gels were run using ampholine carrier specifically for the pH 7-9 region (Fig. 2-B, II). When more total protein, 2 mg instead of 1.0 mg, was used from healthy plants, there was a faint indication of the presence of band B. No differences were found in healthy plants of different ages with regard to band B.

Gels developed on pH gradients from pH 7-9 showed that band A isolated from the pH 3-10 gradients actually consisted of two bands (Fig. 2-B, II).

Bands A and B recovered and electrophoretically purified showed peroxidase activity both from the infected and noninfected material. However, appreciably more protein from healthy tissue was required to show the presence and activity of band B in these tissues.

When gels were developed using 8 M urea, differences in protein patterns between healthy and infected plant tissue appearing early in infection disappeared at later stages, implying that the effects of PEMV were similar to those occurring in senescence.

Aphid extracts.—No differences in soluble protein (Fig. 2-C), peroxidase, acid phosphatase, and esterase patterns were observed with the protein extracts from viruliferous and nonviruliferous green peach aphids. The same was true for these enzymes tested in pea aphid protein extracts. In the soluble protein pattern generated from viruliferous pea aphid extracts, a sharp band was present at pH 5.1 although absent in patterns developed from nonviruliferous aphid extracts (Fig. 2-D). No serological reaction could be obtained with this protein and anti-PEMV antiserum.

DISCUSSION.—The conclusion of Novacky & Hampton (12), that no new isoenzymes were formed as a result of infection or of senescence in the hosts they used, is corroborated by our study of PEMV infection in peas. The increase in peroxidase activity at band B (pH 7.1), in diseased as compared with healthy specimen plants, even 40-50 days after inoculation, indicates an increase in peroxidase due to the presence of PEMV rather than to an accelerated senescence of the tissues. Increased senescence in infected tissue was noted when peroxidase bands appeared in protein extracts from 14-day-old peas inoculated 7 days prior to extraction, but was lacking in extracts (1 mg protein) from healthy plants of the same age. At later stages of infection, these differences disappeared except for band B. Accelerated senescence accompanies PEMV infection in pea tissue, and tends to obscure the effect of the virus per se.

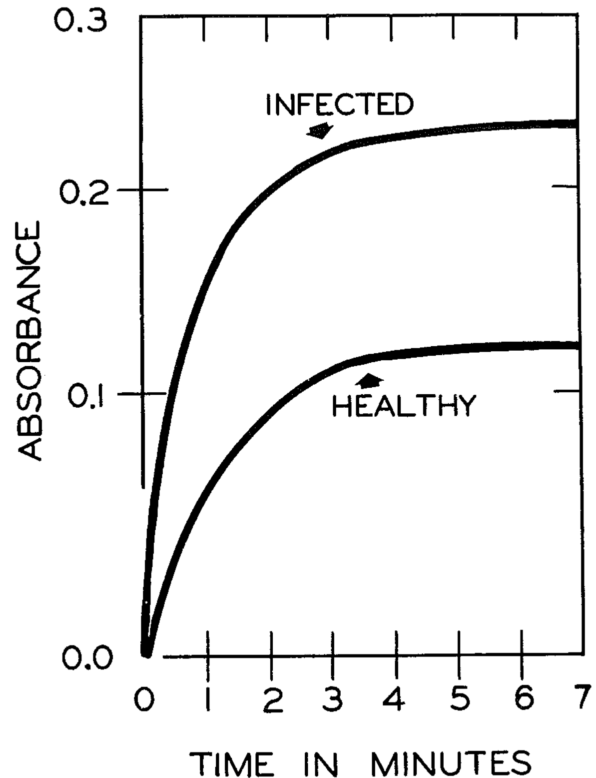
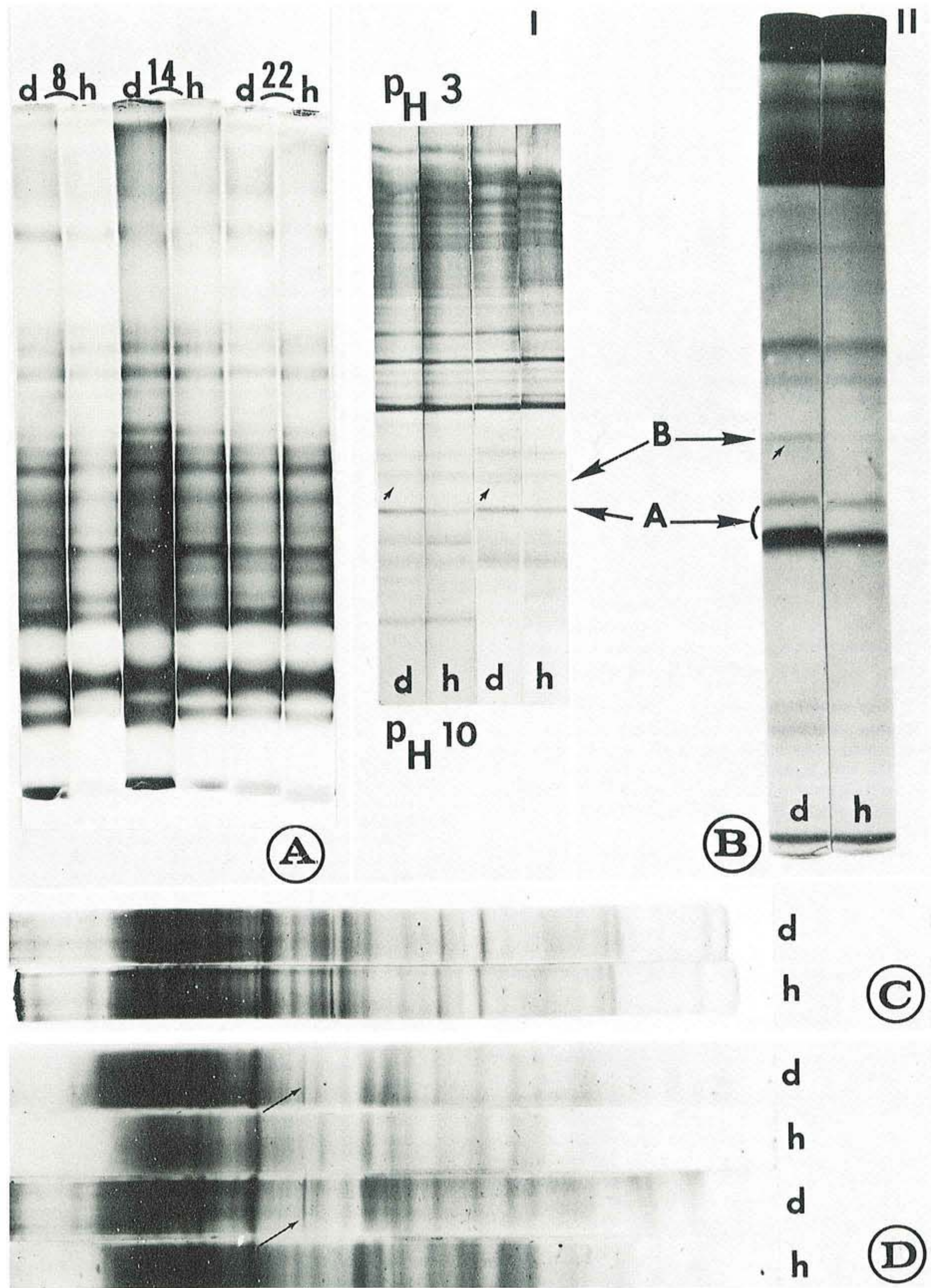


Fig. 1. Peroxidase activity (activity/mg protein) of crude protein extracts from pea enation mosaic virus infected pea tissue 14 days after inoculation and from comparable pea tissue. (0.01 ml crude extract, 0.1 ml of 0.02 M guaiacol, 1 ml 0.38 M H_2O_2, and 3.8 ml of 0.02 M KH_2PO_4 buffer, pH 6. Absorbancy read at 470 nm).

Fig. 2. Protein patterns developed from tissue extracts by isoelectric focusing. Unless noted, equal amounts of protein were used in samples of healthy and diseased tissue. Only those differences are discussed that also showed in densitometer tracings. **A)** Peroxidase patterns in extracts from pea enation mosaic virus (PEMV) infected plant tissue (d) and healthy plant tissue (h). Extracts were made 8, 14, and 22 days after inoculation. **B)** Total protein patterns from PEMV-infected plant tissue extracts and healthy plant tissue extracts; (I) 7 days (left pair); and 14 days (right pair) after inoculation, respectively. A pH gradient from 3 to 10 was used. (II). A pH gradient from 7 to 9 was used with 14-day-old infected tissue extracts and comparable healthy tissue extracts. Note the specific protein (arrow, band B) shown in the gels from infected-tissue extracts at pH 7.2. **C)** Protein patterns from green peach aphid fed on diseased (d) and healthy tissue (h). No difference is visible between the d and h gels. **D)** Protein patterns from pea aphids fed on diseased (d) and healthy tissue (h). A band at pH 5.1 (arrow) is present in the patterns from viruliferous aphids. The top pair of gels (d, h) were loaded with 0.75 mg of protein; and the bottom pair (d, h), were loaded with 1.5 mg protein.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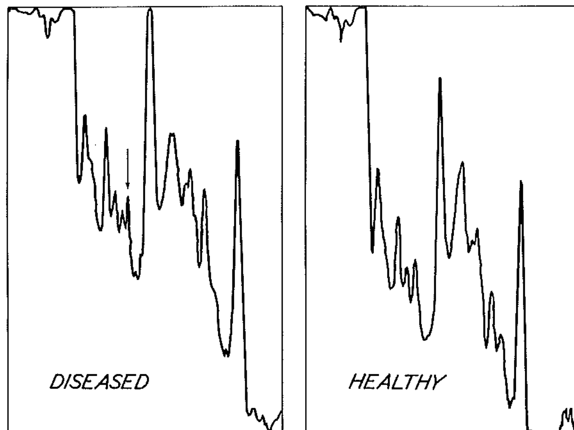

Fig. 3. A densitometer tracing of the basic halves of gels resulting from protein extracts of plants that had been inoculated 14 days prior to extraction and comparable healthy plants. Note the extra peak at the arrow in the gel of the diseased tissue extract which does not appear in the scan of the gels of healthy tissue extract.

Whole virus did not enter the 6% gel to any extent, and the amount of viral subunit protein in 1 mg extracted plant and aphid protein was too small to be detected. Enhancement of peroxidase, acid phosphatase, and esterase activities in diseased plant tissue was striking. However, increases in peroxidase activity in diseased plants have been reported before for plants with fungal diseases (7, 21) and with virus diseases (8, 9, 17).

In our working hypothesis, we assumed that if the metabolic and biochemical changes in plant and insect tissue upon infection with PEMV are similar (as depicted by changes in protein patterns and enzyme activities) we should conclude, since PEMV multiplies in plant tissue, that multiplication of this virus is also taking place in viruliferous insects.

No evidence for this assumption, and therefore, for the multiplication of PEMV in its vectors, was obtained.

Our results may be judged against one of the following alternatives: (i) The observed changes in plant tissues reflect a nonspecific direct reaction to virus multiplication, in which case, a similar reaction should occur in insect tissues sustaining virus multiplication; (ii) the observed changes in plant tissues reflect a plant-specific reaction to virus multiplication, in which case, no similar reaction should necessarily occur in insect tissue sustaining virus multiplication; (iii) multiplication of PEMV does occur in aphids which results in metabolic and biochemical differences between viruliferous and nonviruliferous aphids that are, in some species and not in others, detectable by isoelectric focusing techniques; (iv) multiplication of PEMV does not occur in aphids, but the presence of the virus in some aphid species changes its metabolism as detected by isoelectric focusing. In view of the fact that the changes observed in pea plants upon PEMV infection occur in many plants infected with a wide range of pathogens (including viruses) and in view of ultrastructural studies on PEMV-infected plants and viruliferous and nonviruliferous pea aphids (5) that could not establish a basis for the claim that PEMV multiples in the aphid, the authors tend to favor alternatives ii and iv.

The unidentified protein band in gels from viruliferous pea aphids not present in gels from nonviruliferous aphids cannot be considered to be of viral coat protein origin, not only because this protein (when isolated) did not react with our antiserum (titer to coat protein 64), but the isoelectric point of the anomalous protein is considered too low to be virus coat protein, since Shepherd et al. (15) showed that the subunit protein was highly cationic.

Furthermore, it is unlikely that the small change in protein patterns observed in infected plants would change the metabolism (protein pattern) of the aphid feeding on it, whereas a change in host genus for both aphid species had no influence on the protein patterns of nonviruliferous aphids (de Zoeten & Rettig, *unpublished data*). Therefore, the different protein pattern of the viruliferous pea aphids must depict a specific reaction of the aphid to the presence of the virus, not necessarily to virus multiplication, however.

The fact that the protein patterns of viruliferous and nonviruliferous green peach aphids were identical remains, in the light of the similar PEMV transmission pattern, unexplained. When the virus is propagative or circulative in both species, our techniques showed an effect of the virus only on pea aphids.

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