Hemolysins in Potato Plants and Their Relation to Virus Infection

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Portion of a Ph.D. thesis submitted by the senior author to The Pennsylvania State University.

Contribution No. 734 from the Department of Plant Pathology, The Pennsylvania Agricultural Experiment Station. Authorized for publication 23 May 1973 as Journal Series Paper No. 4456.

Accepted for publication 8 December 1973.

ABSTRACT

It was found that foliage extracts from both healthy potato plants and plants infected with viruses hemolyzed red blood cells from various animal sources. The intrinsic substances present in the extracts and responsible for hemolysis were investigated for possible effect on virus infectivity and pathogenesis.

Extracts from potato plants chronically infected with tobacco mosaic virus (TMV) yellow strain (Y) alone or TMV-U1 strain (U1) plus potato virus X (PVX) lost most of their hemolytic activity, whereas those plants infected with TMV-U1 or PVX alone retained the activity. Extracts from young leaves of plants infected with TMV-U1, or PVX were distinctly higher in hemolytic activity than those from older leaves, and an inverse correlation between numbers of lesions on indicator plants and hemolytic activity of these extracts was observed. This is not the case with extracts from potato plants chronically infected with TMV-Y or TMV-U1 plus PVX.

When extracts of leaves from different positions on a plant infected with PVX were heated at 55 C for 10 min, PVX infectivity increased in all. The increase was greatest in extracts from the lower part of a plant, which also showed the lowest hemolytic activity. Electron micrographs showed similar numbers of PVX particles in extracts from either younger or older leaves. Hemolytic activity was not affected by heat.

It is evident that a virus inhibitor is present in potato plants, and that this inhibitor also possesses hemolytic activity. Since heat reduced the effects of inhibitor on virus infection, but not the hemolytic activity, it is conjectured that the activity of the inhibitor might be related to its direct attachment to virus particles rather than any capability for causing a host-directed inhibition of infection.

Phytopathology 64:714-718.

During an investigation on the possible use of the hemagglutination phenomenon for the detection of plant viruses, it was found that extracts from both virus-infected and noninfected potato plants hemolyzed red blood cells from various warm-blooded animals including humans (8). The substances which hemolyze red blood cells are called hemolysins. They have been identified as glycosides, and are known to occur in a wide variety of plants (2). Limited studies of hemolysins with regard to occurrence, properties, and uses have been made because of their toxicological importance when present in plant foodstuffs. The significance of these substances in plants is still an open question. Recently, hemolysins have been reported to provide the basis of resistance in legume seeds to attack by insects (1).

This report presents the results of experiments on hemolysins in potato plants and their relation to virus infection.

MATERIALS AND METHODS.—Erythrocyte suspensions were prepared by the method of Casals (5). Freshly drawn blood (8.5 ml) was poured into a vial containing 1.5 ml of acid-citrate-dextrose (ACD). The cells were washed and compacted four times by centrifugation at 300-*g* in dextrose-gelation-veronal (DGV) at 4 C; the first time by adding 1 vol of whole blood to 2.5 vol of DGV and subsequently with 3 vol of DGV per original vol of whole blood. After the final wash, the cells were again suspended in 3 vol of DGV. These stock suspensions could be stored at 2-4 C for at least 3 wk without deterioration. Tenfold dilutions of the stock suspensions were used in the tests.

Plant extracts were made by the trituration of 1 g of plant tissue with 4 ml 0.1 M PO_4 buffer, pH 7, and clarified by centrifugation for 5 min at 40,000 *g* in a Spinco L-2 ultracentrifuge. The supernatant extracts were used undiluted or diluted in 0.1 M PO_4 buffer by pipetting to the shallow wells in plastic spot plates, after which an equal amount of the red blood cell suspension was added. In most cases, the total volume was 0.6 ml. The ingredients were mixed by tapping the edges of the plates, and the mixtures were incubated at room temp until the reactions were complete.

The highest dilutions at which the red blood cells were hemolyzed were recorded when the red blood cells in the control wells settled. Hemolysis was indicated when the mixture became bright red and there was limited cell sedimentation.

Potato plants used in this investigation were greenhouse-grown from tubers of a selected virus-free but tobacco mosaic virus (TMV)-susceptible potato line, 1-68-6XH-8′ (4). When plants were 15.2 cm (6 inches) high, three each were inoculated with the TMV-U1 strain, a TMV yellow strain,a PVX isolate or TMV-U1 plus PVX. Later, tubers produced by these systemically infected plants were harvested and stored at about 40 F. When they broke dormancy, plants grown from them were used for additional experiments. Plants free of virus served as controls.

Plant extracts were prepared from leaf samples taken from the terminal, including unfolding leaves, the middle, and the lower parts of plants, unless stated otherwise. Infectivity of TMV-U1 and TMV-Y in extracts were quantitatively determined on *Nicotiana tabacum* L. 'Wis. Havana 425'. *Gomphrena globosa* L. was used as an assay host for PVX.

The method used in the electron microscopic

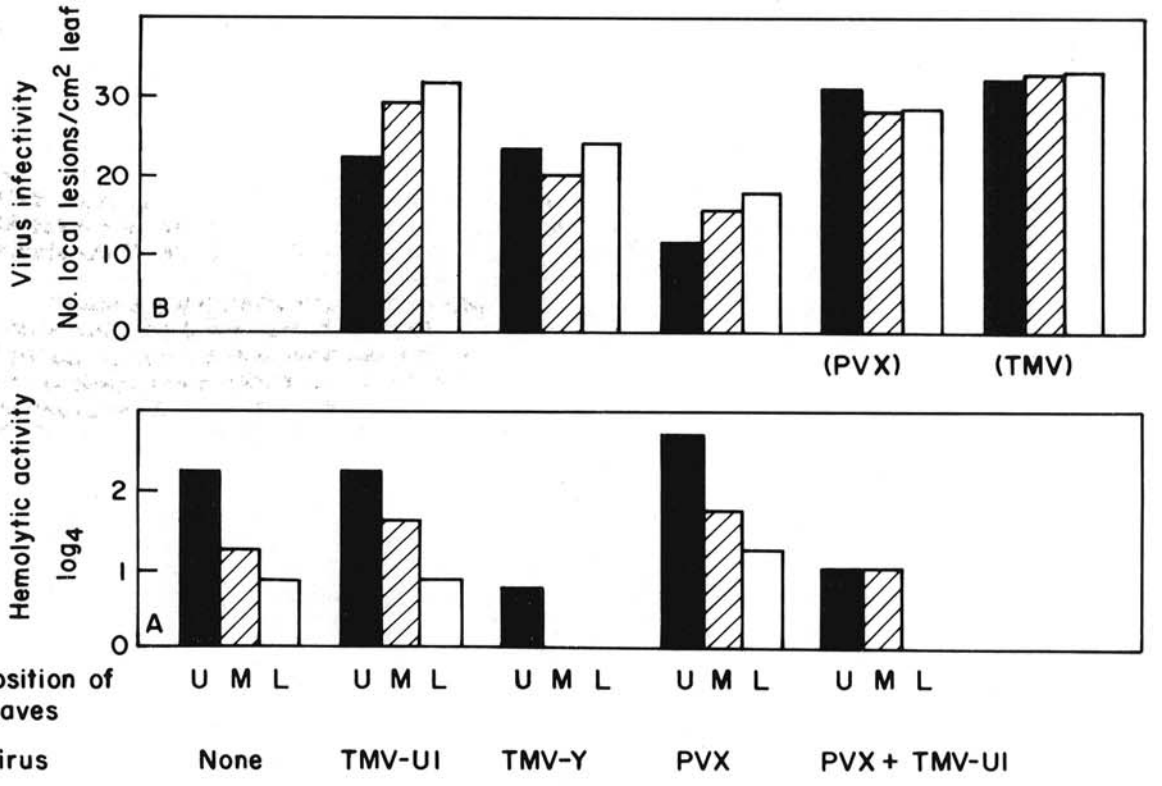

Fig. 1-(A, B). **A)** Hemolytic activity and **B)** virus infectivity of leaf extracts of different positions on potato plants (1-68-6XH-8′) infected with viruses and noninfected. There were significant differences in hemolytic activity ($P = 0.01$) among extracts from different positions on plants infected with TMV-U1, or PVX, or noninfected plants, but not for those plants infected with TMV-Y or PVX plus TMV-U1. There also was a significant difference ($P = 0.05$) in virus infectivity among extracts from different positions on plants infected with TMV-U1 or PVX, but not those from plants infected with TMV-Y or TMV-U1 plus PVX. (PVX) and (TMV) indicate respective infectivity of PVX and TMV-U1 in extracts of leaves from potato plants infected with a combination of PVX and TMV-U1. Leaf position is designated as U, upper; M, middle; and L, lower part of the plant.

examination of leaf extracts to determine virus concn was according to Kellenberger and Arber (7) with some modification. A 0.1-ml portion of an extract was mixed with 0.1 ml of an aqueous suspension of latex spheres (diam 0.088 μ) previously adjusted to 6×10^{10} spheres/ml. One drop of this virus-latex sphere suspension was mixed with one drop 2% aqueous solution of phosphotungstic acid, pH 6.4, and placed on a copper grid. The excess suspension was absorbed with filter paper and the specimen grid was air-dried before examination with a Hitachi HU-11E electron microscope operated at 75 KW with 20- to 30-μm objective aperture. Electron micrographs of eight different fields at ×5,300 were made for each sample. Latex spheres and all virus particles in each field were counted and the concn of virus was calculated.

RESULTS.—*Distribution of hemolysins in potato foliage.*—Distribution of hemolysins in individual virus-free and virus-infected plants was made by testing the activity of leaf extracts from different positions, starting from the top to the bottom. The concn of hemolysins was found to vary with the age of the tested leaf. Invariably, the hemolysin content of younger leaves, including axillary buds, at any position on a plant, was higher than that of older leaves (Fig. 1 A.).

Effects of virus infection on hemolytic activity of potato plants and the relation between activity and virus infectivity.—No significant differences were observed in the hemolytic activity of extracts from plants primarily infected with a virus or viruses compared to noninfected plants. However, there was a tendency for an inverse correlation of lesion numbers and hemolytic activity of extracts from TMV- or PVX-infected plants, or plants infected with both viruses; i.e., fewer lesions were observed on leaves inoculated with extracts with highest hemolytic activity, while more lesions occurred on leaves inoculated with extracts with the lowest hemolytic activity.

The possible effect of chronic virus infection on hemolytic activity was then investigated. Extracts from plants grown from tubers produced by systemically infected plants used in previous experiments were examined as before. It was found that the hemolytic activity of extracts from TMV-U1- or PVX-infected plants was significantly increased when compared to noninfected plants. The number of lesions on Havana 425 or *G. globosa* induced by TMV-U1 and PVX, respectively, were fewer when the leaf extracts were prepared from upper leaves than when the extracts were from lower leaves. As before, the highest hemolytic activity was shown in extracts from upper leaves and lowest activity from the lower leaves. While leaf extracts from upper part of TMV-Y-infected plants and upper and middle parts of PVX and TMV-U1 doubly infected plants showed some hemolytic activity, it was greatly decreased compared to those from plants of healthy, TMV-U1- or PVX-infected plants. No statistical difference in number of lesions was observed when extracts from leaves at different positions on the plants infected with TMV-Y or TMV-U1 plus PVX were inoculated on the leaves of indicator plants (Fig. 1).

Electron microscopic examination of PVX concentration in extracts from leaves at different positions on potato plants.—To ascertain whether reduced infectivity was due to a lower concn of virus in the extracts, an electron microscopic examination was made to determine virus concns from different parts of infected plants. Plants were grown from tubers infected with PVX. One month after planting, samples were taken from the upper and lower leaves and extractions were made as before. Concurrent with electron microscopic examinations to determine virus concns in extracts, examinations of hemolytic activity and PVX infectivity, as indicated by the numbers of local lesions on *G. globosa*, were made. Results of hemolytic activity and PVX infectivity were the same as in the previous tests. No difference in virus concn in extracts from leaves at different positions on the plant was observed (Table 1).

Influence of heating on the hemolytic activity and infectivity of extracts from PVX-infected plants.—In preliminary tests it was found that heating extracts from PVX-infected plants enhanced infectivity as determined by inoculations to *G. globosa*. When partially purified PVX was mixed with leaf extracts from healthy potato plants, virus infectivity was also greatly inhibited, but heating these mixtures to 55 C for 10 min was found to partially restore infectivity.

In the preceding tests, an inverse correlation between infectivity and hemolytic activity of leaf extracts from different positions on virus-infected plants was observed. In addition, as determined by electron microscopy, there was no difference in virus concn in extracts from leaves at different positions on a plant. It was thus apparent that extracts from potato foliage contained some substance inhibitory to virus infection.

Tests on effects of heating on hemolytic activity and infectivity of extracts from PVX-infected plants were made in order to elucidate the possible relation of hemolytic activity to infectivity of extracts. The hemolytic activity was not changed by heating. However, the PVX-

TABLE 1. Hemolytic activity, infectivity, and virus concn of leaf extracts from the upper and lower parts of PVX-infected potato plants

Sample positon	Hemolytic[a] activity ($\log_4$ dilutions)	Infectivity[b] (no. lesions/ half leaf)	Virus concn[a] avg (particles/ml $\times 10^{10}$)
Upper	1.6 ± 0.29	39.8 ± 5.54	3.97 ± 1.12
Lower	1.0 ± 0.00	61.7 ± 5.21	4.03 ± 0.93

[a]Mean of three plants.
[b]Mean of six half-leaves from each of three plants.

infectivity of extracts from different positions of a plant, invariably increased in heated extracts compared to nonheated extracts, and the increase was greater in extracts from the lower than from the upper parts (Fig. 2).

DISCUSSION.—In the present study, hemolytic activity was found to be higher in extracts from younger than from older leaves. If potato plants were infected with PVX or TMV-U1, virus infectivity was lower in extracts from younger than from older leaves. Electron microscopic examinations, however, indicated no difference in numbers of virus particles between extracts from leaves of different ages. Hemolytic activity was extremely low or absent in extracts from leaves of potato plants infected with TMV-yellow or TMV-U1 plus PVX. In these plants there was no statistical difference in virus infectivity between extracts from younger and older leaves, in contrast with those of PVX- or TMV-U1-infected plants.

Extracts heated to 55 C for 10 min, from PVX-infected plants or from noninfected plants to which PVX was added, were more infectious on *G. globosa*. Both Blaszczak et al. (3) and Hooker and Kim (6) also found that the virus inhibitory effects of potato extracts was reduced by heat at 55-60 C for 10 min. No explanation for this phenomenon was given. Recently, Mayhew and Ford (9) have presented strong evidence that an inhibitor produced by *Physarum polycephalum* Schw. altered TMV particles directly and interfered with normal replication of virus in the host cell. Since this inhibition could be reversed by dilution and treatment at high temp, they conjectured that the virus particles were coated by the inhibitor, but that the coating was only weakly chemically bonded. This might also explain the results reported here, since the inhibitor in potato foliage extracts was inactivated by heat. To explain the greater increase of PVX infectivity in the heated extracts from lower leaves over the heated extracts from upper leaves, it might be conjectured that 55 C for 10 min was not sufficient time to release all particles from bonding in the upper extracts which were also shown to have the higher concn of hemolysins.

Shepard (10) reported that hemagglutinins in potato tubers interfere with serological reactions in single-radial diffusion systems by agglutinating or precipitating antiserum or normal serum. At this time it is not known whether the hemolysins present in potato foliage might also interfere with serological reactions. It might merit investigation.

Hooker and Kim (6) studied inhibitors of PVX in potato leaves because of their possible involvement in virus resistance. They could find no evidence that inhibitors from the different resistant types of potato differed in quantity or in quality. It is apparent that these intrinsic substances from potato foliage lyse red blood cells and also act as inhibitor of virus infection when plants are mechanically inoculated with extracts containing these substances. However, these substances do not appear to play a significant role in the resistance of potato varieties to virus infection. The fact that the concn of inhibitors varies with the age of the leaf tissue and in turn affects the infectivity of extracts demonstrates the importance of consistency in tissue sampling when attempting to quantify virus concns.

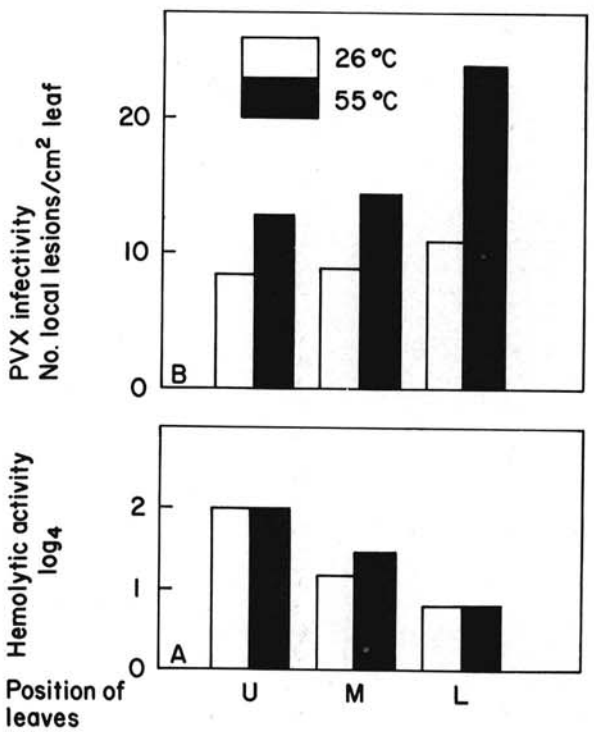

Fig. 2-(A, B). Influence of temp on **A)** hemolytic activity and **B)** infectivity of extracts from different parts of a potato plant (1-68-6XH-8') infected with PVX. Effects of heat treatment on PVX infectivity of extracts were significant ($P = 0.01$). No differences were noticed between nonheated and heated samples for hemolytic activity. There were significant differences ($P = 0.01$) among extracts of leaves from different positions on the plant in PVX infectivity. Leaf position is designated as U, upper; M, middle; and L, lower part of the plant.

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