ISOLATION AND PARTIAL CHARACTERIZATION OF A LARGE MOLECULAR WEIGHT VIRUS INHIBITOR FROM PEPPER, CAPSICUM FRUTESCENS L.

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ABSTRACT

The purification of an active carbohydrate and protein containing plant virus inhibitor from a high molecular weight fraction of pepper, Capsicum frutescens L. var. California Wonder, is described.

The crude juice was stabilized with ascorbate in an attempt to prevent oxidation of polyphenols to quinones and covalent bonding to proteins. Treating the extract with Polyclar A. T. was effective in removing polyphenols.

Gel filtration of the extract on Sephadex G-75 gave a fraction which contained material with molecular weights greater than 50,000 daltons. This fraction contained both carbohydrate and protein. Its disc gel electrophoresis pattern contained several bands which stained for both carbohydrate and protein. High levels of activity were found for the fraction and for one of the bands on the acrylamide gel. The inhibitory material in this band was also found to be heat-stable.

The gel filtration fraction was separated by ion-exchange chromatography into acid, basic and neutral fractions. The neutral fraction was highly active and contained both protein and carbohydrate. Its disc electrophoretic pattern was similar to the pattern of the gel filtered material. The band which corresponded to the heat-stable active band assayed after disc electrophoresis of the gel filtered material was found to be highly active.

Sephadex G-200 chromatography of the neutral fraction produced three fractions, each containing different ratios of protein to carbohydrate. Two of these fractions contained heat-labile

inhibitory material. The third fraction contained heat-stable inhibitory material.

An active fraction that gave a single band upon disc electrophoresis was obtained by calcium phosphate chromatography of the Sephadex G-75 gel filtration fraction. This band which corresponded to the active bands assayed after Sephadex G-75 gel chromatography, ion-exchange chromatography and disc electrophoresis was also active.

Quantitative amino acid and qualitative sugar analysis were performed on the purified material. Large quantities of serine, glycine, and alanine, together with smaller amounts of the aromatic amino acids were found. The major sugar components were galactose and arabinose. Glucose, ribose, and xylose were present in trace amounts.

A method for analysis of inhibition data is also described. The plot of

$$\log_{10} (\text{inhibitor concentration}) \quad \text{versus}$$

$$\log_{10} \frac{(\text{fractional degree of inhibition})}{(1\text{-fractional degree of inhibition})}$$

gave a straight line. This plot will facilitate the determination of the 50 per cent inhibition level of activity.

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INTRODUCTION

Substances which affect plant virus infection have been studied extensively and have been reviewed by Bawden (1964). These substances fall into two groups, inactivators and inhibitors. Inactivators inhibit infection by acting directly on the virus particles while inhibitors inhibit by acting on the host plant.

Plant extracts can contain both inactivators and inhibitors. Tannins and polyphenols are found in most plant extracts. Tannins and polyphenols which have been oxidized to quinones are able to bind to proteins altering their chemical and physical properties. These substances have been shown to inactivate virus particles (Cadman, 1959; Hampton and Fulton, 1961). Plant proteins on the other hand, act as inhibitors. They prevent the absorption and penetration of the virus into the host cell by blocking the receptor sites on the host cell. In addition, there are indications that the proteins can modify cell metabolism preventing infection and/or virus multiplication (McKeen, 1956; Ragetli and Weintraub, 1962; Apablaza and Bernier, 1972).

The inhibitory nature of pepper juice has been examined by McKeen (1956) and Apablaza and Bernier (1972). McKeen reported that pepper juice inhibited the production of local lesions by cucumber mosaic virus on cowpea (Vigna sinensis Savi). The juice was inhibitory when applied either with the virus or to the upper or lower leaf surface before inoculation of the upper leaf surface. The juice was not inhibitory when applied one or two hours after inoculation. The inhibitory component was partially characterized

and found to be non-dialyzable and heat-labile. Being non-dialyzable the inhibitor should not be able to penetrate the cell membranes when sprayed on the lower leaf surface. In order to account for the activity observed when the upper surface is inoculated with virus, the author suggested that the protein may cause changes at the epidermal surface which alters the cell metabolism preventing virus multiplication. Apablaza and Bernier examined the inhibitory effects of pepper juice using tobacco mosaic virus (TMV) on Pinto bean (Phaseolus vulgaris L.var. Pinto). The juice was inhibitory when mixed with the virus or applied to the lower leaf surface before inoculation of the upper surface. In the latter assay the effect was present in the untreated leaf as well as the treated leaf. Unlike McKeen they found the juice was inhibitory when applied after inoculation. Upon boiling to determine heat stability, the pepper extract retained its activity as measured by the mixed inoculum assay and the lower leaf surface assay but showed a decrease in activity when applied after inoculation. The authors suggested that these results may indicate the presence of more than one inhibitor. Using membrane filtration the authors separated the extract into two active heat stable fractions, one with a molecular weight greater than 50,000 daltons and one with a molecular weight range 1,000 - 50,000 daltons. The high molecular weight pepper fraction was found to be active when applied to the lower leaf surface. Like McKeen the authors proposed that the high molecular weight fraction inhibits virus infection when applied to lower leaf surfaces by altering the metabolism of the leaf cell so that virus particles cannot multiply. Noting that McKeen found the pepper inhibitor to be

heat-labile, Apablaza and Bernier suggested that the discrepancy might be a result of the different virus host system. They also felt that the decrease in activity of the boiled extract as measured by the after inoculation assay may be due to the presence of a highly active partially heat-stable inhibitor. In unpublished results the pepper extract was still highly inhibitory at a 1:500 (v/v) dilution in the mixed inoculum assay. They concluded that boiling might not reduce the concentration of inhibitor sufficiently to affect the level of inhibition produced by the mixed inoculum assay but may do so when applied after inoculation.

Apablaza (1968) obtained a neutral fraction from the high molecular weight membrane filtered material by ion-exchange chromatography. This fraction, which contained most of the original activity gave a positive test for both carbohydrate and protein. The author felt the active factor could be a glycoprotein and treated the fraction with protein degrading enzyme and a carbohydrate degrading enzyme. Treatment with either enzyme resulted in the loss of all activity. These results further confirmed the glycoprotein nature and indicated the intact molecule may be necessary for activity.

This study was undertaken to provide more information on the nature of the inhibitory substance in the high molecular weight fraction of pepper. In particular it was desired to:

1) determine whether the activity in the high molecular weight fraction is due to protein bound polyphenols;
2) investigate the presence of a heat-labile inhibitor whose activity is overshadowed by the heat stable

inhibitor;

3) isolate and characterize the glycoprotein inhibitor;

4) provide a graphical method of estimating activity based on the local lesion bioassay.

This work is divided into three sections. The first describes the isolation of different inhibitor containing fractions from pepper. The second describes the isolation of a protein-carbohydrate containing inhibitor in a pure form. The third section deals with the application of statistical analysis of lesion numbers to the TMV-Pinto bean system.

LITERATURE REVIEW

There are two ways by which inhibition of virus infection can be achieved. In one instance substances can inactivate the virus particle. Such substances are referred to as inactivators. In the second case the substance can act on the host plant, blocking infectable sites or altering the cells' resistance. These agents are referred to as inhibitors.

Tannin in strawberry (Fragaria chiloensis L.) was shown to be an inactivator of tobacco mosaic virus (Bawden and Kleczkowski, 1945). The tannin was liberated by maceration of the leaves in quantities sufficient to precipitate all the native protein as well as any virus added to the supernatant. Cadman (1959) examined the tannins in Raspberry (Rubus idaeus L.) and found their action similar to tannic acid. The degree to which the virus infection was inhibited depended on the virus and not the species of the test plant. Some viruses formed irreversable complexes with Raspberry tannins and tannic acid while others formed complexes which were readily reversed by dilution or increase in pH. Cadman found that the inhibitory activity of the sap was not affected by heating in a boiling water bath for 10 minutes but was lost upon dialysis. Cadman also showed that "non-tanning" phenolic compounds which occur in the saps of many plants did not affect virus infectivity. The "non-tanning" phenolics tested included quercitrin, catechin, chlorogenic, cinnamic, gallic and protocatechuic acids and leuco-anthocyanim. Hampton and Fulton (1961) on the other hand showed that polyphenols can be active in their oxidized forms. The

oxidation of polyphenols by polyphenol oxidase results in the formation of o-quinones. Hampton and Fulton found that prune dwarf and sour cherry necrotic ring spot virus were inactivated by o-quinones but not by the reduced polyphenol. Once the virus was inactivated infectivity could not be restored by addition of reducing agents. They also found that the serological properties and gross structure of the virus were not altered by inactivation with o-quinones. Mink (1965) found similar results with tulare apple mosaic virus. He showed that the virus can be inactivated immediately by substituted o-quinones. Recently Mayhew and Ford (1971) have isolated an inactivator of TMV from Physarum polycephalum. This inactivator has the properties of a polysaccharide and a molecule weight of 35,000 to 55,000 daltons. Infectivity of the virus-inhibitor complex was restored by dilution or by heating at high temperatures. Treating the polysaccharide alone with high temperatures had no effect on inhibition. The inactivator was active when mixed with the virus or applied to the upper leaf surface of bean before and after inoculation. The polysaccharide was not inhibitory when applied to the lower leaf surface before inoculation of the upper surface with virus. On the basis of electron micrographs the authors suggested that the inactivator coats the TMV particle and prevents normal coat stripping.

Polysaccharides from fungi on the other hand act as inhibitors. Trichothecium roseum Link was the source of two heat-stable substances that inhibited infection of Nicotiana glutinosa L. by TMV and of French bean (Phaseolus vulgaris L.var. Prince) by tobacco necrosis virus (Bawden and Freeman, 1952). The authors isolated

a dialyzable component, trichothecin, a sometimes phytotoxic compound and an non-dialyzable component, a polysaccharide. The polysaccharide contained 60-70 percent reducing sugars (as glucose) and 1.1-1.4 percent nitrogen. The main sugar component was D-galactose. When mixed with the virus or applied to the leaf surface before inoculation the polysaccharide was inhibitory but when applied after inoculation or to the lower leaf surface it was not. The polysaccharide did not combine with the virus *in vitro*. The authors suggested that the lack of activity when applied to the lower leaf surface was due to its large size and inability to penetrate into the leaf cell. They also suggested that the inhibitors act by altering the leaf cell metabolism such that the introduced virus cannot multiply and are inactivated.

A polysaccharide inhibitor has also been isolated from a fungus, *Phytophthora infestans* Mont. (Hodgson *et al.*, 1969; Singh *et al.*, 1970; Wood *et al.*, 1971). This polysaccharide was also found to be non-dialyzable. It contained less than 0.1 percent protein and upon hydrolysis it yielded only glucose. The inhibitor was not affected by autoclaving. The polysaccharide inhibited the development of local lesions on *Nicotiana tabaccum* L. when mixed with the virus or applied before the virus. The polysaccaride was not effective when applied to the under leaf surface and only partly effective applied after inoculation. The authors found that the polysaccharide was not translocated and remained confined to the epidermal layer. They suggested that the polysaccharide inhibited virus entry by modifying infectable sites. They also found that although lesion formation could be completely inhibited there was still some virus

penetration and multiplication as shown by serological tests. Recent work on the polysaccharide (Wood et al.,1971) showed it to be a water soluble B(1-3) linked D-glucan.

Simple sugars have been shown to act as inhibitors. Subbarayudu and Wilcoxson (1967) found that mannose inhibited the infection of Gomphrena globosa L. by red clover vein mosasic virus. The extent of inhibition depended only on the mannose concentration and could be reversed by dilution. Concentrations of mannose between 1.5 percent and 7.5 percent were most effective in inhibiting infection when mixed with the virus before inoculation. Mannose was also effective when supplied to the leaves through the stems or applied to the lower leaf surface 24 hours before inoculation with the virus. With foliar application of mannose before inoculation the effect was not apparent until an hour after application and was lost after 24 hours. The authors also found in preliminary experiments that arabinose, xylose, glucose, sucrose, maltose, lactose and galactose may be inhibitors. The authors suggested that mannose acted upon the plant cell and altered the resistance of the cell, possibly by changes in the ectodesmatas. Jong-ho and Sehgal (1969) also found mannose to be inhibitory. One to 5 percent mannose reduced the infection of Sorghum bicolor Moench by maize swarf mosaic virus.

While the type of inhibitors found in fungi were reported to be polysaccharides the inhibitors isolated from higher plants were proteinaceous. Ragetli and Weintraub (1962) isolated a potent inhibitor from carnation (Dianthus caryophllus L.). They showed it to be a protein and determined the amino acid content.

Leucine(s), serine, and glycine were present in abundance with lesser amounts of threonine, alanine, lysine, valine, and proline and only traces of tyrosine and arginine. No ribonuclease activity was associated with the inhibitor. These workers felt that the inhibitor acted by competing for infectable sites via the E-amino groups of lysine. This view was supported by the decrease in infectivity when the inhibitor was treated with reagents which block the E-amino groups.

An inhibitor from Phytolacca esculenta L. was isolated and purified by Kassanis and Kleczkowski (1948). They found the inhibitor to be non-dialyzable, heat-labile and to contain 14-15 percent nitrogen and 8-12 percent carbohydrate. The carbohydrate was not separated from the protein when the purified inhibitor was precipitated with either ammonium sulfate or trichloroacetic acid. The authors suggested that, since the carbohydrate appeared to be an integral part of the active material, the inhibitor could be termed a glycoprotein.

More recently Wyatt and Shepherd (1969) have isolated this inhibitor in a more purified form and found less than 1 percent carbohydrate associated with it. These workers further purified the material isolated by the method of Kassanis and Kleczkowski on carboxymethyl cellulose. This process removed inactive material and most of the carbohydrate. The final product was a highly basic protein containing 12 percent lysine by weight and had a molecular weight of approximately 13,000 daltons. The potency of the inhibitor was increased four fold by their purification. The inhibitor lost its activity when the E-amino groups were blocked by succinylation

as did the inhibitor from carnation (Ragetli and Weintraub, 1962). Wyatt and Shepherd suggested that the loss of activity could be due to blocking of the E-amino groups or also could be due to conformational changes induced by the succinylation.

McKeen (1956) partially characterized an inhibitory component of pepper juice. The inhibitor was found to be non-dialyzable and heat-labile. It inhibited the infection of cowpea plants by cucumber mosaic virus when applied either with the virus or to the upper or lower leaf surfaces before inoculation. The inhibitor was not effective when applied one to two hours after inoculation. The author concluded from these results that the inhibitor acted upon the infection process rather than the virus multiplication. Also, presumably, because of its non-dialyzable nature the inhibitor was not able to penetrate the leaf tissue when sprayed on the lower leaf surface. To account for the activity observed in this case, the author suggested that the inhibitor may cause changes at the epidermal surface which alters the cell metabolism preventing virus multiplication.

Apablaza and Bernier (1972) found that extracts from pepper, geranium (Pelargonium hortorium B.) and jimsonweed (Datura stramonium L.) when applied to Pinto bean leaves inhibited local lesion formation by TMV. This occurred when the extracts were mixed with the inoculum, sprayed on the upper leaf surface after inoculation or sprayed on the lower leaf surface before inoculation of the upper leaf surface. In the latter assay the extracts also inhibited lesion development on the opposite untreated primary leaf. The geranium and pepper extracts were separated into two active heat-stable

fractions; one with a molecular weight greater than 50,000 daltons and one with a molecular weight range of 1,000-50,000 daltons. The inhibitory activity of the jimsonweed extract was found in the higher molecular weight fraction only. The pepper and jimsonweed high molecular weight fractions were still inhibitory when applied to the lower leaf surface even though, presumably, the high molecular weight components would be unable to penetrate the leaf cell membranes. The authors suggested that the high molecular weight components are capable of inducing a systemic resistance and most likely act, not by blocking virus receptor sites,but by altering the metabolism of the cell so that introduced virus particles cannot multiply.

Apablaza (1968) partially purified the fraction from pepper containing material with a molecular weight greater than 50,000 daltons. He isolated a neutral fraction by ion-exchange chromatography which comprised most of the activity and which contained both protein and carbohydrate. Treatment of this fraction with either a protein degrading enzyme or a carbohydrate degrading enzyme resulted in the loss of all inhibitory activity. The author felt that the active factor could be a glycoprotein and that the intact molecule may be necessary for activity.

Inhibitory substances are not always products of a healthy plant. Formation of inhibitors in some plants can be induced by virus infection. Sela et al.,(1966) and Kimmins (1969) have isolated and identified virus inhibitors from virus infected plants.

Sela et al.,(1966) isolated their "antiviral factor" by ion-exchange chromatography and phenol extraction from Nicotiana