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

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Adrian C. Fesser

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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

glutinosa L. leaves infected with TMV. The purified active material was identified as RNA. The activity of the inhibitor was not affected by incubation with trypsin or pronase but was completely lost when incubated with ribonuclease. The procedure of testing consisted of mixing the inhibitor with the inoculum before inoculation. RNA from healthy plants was used as the control. The control showed no antiviral activity.

Kimmins (1969) also showed the virus inhibitor from infected P. vulgaris, C. amaranticolor, N. glutinosa, and D. stramonium was RNA. He isolated a highly purified fraction from the leaves of healthy and infected plants by phenol extraction and partition on a liquid two phase system. The RNA fractions obtained were further fractionated into three peaks on Sephadex G-200. All three types of RNA from healthy and infected D. stramonium, N. glutinosa, P. vulgaris and C. amaranticolor showed inhibition when mixed with TMV and inoculated onto Phaseolus vulgaris var. Prince. The high molecular weight RNAs from both the healthy and infected plants gave approximately the same level of inhibition. The lower molecular weight RNA from the infected plants showed the greatest amount of inhibition. This low molecular weight RNA gave a positive reaction with orcinol reagent and had an absorption spectra characteristic of a mucleic acid. Inoculation with ribonuclease removed all activity, thus confirming the inhibitor was RNA. Kimmins also found activity in the protein fraction of the extracts. It is likely that these proteins are the inhibitors which have been reported by previous workers.

SECTION I

Isolation of Inhibitor Containing Fractions from Pepper Juice

Introduction

Polyphenolic compounds have been shown to inhibit plant virus infection (Cadman, 1959; Hampton and Fulton, 1961), bind to proteins and cause protein precipitation (Loomis, 1969). The presence of polyphenols in pepper juice was indicated by absorption at 340 nanometers. Extracts of pepper leaves darkened upon standing and a brownish precipitate was formed. It was felt that these changes were due to oxidation of polyphenols. To avoid possible interference by polyphenols or their oxidation products the pepper juice was treated with a reducing agent and a phenol complexing agent.

Apablaza and Bernier (1971) isolated a heat-stable inhibitory fraction from pepper which contained material with a molecular weight greater than 50,000. These workers also suggested that there may be more than one inhibitor present in this fraction. The aim of this study was to provide more information on the number and nature of inhibitory substances in the high molecular weight fractions of pepper extract.

Materials and Methods

Preparation of Pepper Extracts Treated with Polyclar AT. The extracts were prepared from pepper plants (Capsicum frutescens L. var. California Wonder) grown in the greenhouse. The leaves and fleshy parts of the stems were ground in a Waring blender with 5 ml of 0.1 M phosphate buffer pH 5.9 per gram of fresh tissue. One half gram of purified Polyclar AT (Loomis, 1969) per gram of fresh tissue was added, with gentle stirring, to the homogenate. The mixture was filtered through four layers of cheesecloth. The same amount of Polyclar AT was added to the filtrate. The mixture was stirred and filtered as described above. The filtrate was centrifuged at 30,000 g for twenty minutes. Another extract was prepared without the addition of Polyclar AT.

One gram of Polyclar AT was added to ten ml of a solution containing 2 mg bovine serum albumin (BSA) per ml. The mixture was centrifuged at 30,000 g for 10 minutes and the supernatant decanted. The precipitate was extracted with 10 ml of distilled water and centrifuged. The supernatants were combined and made to 25 ml with distilled water.

The ultra-violet absorption spectra of the pepper extracts and BSA solutions were recorded on a Cary 15 spectrophotometer.

Sephadex G-75 Gel Filtration of Pepper Extracts. The pepper extracts were prepared as described above using 0.1 M ascorbate in 0.1 M phosphate buffer pH 5.9 as the extracting buffer.

Fifty ml of extract were concentrated to 5 ml by membrane filtration in a Diaflo cell (Amicon Corporation) with a Um-2

membrane which retains material with a molecular weight greater than 1,000 daltons and applied to a 2.6 x 29 cm Sephadex G-75 column. The column was eluted with 0.05 M phosphate buffer pH 5.9 containing 0.2 percent sodium azide and 5 ml fractions were collected. Each fraction was analyzed for protein, carbohydrate, and inhibitory activity. Fractions 10-15 inclusive were pooled and designated fraction A. This fraction was examined by disc electrophoresis.

Ion-exchange Chromatography of Gel Filtered Material. Extraction A from the Sephadex G-75 column was passed consecutively through a 1.1 x 9 cm Rexyn 101 (H^+) and a 1.1 x 9 cm Dowex 10 x 8 (formate) ion-exchange column. The columns were eluted with 40 ml of water and the washings were added to the effluent. This volume comprised the neutral fraction. The cationic column was eluted with 2N hydrochloric acid and the anionic column was eluted with 4N formic acid. These effluents, comprising the cationic and anionic fractions, were neutralized and dialyzed against distilled water.

The anionic, cationic and neutral fractions were freeze-dried and then taken up in 1 ml of water. The fractions were examined on disc electrophoresis. The neutral fraction was tested for inhibitory activity.

Sephadex G-200 Chromatography of the Neutral Ion-Exchange Fraction. Two ml of neutral fraction containing 4 mg of protein was applied to a 2.6 x 4.8 cm Sephadex G-200 column. The column was eluted with 0.05 M phosphate buffer pH 5.9 containing 0.2 percent sodium azide and 5 ml fractions were collected. These fractions were analyzed for carbohydrate and protein. Fractions 25, 26 and

27, fractions 33, 34 and 35, and fractions 44-58 inclusive were each pooled and designated fractions I, II, and III respectively. These pooled fractions were analyzed for inhibitor activity.

Assays. Total sugar content was determined by the method of Dubois et al. (1958) using D-glucose as a standard.

Protein content was determined by the method of Lowry et al. (1951). Bovine serum albumin was used as the standard.

Disc electrophoresis was performed on 7 percent polyacrylamide gels at pH 8.9 according to the procedure of Smith (1968). Samples for electrophoresis were dialyzed if necessary, and freeze-dried. One hundred to two hundred micrograms of protein in 50-100 microliters were placed on each gel. The gels were stained for protein with 1 percent amido black containing 7 percent acetic acid and destained electrolytically. Staining for carbohydrate was done by the Schiff-periodic acid method (Zacharius et al.,1969).

The material for inhibition assay was extracted from unstained gels. The gels were either used immediately or frozen. The desired area was excised with a razor blade using a stained gel as a guide. A piece of equal size, cut from a region which did not stain, served as a control for the assay. The slices were placed in separate small dialysis bags with 1 ml of water. The gel pieces were mashed by gently squeezing the bag. After overnight dialysis against water the suspensions were filtered through glass wool and the clear filtrates were used for the assay.

Inhibitory activity was measured by the local lesion assay method on bean plants (Phaseolus vulgaris L. var. Pinto UI III) with purified tobacco mosaic virus (TMV) as described by Apablaza

and Bernier (1972). The lesion numbers were analyzed statistically by the method of Kleczkowski (1955).

Qualitative analysis of fraction A for polyphenolic compounds was performed using thin layer chromatography on silica gel plates as described by Randerath (1966). The plates were developed with benzene/dioxane/acetic acid (90:25:4). The compounds were detected by spraying the plates with tetrazotized benzidine. Samples for analysis were hydrolyzed in 1N hydrochloric acid for 1 hour at 100°C. The hydrolysate was filtered and taken to dryness under vaccum.

Results

Effect of Treating Pepper Extracts with Polyclar AT. The ultra-violet absorption spectra of a fresh extract before and after Polyclar AT treatment are shown in Figure 1. The absorption at 340 nm, present in the untreated extract is absent in the treated extract. The Polyclar AT also reduced the absorption at 280 nm. No change was found in the absorption spectra of the BSA solutions (Figure 2).

Ascorbate containing extracts treated with Polyclar AT were a light yellow colour and gave little or no precipitate upon storage.

Pepper extracts could only be concentrated tenfold by membrane filtration. Concentrating these solutions further caused precipitation of material.

Isolation of an Active Fraction by Sephadex G-75 Gel Filtration. The concentrated pepper material was separated into two components as shown in Figure 3. The first, fraction A (fractions 10-15) was eluted at the void volume. The second, fraction B (fractions 24-36) only partly shown, was eluted at 2.6 times the void volume.

High levels of activity were found in all the fractions of the first component (Figure 3).

Electrophoresis of fraction A gave several bands which stained for both protein and carbohydrate (Figure 5). The band indicated by the arrow was assayed and gave greater than 90 percent inhibition. Boiling this material for 10 minutes reduced the level

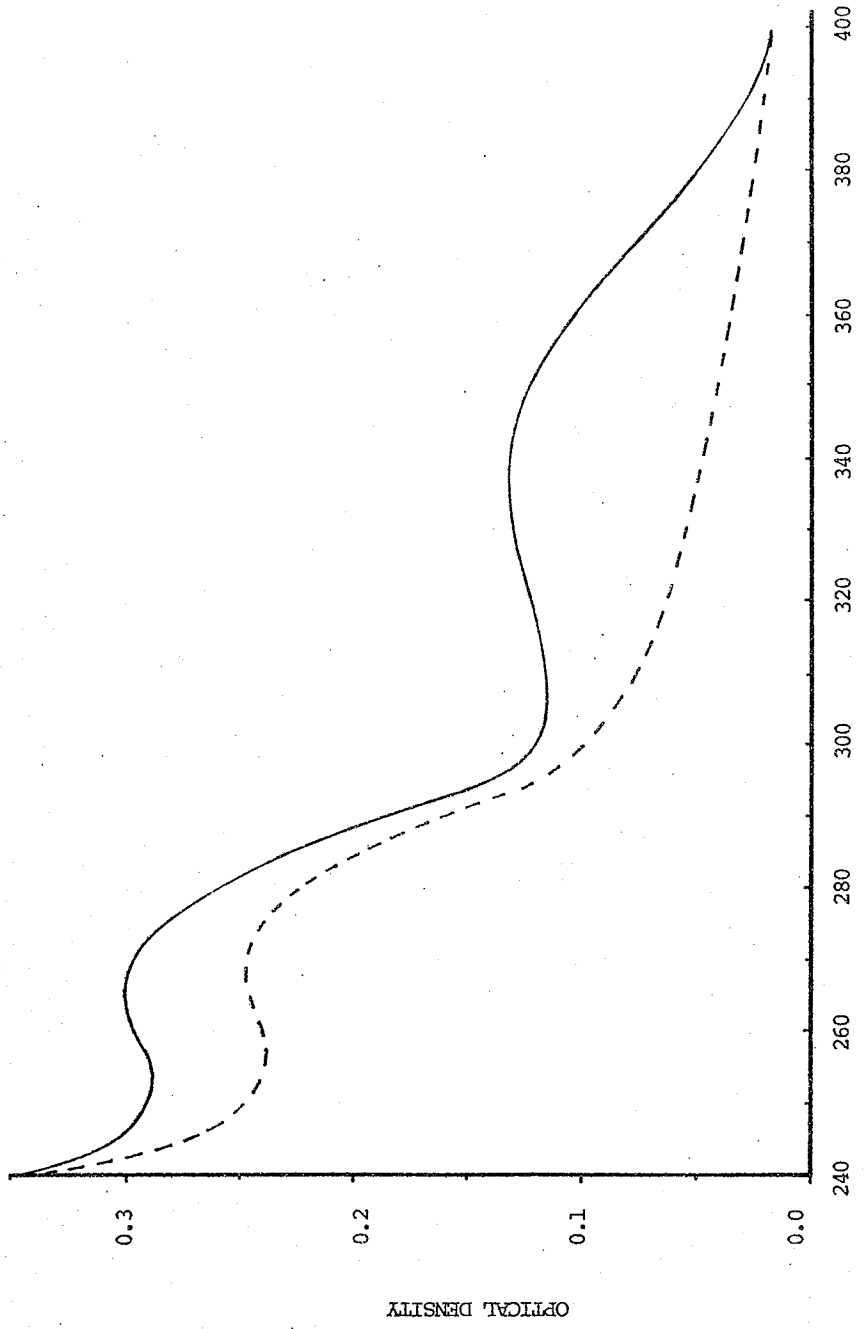


FIGURE 1.) ULTRA-VIOLET ABSORPTION SPECTRA OF FRESH EXTRACT BEFORE AND AFTER EXTRACTION WITH POLYCLAR AT. ——, BEFORE; - - -, AFTER.

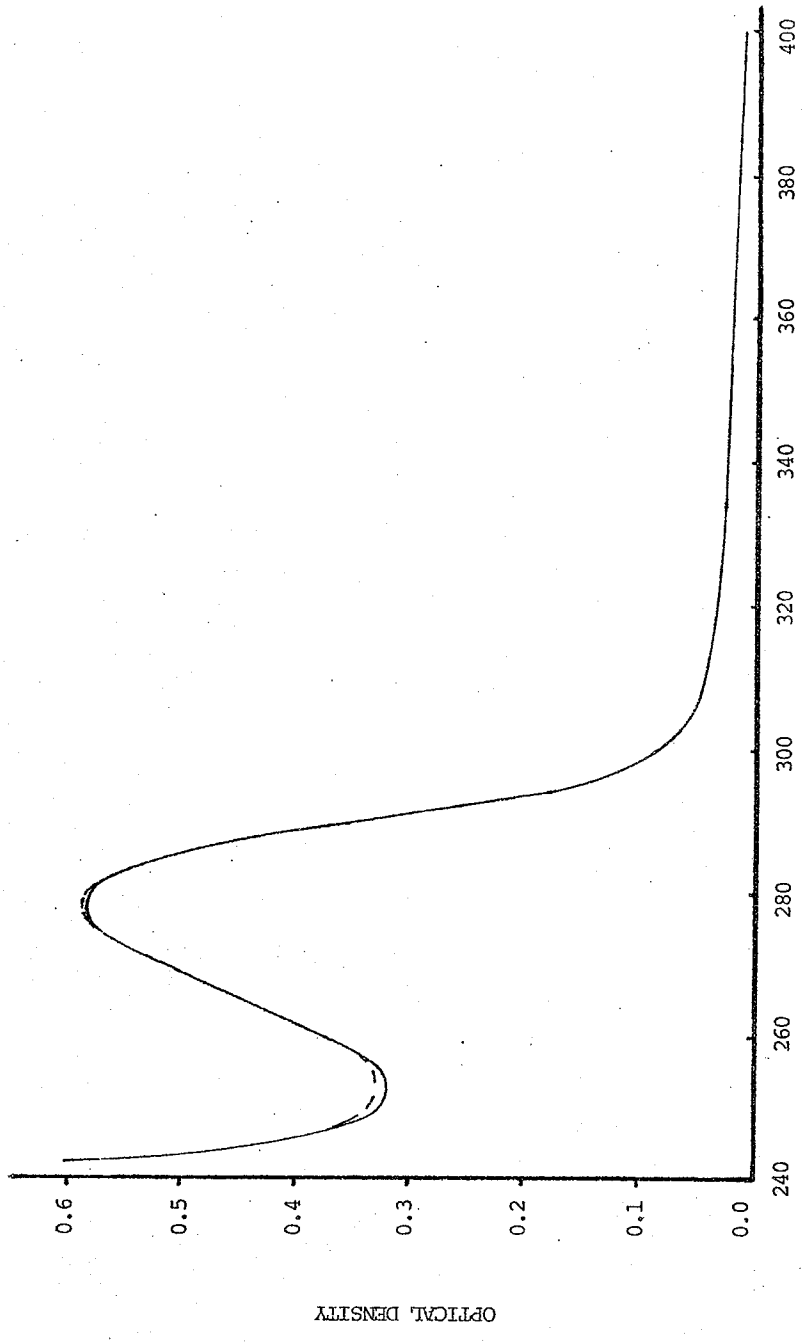


FIGURE 2.) ULTRA-VIOLET ABSORPTION SPECTRA OF BSA SOLUTION BEFORE AND AFTER TREATMENT WITH POLYCLAR AT. ——— , BEFORE; - - - , AFTER.

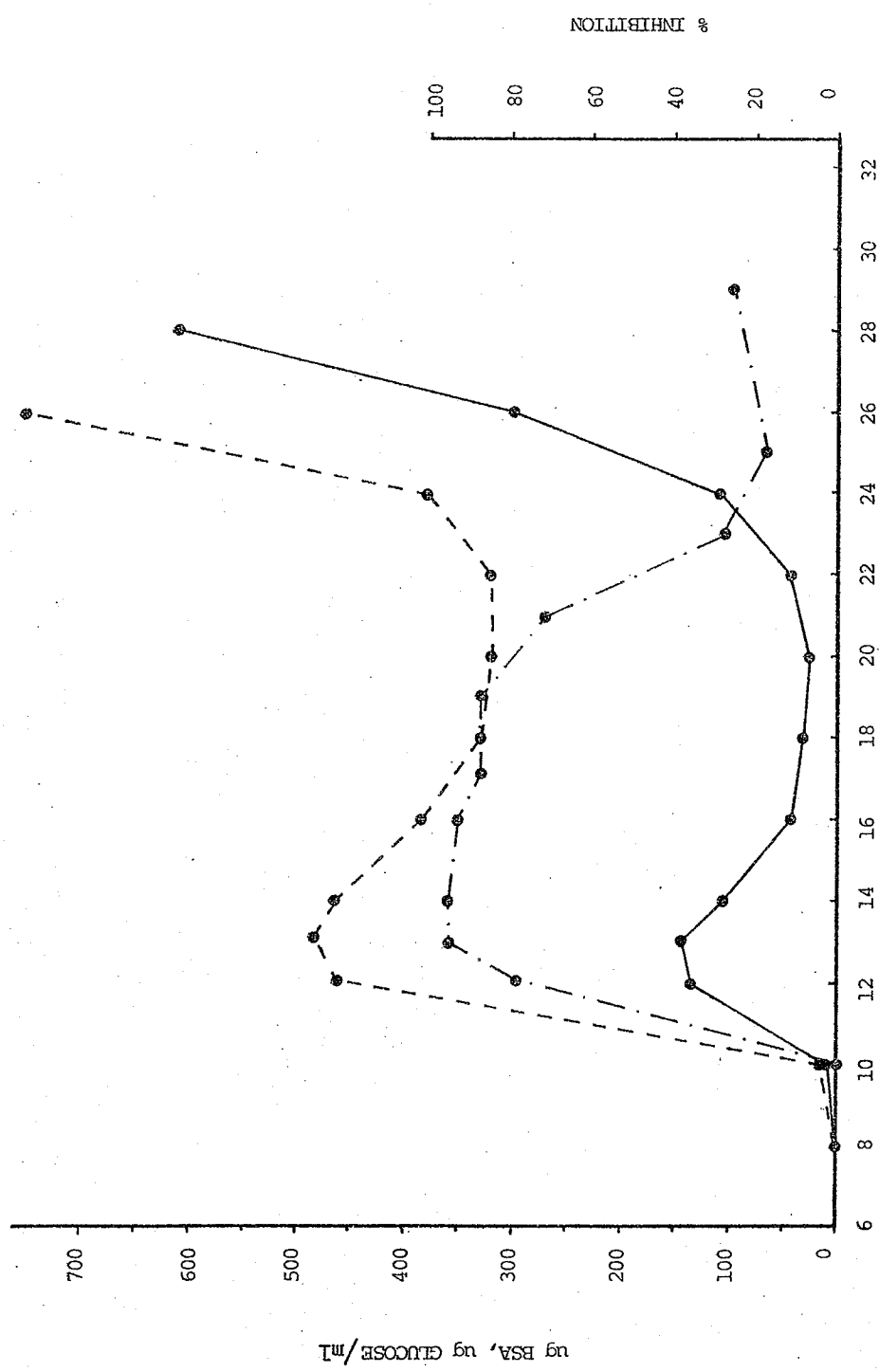


FIGURE 3.) CHROMATOGRAPHY OF PEPPER EXTRACT ON SEPHADEX G-75. ●——●, NEUTRAL SUGARS; ●– –●, PROTEIN; ●– · –●, INHIBITORY ACTIVITY.

of activity to about 75 percent.

Polyphenolic compounds were not detected in the hydrolysate of fraction A material by thin layer chromatography.

Ion-Exchange Chromatography of Fraction A. Ion-exchange chromatography separated fraction A into acid, basic and neutral fractions.

The neutral fraction was highly active and contained protein and carbohydrate in a ratio of approximately 2:1.

The electrophoretic pattern of the ion-exchange fractions were similar to the pattern of fraction A (Figure 5). The electrophoretic pattern of the neutral fraction contained the same number of bands which stained for both carbohydrate and protein as was found for fraction A. The band, indicated by the double arrow, which corresponds to the active band assayed after disc electrophoresis of fraction A also showed greater than 90 percent inhibitory activity.

Sephadex G-200 Gel Filtration of the Neutral Ion-Exchange Fraction. The neutral fraction was separated into three components by Sephadex G-200 gel filtration as shown in Figure 4. The three components were eluted at 1.4, 1.8 and 2.7 void volumes respectively. Fraction I (fractions 25-27 inclusive) and fraction III (fractions 44-58 inclusive) showed moderate levels of inhibitor activity (Figure 4). A high level of inhibition was found for fraction II (fractions 33-35 inclusive). Boiling each fraction for 10 minutes reduced the activity of all the fractions. The level of inhibition activity of fraction II was reduced to approximately 50 percent.

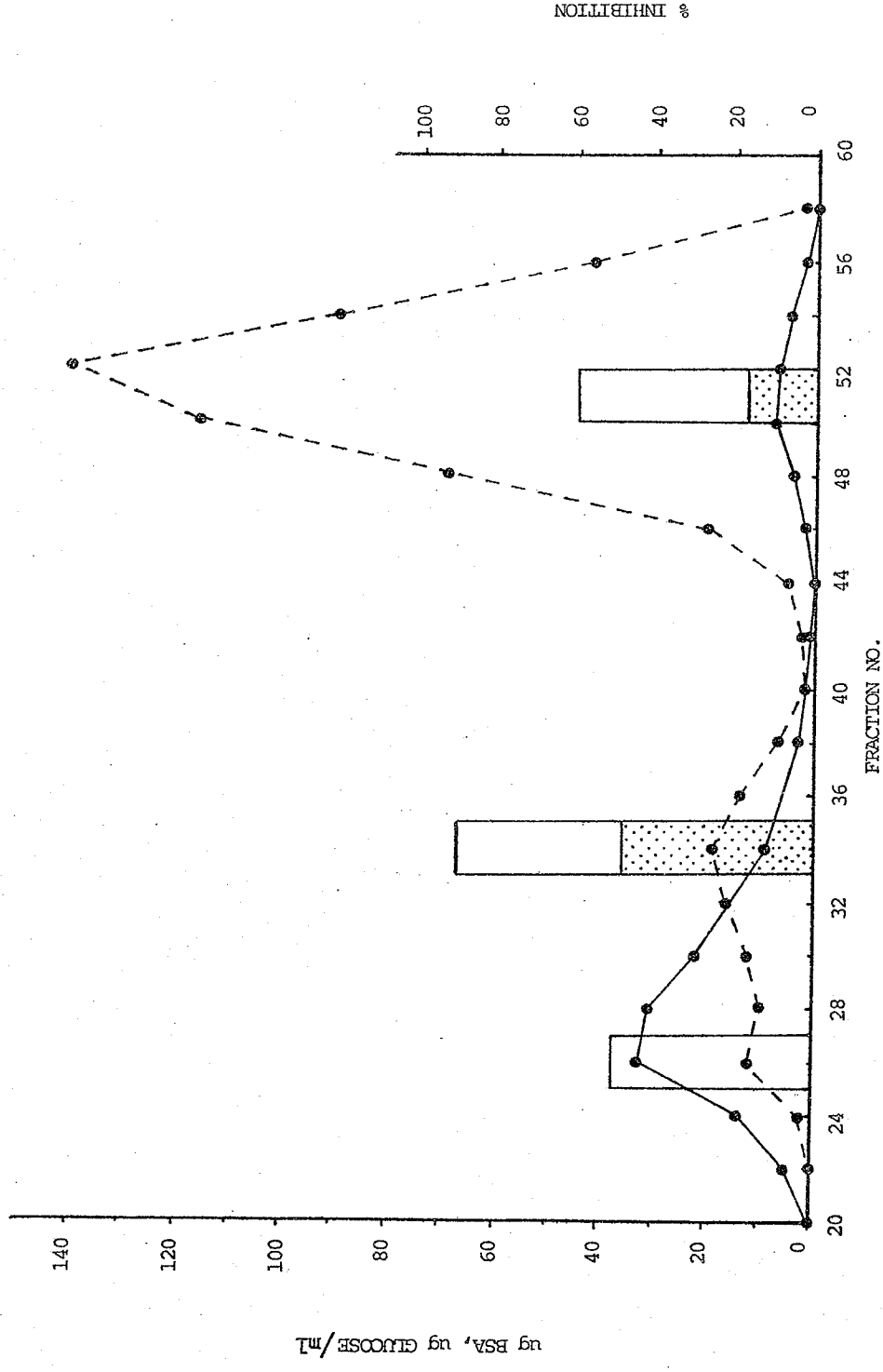


FIGURE 4.) CHROMATOGRAPHY OF HIGH MOLECULAR WEIGHT MATERIAL ON SEPHADEX G-200. ●—●, NEUTRAL SUGARS; ●—●, PROTEIN. INHIBITORY ACTIVITY: ▭, UNBOILED; ⁘, BOILED.

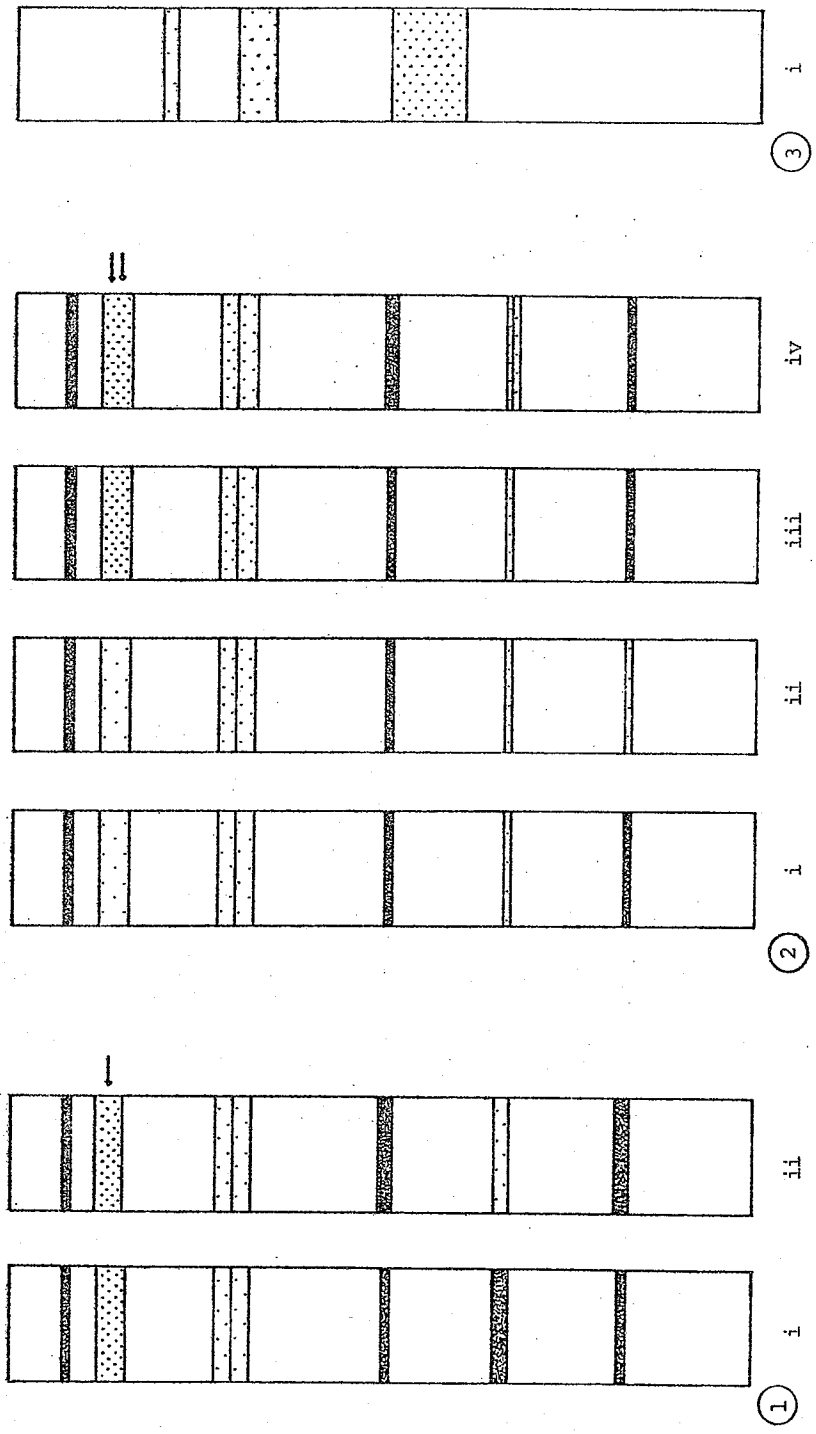


FIGURE 5.) ELECTROPHORESIS OF VARIOUS FRACTIONS. 1) FRACTION A STAINED FOR CARBOHYDRATE (i) AND PROTEIN (ii).
2) ION-EXCHANGED FRACTIONS: ANIONIC FRACTION (i), CATIONIC FRACTION (ii), NEUTRAL FRACTION (iii), STAINED FOR PROTEIN; NEUTRAL FRACTION STAINED FOR CARBOHYDRATE (iv). 3) SEPHADEX G-200 GEL FILTERED FRACTION 3 (i), STAINED FOR PROTEIN. THE BANDS INDICATED BY THE ARROWS WERE EXCISED AND TESTED FOR ACTIVITY.
▭, WEAKLY STAINED; ▩, STRONGLY STAINED.

Electrophoresis of the material in fraction III gave three bands staining for protein.

Discussion

Polyclar AT was effective in removing polyphenolic compounds. The absorption peak at 350 nanometers, representing polyphenols, was not present in the treated juice. Although the treatment also reduced the absorption at 280 nanometers, it was felt that this did not represent a loss of protein since the Polyclar AT had not removed any protein from the BSA solution.

Gel filtration serves a dual purpose. At the same time as the pepper extract is separated into two fractions the unbound polyphenols and ascorbate are removed from the first fraction.

Bound polyphenols were not detected in the material of fraction A and thus it is not likely that the activity of this fraction was due to these components.

The material eluted at the void volume (fractions 10-15) from the Sephadex G-75 column has a molecular weight greater than 50,000 daltons based on the exclusion limit of the column. This fraction contains protein and carbohydrate and high levels of activity were found throughout this fraction. The activity in the carbohydrate-protein region could be due to polysaccharides, proteins, or glycoproteins.

The presence of material which stained for both protein and carbohydrate on the polyacrylamide gels indicated possible protein-carbohydrate complexes. The heat-stable active material assayed after disc electrophoresis may be partially or wholly responsible for the heat-stable activity previously reported by Apablaza and Bernier (1972). The fact that this active material retained its

carbohydrate component after treatment with strong ion-exchangers indicated a covalent complex rather than an ionic one.

The neutral fraction after gel filtration and ion-exchange chromatography contained at least three inhibitors as evidenced by the presence of heat-labile and heat-stable activity in the G-200 fractions (Figure 4). The presence of heat-labile inhibitors has been suggested by previous workers (McKeen, 1956; Apablaza and Bernier, 1972).

There is some loss in the level of activity of fraction II which was assumed to contain the heat-stable material. Since fractions I and II were not well separated, the loss in activity may be due to the presence of heat-labile material from fraction I.

Fraction III appeared to be a protein and would be expected to be heat-labile. This material may correspond to the non-dialyzable heat-labile proteinaceous inhibitor reported by McKeen (1956). Even though disc electrophoresis of this material gave three bands it was felt that the material may be homogenious. The staining and distribution of the bands suggested a monomer, dimer, trimer relationship. No attempt was made to further characterize this fraction.

SECTION II

Isolation and Characterization of a High Molecular Weight Virus Inhibitor from Pepper (*Capsicum frutescens* L.) var. California Wonder.

Abstract

A high molecular weight component that inhibits infection of Pinto bean leaves by tobacco mosaic virus (TMV) has been purified from pepper plants by gel filtration and calcium phosphate chromatography. Polyacrylamide gel electrophoresis of the purified material gave a single band when stained for protein. This band was highly inhibitory when mixed with the virus inoculum. All active fractions and the final product contained both carbohydrate and protein, suggesting that the active component may be a glycoprotein.

Quantitative amino acid and qualitative sugar analyses were performed. The purified product was found to be rich in glycine, serine, and alamine. The major sugars present were galactose and arabinose.

Introduction

Reports of substances in the juice of virus infected plants which affect the transmission of the virus to healthy plants are common in literature (Bawden, 1964; Kimmins, 1969; Nagaich and Singh, 1970). These substances are normal plant components (Bawden, 1964; Apablaza and Bernier, 1972; Apablaza, 1968; Wyatt and Shepherd, 1969; Ragetli and Weintraub, 1962) or can be induced in the plant as a result of virus infection (Kimmins, 1969; Nagaich and Singh, 1970). Some substances such as tannins and oxidisable polyphenols act directly upon the virus and are termed inactivators (Bawden, 1964; Hampton and Fulton, 1961; Cadman, 1959). Other substances which act upon the host plant rather than the virus are termed inhibitors. The majority of the inhibitors are proteinaceous in nature and are considered to cause inhibition by competing with the virus for infectable sites or by altering the metabolism of the cell so that infection and/or multiplication of the virus cannot occur (Bawden, 1964; Apablaza and Bernier, 1972; Wyatt and Shepherd, 1969; Ragetli and Weintraub, 1962; McKeen, 1956).

Pepper contains plant components which inhibit virus infection. McKeen (1956) reported isolating an active component of pepper juice which was heat-labile and non-dialyzable. This active component inhibited lesion formation by cucumber mosaic virus on cowpea when applied either with the virus or to the upper or lower leaf surface before inoculation of the upper leaf surface. The active substance, being non-dialyzable, should not pass through

the cell membrane. Therefore the author suggested that the lower leaf application was inhibitory because the inhibitor caused changes at the epidermal surface which induced alterations in the metabolism of the leaf cells.

Apablaza and Bernier (1972) also working with pepper, used membrane filtration to obtain a fraction containing material with molecular weight greater than 50,000 daltons. This material was found to be heat-stable. It inhibited lesion formation by TMV on Pinto bean leaves when mixed with the virus, sprayed on the upper leaf surface after inoculation, or sprayed on the lower leaf surface before inoculation. In the last case inhibition of infection was also found on the opposite unsprayed leaf which would indicate the induction of a systemic type of resistance. The authors felt that this material would be unable to penetrate uninjured tissue because of its large molecular weight. They suggested that the inhibitor brought about its effect by altering the metabolism of the cell. This fraction was further purified using ion-exchange chromatography (Apablaza, 1968). The greatest activity was found in the neutral fraction. The partially purified product which contained both carbohydrate and protein, lost all activity upon treatment with protein or polysaccharide degrading enzymes. The author suggested that the active component may be a glycoprotein and that the intact molecule was necessary for activity. The partially purified product was still able to induce systemic resistance when sprayed on the lower leaf surface before inoculation.

Few of the non-dialyzable or large molecular weight inhibitors of plant viruses have been fully characterized in chemical nature or

mode of action (Ragetli and Weintraub, 1962; Bawden and Freeman, 1952; Singh et al., 1970). Those that have been are either proteins or polysaccharides. This paper describes the purification of an active carbohydrate and protein containing component of the high molecular weight fraction from pepper. The sugar and amino acid composition of the purified material is also given.

Methods

Preparation of Crude Extract

The extract was prepared from pepper plants grown in the greenhouse or obtained in bulk from truck gardens. The leaves and other fleshy parts of the plant were ground in a Waring blender with 5 ml of 0.1 M phosphate buffer pH 5.9 containing 0.1 M ascorbic acid, per gram of fresh tissue. One half gram of purified Polyclar AT (Loomis, 1969) per gram of fresh tissue was added, with gentle stirring, to the homogenate. The mixture was filtered through four layers of cheesecloth. The same amount of Polyclar AT was added to the filtrate. The mixture was stirred and filtered as previously described. The yellowish supernatant was decanted and the precipitate was discarded.

Purification of Inhibitor

Fifty ml of extract were concentrated to 5 ml by membrane filtration (1,000 molecular weight limit) and applied to a 2.5 x 29 cm Sephadex G-75 column. The column was eluted with 0.05 M phosphate buffer pH 5.9 containing 0.02 percent sodium azide. Five ml fractions were collected and each fraction was analyzed for carbohydrate, protein, and inhibitory activity. The carbohydrate containing material eluted at the void volume (fractions 10-15), were pooled and designated fraction A. Fraction A was examined on disc electrophoresis.

Fraction A was dialyzed overnight against distilled water and then was centrifuged to remove any precipitate. The supernatant was adjusted to 0.01 M phosphate with 0.2 M phosphate buffer pH 5.9. The sample was concentrated to 5 ml by membrane filtration (10,000 molecular weight limit) and then it was applied to a 1.3 x 16 cm calcium phosphate column (Siegelman et al., 1965) previously equilibrated with 0.01 M phosphate buffer pH 5.9. Five ml fractions were collected. The column was washed first with 30 ml of 0.01 M phosphate buffer pH 5.9 to remove any non-adhering material and then with a linear gradient of phosphate buffer, from 0.01 M to 0.5 M. The fractions were tested for carbohydrate and protein. The composition of the carbohydrate containing peaks were examined by disc electrophoresis.

Assays

Total sugar content was estimated by the phenol-sulphuric acid method (Dubois et al., 1958) using D-glucose as a standard. Qualitative analysis of individual sugars was performed using thin layer chromatography. The samples were hydrolyzed for two hours in 1 N H_2SO_4, neutralized with solid $BaCO_3$ and centrifuged. The supernatant was passed through two small ion-exchange columns, Dowex 50 x 2 (H^+) and Dowex 10 x 2 (Cl^-) and then taken to dryness under reduced pressure. The residue was dissolved in a minimum amount of water and spotted on Keeselguhr G plates buffered with 0.1 M phosphate buffer pH 5.0 (Lewis and Smith, 1969). The plates were developed with butanol, acetone, water (4:5:1, v/v/v) and sprayed

with a solution of 2 ml aniline, 2 g diphenylamine and 10 ml of concentrated phosphoric acid in 100 ml of acetone. The sprayed plates were heated at 100°C for 10 minutes.

The total protein content was determined by either measuring the optical density at 280 nm or by using the phenol reagent method of Lowry et al., (1951). Bovine serum albumin was used as the reference standard.

Quantitative amino acid analysis was performed on a Beckman 121 automatic amino acid analyzer. The samples were hydrolyzed with 6 N HCl in evacuated tubes at 110°C for twenty-four hours.

Disc electrophoresis was performed on polyacrylamide gels at pH 8.9 (Smith, 1968). Samples for electrophoresis were dialyzed, freeze-dried and redissolved in distilled water. One to two hundred micrograms of protein in 50-100 microliters were placed on each gel. The gels were stained for protein with 1 percent amido black in 7 percent acetic acid. Staining for carbohydrate was done by the Schiff-periodic acid method (Zacharius et al., 1969). The material for inhibition assays was extracted from unstained gels. The gels were either used immediately or frozen. The desired area was excised with a razor blade using a stained gel as a guide. A piece of equal size was cut from the same gel in a region which did not show any staining. This piece served as the control for the assay. The slices were placed in separate small dialysis bags with 1 ml of water. The gel pieces were mashed by gently squeezing the bag between the fingers. After overnight dialysis against water, the suspensions were filtered through glass wool and the clear filtrates were used for the assay.

Inhibitory activity was measured by the local lesion assay method on bean plants (Phaseolus vulgaris L. var. Pinto UI III) with purified TMV as described by Apablaza and Bernier (1972). The lesion numbers were analyzed statistically by the method of Kleczkowski (1955).

Results and Discussion

The results of gel filtration of the pepper extract on Sephadex G-75 are shown in Figure 1. The fractions 10-18 inclusive, were eluted at the void volume and represent material with a molecular weight greater than 50,000 daltons. Fractions 22-44 inclusive, only partly shown, were eluted at 2.6 void volumes and represent the lower molecular weight materials.

The higher molecular weight material, fractions 10-15 inclusive, contains protein and carbohydrate. The high levels of activity are found throughout this region. The activity in this carbohydrate-protein region could be due to polysaccharides, proteins, or gylcoproteins.

Calcium phosphate chromatography of fractions 10-14 inclusive from the Sephadex column produced three fractions (Figure 2). The first fraction, fraction A′, was eluted off with the wash buffer and contained both carbohydrate and protein. The second fraction, fraction B′, was eluted early in the phosphate concentration gradient and also contained carbohydrate and protein. The last fraction, fraction C′, came off the column as a broad peak and contained mostly protein and a little carbohydrate.

Electrophoresis of fraction 10-14 inclusive from the Sephadex column gave several bands which stained for both carbohydrate and protein (Figure 3). The band indicated by the arrow was excised and gave greater than 90 percent inhibition. Boiling the sample for 10 minutes reduced the inhibition level to about 75 percent. This

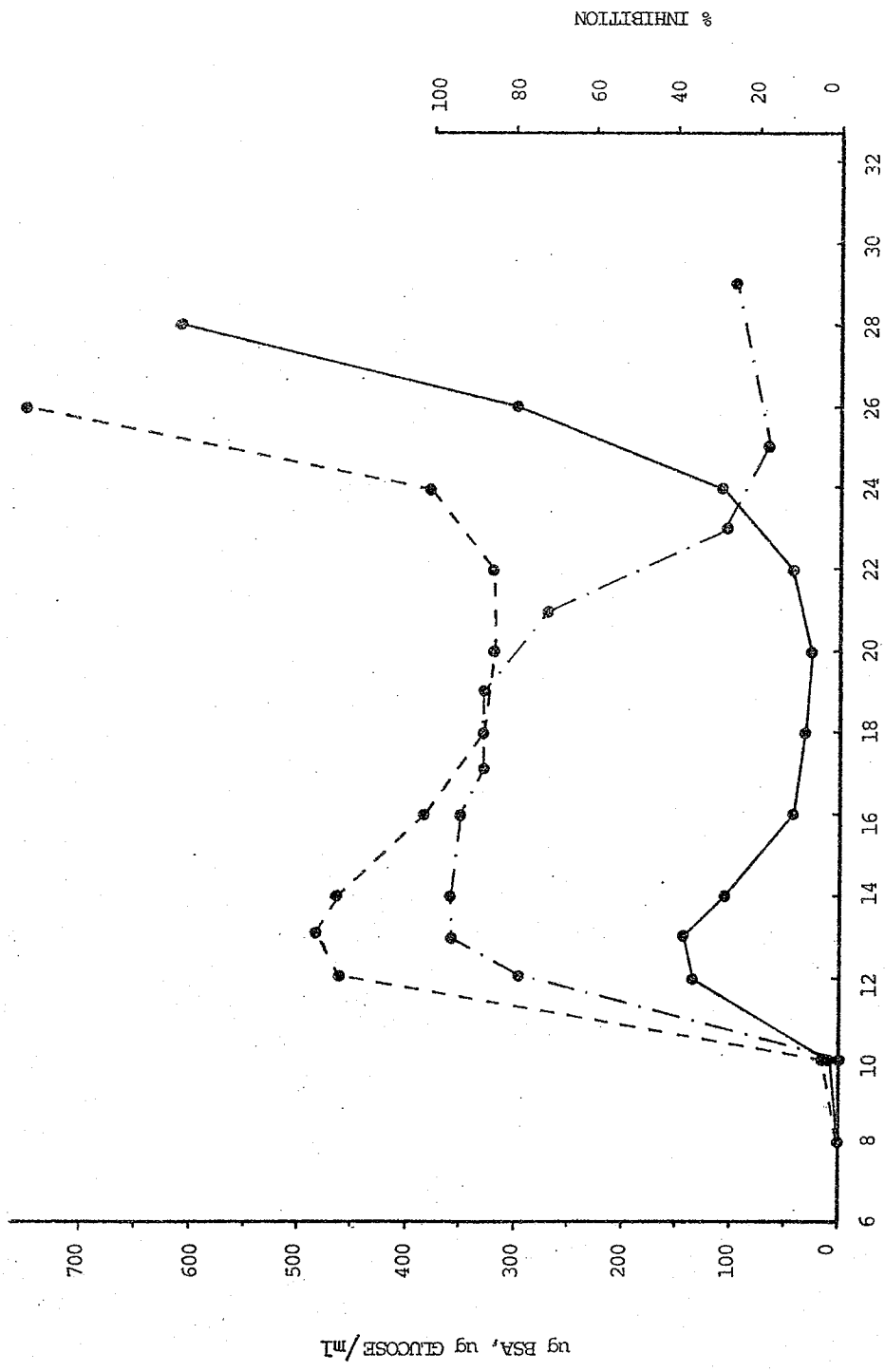


FIGURE 1.) CHROMATOGRAPHY OF PEPPER EXTRACT ON SEPHADEX G-75. ●——●, NEUTRAL SUGARS; ●– –●, PROTEIN; ●— · —●, INHIBITORY ACTIVITY.

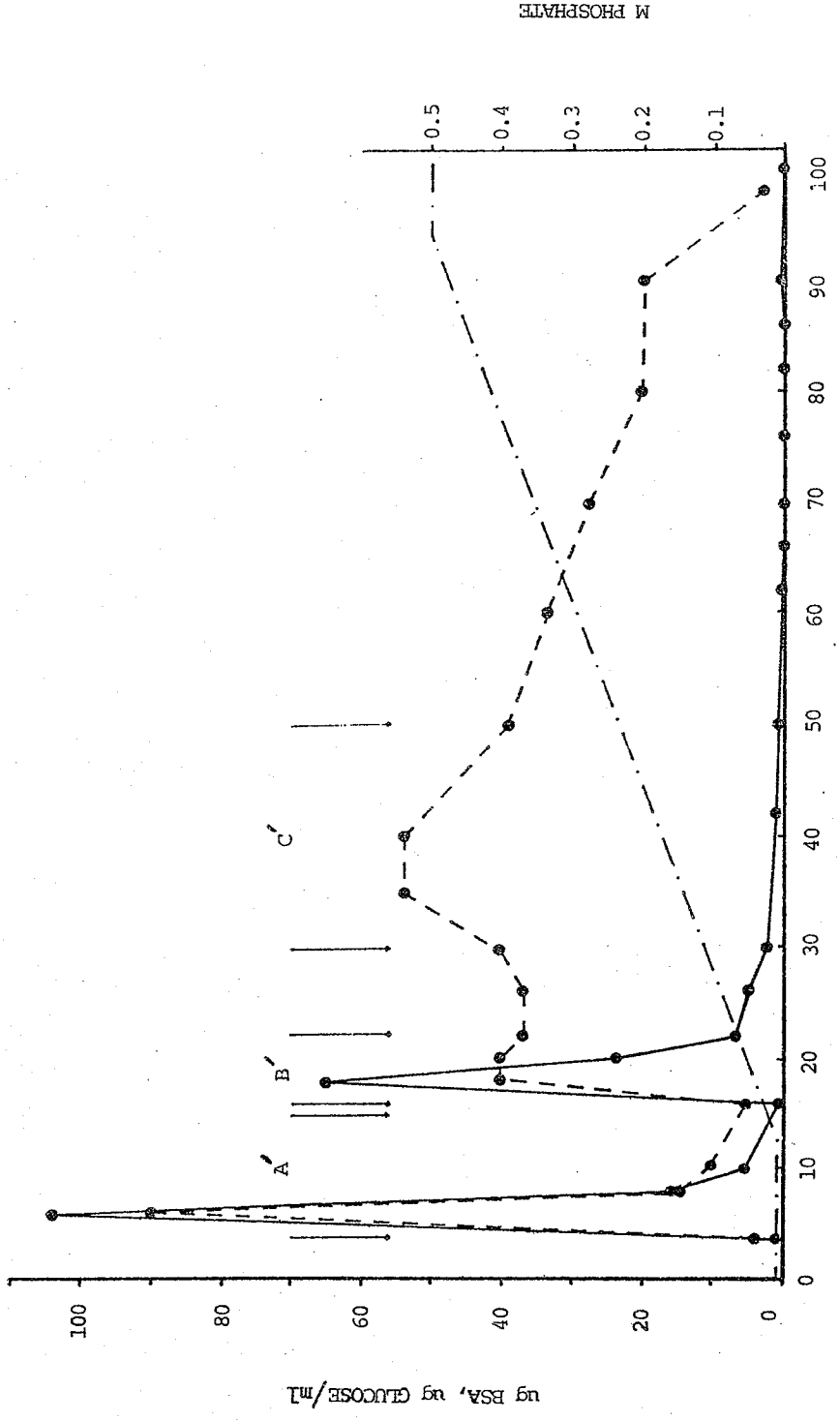


FIGURE 2.) CHROMATOGRAPHY OF HIGH MOLECULAR WEIGHT MATERIAL ON HYDROXYAPATITE. ●——●, NEUTRAL SUGARS; ●- - -●, PROTEIN; —·—, PHOSPHATE CONCENTRATION; ↓ ↓, POOLED FRACTION.

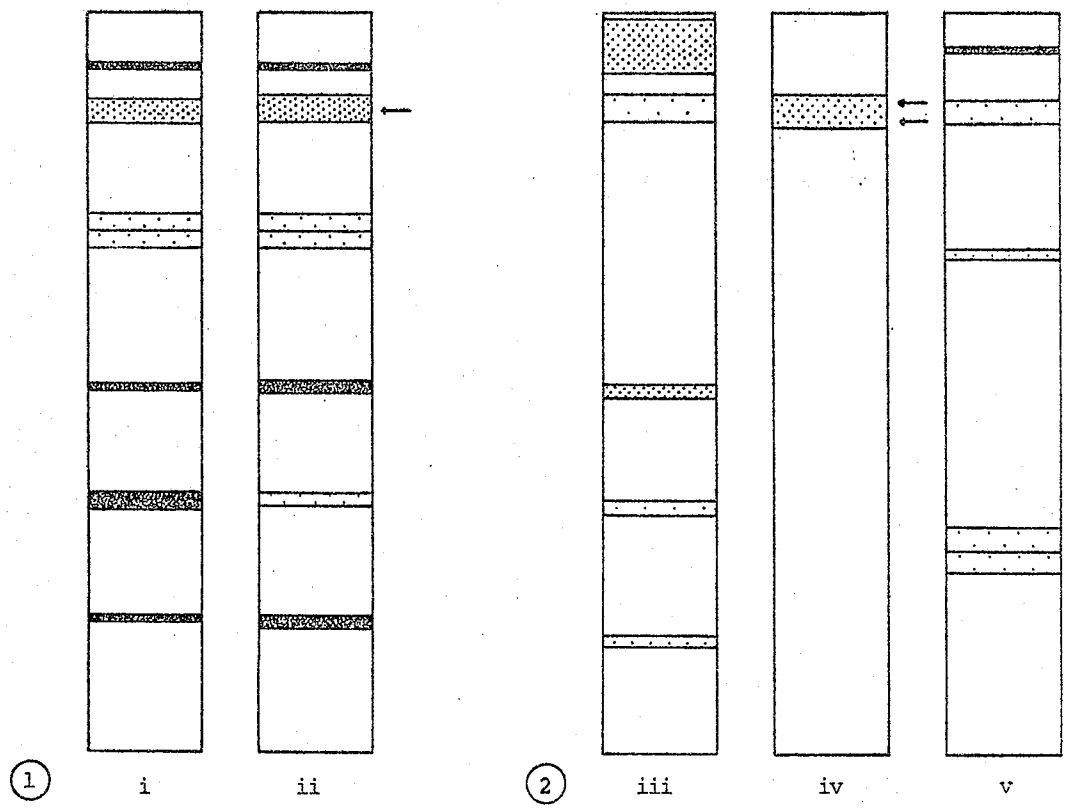


FIGURE 3.) ELECTROPHORESIS OF VARIOUS FRACTIONS. 1) HIGH MOLECULAR WEIGHT FRACTION FROM SEPHADEX G-75 GEL FILTRATION STAINED FOR CARBOHYDRATE (i) AND FOR PROTEIN (ii). 2) FRACTIONS FROM CALCIUM PHOSPHATE COLUMN CHROMATOGRAPHY STAINED FOR PROTEIN; FRACTION A′ (iii), FRACTION B′ (iv), FRACTION C′ (v). ⋯⋯, WEAKLY STAINED; ▒▒▒, STRONGLY STAINED. THE BANDS INDICATED BY THE ARROWS WERE EXCISED AND TESTED FOR INHIBITOR ACTIVITY.

result suggests that this material was one of the heat-stable active components previously reported (Apablaza and Bernier, 1972). The electrophoretic patterns of fractions A′, B′, and C′ from the calcium phosphate column are shown also in Figure 3. Several bands were obtained in the electrophoretic pattern of fractions A′ and C′. A single band was obtained with fraction B′. This band which corresponds to the active band assayed after Sephadex chromatography and disc gel electrophoresis also showed greater than 90 percent inhibitory activity. The apparently homogeneous material of fraction B′ was subjected to quantitative amino acid analysis. The amino acid composition of the pepper material and several other related substances is shown in Table 1. The pepper material is relatively low in aromatic amino acids and relatively high in serine, glycine and alanine. The lysine and methionine content of the pepper material is similar to the pokeweed inhibitor. In addition the pepper material has a high histidine content similar to other glycoproteins. The sugars present in the pepper material were galactose, arabinose and faint traces of glucose and xylose.

Galactose and arabinose are sugars commonly found in glycoproteins of plant and animal origin (Pomeranz, 1971); Neuberger et al., 1966). The inhibitory polysaccharide from T. roseum was found to contain only galactose (Bawden and Freeman, 1952). No attempt was made in this study to determine whether the carbohydrate component of the pepper inhibitor was responsible for activity. Previous work (Apablaza, 1968) using enzymes on the high molecular weight fraction from pepper has indicated that the intact molecule is needed for activity.

TABLE I

COMPOSITION OF PEPPER GLYCOPROTEIN

AND OTHER PROTEINS

Amino Acid	MOLE PERCENT				
	Pepper Glycoprotein	Pokeweed[1] Inhibitor	Soy Bean[2] Hemaggultinin	Kidney Bean[3] Glycoprotein	Ovalbumin[4]
Ala	12.5	7.1	8.6	5.9	9.3
Arg	5.8	3.5	2.6	3.9	4.0
Asp	7.6	12.4	14.0	16.4	8.5
Glu	6.6	8.8	7.5	6.8	13.8
Gly	12.2	7.1	5.8	5.1	5.0
His	2.3	0.9	2.0	1.5	1.9
Leu	4.1	5.3	8.6	5.6	8.5
Ileu	3.0	8.0	5.4	5.0	6.6
Lys	7.9	10.6	5.8	4.0	5.3
Met	4.6	2.7	0.9	1.1	4.2
Phe	1.9	2.7	5.3	5.8	5.6
Pro	3.1	4.4	6.7	2.7	3.7
Ser	14.5	8.0	10.9	14.7	9.5
Thr	8.2	8.8	7.1	8.9	4.2
Tyr	1.4	4.4	2.3	3.4	2.4
Val	4.1	5.3	6.4	9.0	7.4

[1]Wyatt and Shepherd (1969)

[2]Lis et al. (1966)

[3]Pusztai (1966)

[4]Neuberger and Marshell (1966)

Carbohydrate-protein complexes can form in solution but they are not stable. Initially Kassanis and Kleczkowski (1948) described a protein and carbohydrate containing inhibitor, isolated from pokeweed, as a glycoprotein. Recently Wyatt and Shepherd (1969) managed to remove all but less than 1 percent of the carbohydrate from the pokeweed inhibitor using carboxymethyl Sephadex. Material from pepper that has been put through strong anion and cation exchanges retains activity and contains both carbohydrate and protein (Section I, p.22). Retention of both components suggests a stable carbohydrate-protein complex and strengthens the assumption that the material is a glycoprotein.

The mode of action of the inhibitor is not known. The pepper inhibitor is similar to the Pokeweed and trichothecium inhibitor in acting on the host plant rather than the virus. Like the pokeweek inhibitor, it does not inhibit infection in the host species of its origin.

This isolation and purification of a large molecular weight inhibitor from pepper will allow a more exact determination of the protein-carbohydrate nature of the inhibitor and facilitate research on its mode of action.

SECTION III

Inhibitor Analysis

Introduction

A large variation in the number of local lesions produced by replicate leaves was encountered with the Pinto bean-TMV system. A sizeable variation in the value of the average number of lesions per leaf was also found for duplicate treatments. Statistical analysis cannot be applied directly to the lesion numbers for, according to Kleczkowski (1955) their standard errors depend upon their magnitude and their frequency distribution is not normal. The transformation proposed by Kleczkowski (1955) converts the actual lesion numbers to a normal distribution. This transformation compensates for the variation in lesion numbers and allows for a more accurate calculation of the mean. In addition the transformation makes the standard errors constant. Normal statistical analysis can be applied to the transformed data. More uniform results were found when using the transformation. However, the large variation between duplicates still remained. Comparison of the activities of inhibitors from different sources is difficult due to the lack of a standard unit of activity. It was felt that a unit compariable to an LD_{50} unit would be desirable. In the case of an inhibitor (or inactivator) this unit would be the amount of material required for 50 percent inhibition. The activity would be determined by serial dilutions, but the large variations in the inhibition level obtained with the same amount of material indicated that a graphical method was needed in order to obtain a good estimate of the 50 percent inhibition amount.

This work describes the application of Kleczkowski's transformation to the Pinto bean-TMV lesion numbers. Also described is a straight line plot for the level of inhibition as a function of inhibitor concentration.

Materials and Methods

Purification of Virus. The TMV used for these assays was prepared by the method of Gooding and Herbert (1967). The procedure was performed at room temperature. Systemically infected Nicotiana tabacum var. 402 leaves were homogenized in 1 ml/g fresh tissue of 0.5 M. Na_2HPO_4-KH_2PO_4 buffer (pH 7.2) containing 1 percent 2-mercapto-ethanol. The homogenate was strained through cheesecloth. Eight ml of n-butanol/100 ml of extract were added to the filtrate. After coagulation of the chloroplasts, the suspension was stirred for an additional 15 minutes and then centrifuged for 30 minutes at 10,000 g. The supernatant was decanted and retained. While stirring 4.0 g of polyethylene glycol (M. W. 6,000)/100 ml of solution was added to the supernatant. After the PEG had dissolved the solution was centrifuged for 15 minutes at 10,000 g. The supernatant was discarded, the pellet was suspended in 20 ml of 0.01 M phosphate buffer (pH 7.2)/100 ml of initial extract and the solution was centrifuged again at 10,000 g for 15 minutes. The supernatant was decanted, 0.4 g NaCl and 0.4 g PEG/10 ml supernatant was added with stirring. After the NaCl and PEG had dissolved, the suspension was centrifuged at 10,000 g for 15 minutes and the supernatant was discarded. The pellet was resuspended in 2 ml of 0.01 M phosphate buffer for each 100 ml of extract and then centrifuged at 10,000 g for 5 minutes. The milky white supernatant was primarily a solution of TMV. From the final weight of the pellet the approximate concentration of the TMV was calculated to be 108 mg/ml.

Inhibition Assay. Inhibition activity was measured using the quantitative local lesions method with TMV on bean plants. The primary leaves of young plants were dusted with 600 mesh carborundum and then rubbed with a pipe cleaner that had been dipped in the inoculum. The inoculum was prepared by mixing 5 ml of inhibitor solution with 5 ml of TMV solution. In the control inoculum 5 ml of buffer replaced the inhibitor solution. The concentration of the TMV solution was previously adjusted in a separate experiment so that the control inoculum gave approximately 200 lesions per leaf. A set of sixteen leaves were used for each treatment. A separate set was used as the control. The treatments and controls were performed in duplicate. The leaves were harvested after 10-14 days and the lesions counted.

Transformation of Lesion Numbers. The lesion numbers were statistically analyzed by the method of Kleczkowski (1955).

The transformations used were as follows, where X is the lesion number and Z is the transformed lesion number:

$$Z = \log_{10} \tfrac{1}{2}(X + C) \qquad (1)$$

$$Z = \log_{10} \tfrac{1}{2}(X + C + \sqrt{X^2 + 2CX}) \qquad (2)$$

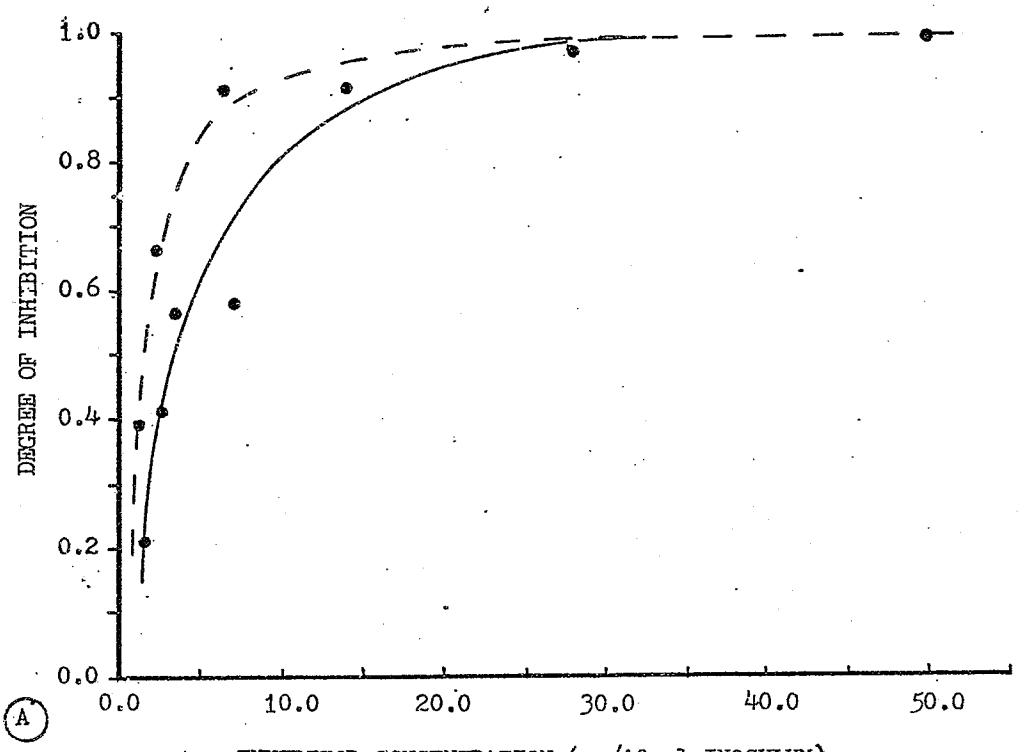
Equation (1) was used when the mean of the untransformed lesion numbers was greater than 10 and equation (2) when the mean was less than or equal to 10. The value of C was determined by plotting the standard errors of untransformed numbers of lesions, whose means are greater than 10, against the means. A regression

line was drawn through the points. The value C is the value of the point of intersection of the regression line and the x-axis.

Results

Application of the Kleczkowski Transformation. The value of C obtained for 51 treatments and controls was 19.12. The slope of the regression line was 0.46 and the correlation coefficient was 0.904.

Plots Relating Inhibition Level to Concentration of Inhibitor. The plots of the two sets of inhibitor data are shown in Figure 1. The first curves, plot A, are non-linear and appear to be sections of a square hyperbola. The second curves are linear. The fit of straight lines to these curves by the least squares method have correlation coefficients greater than 98 percent.



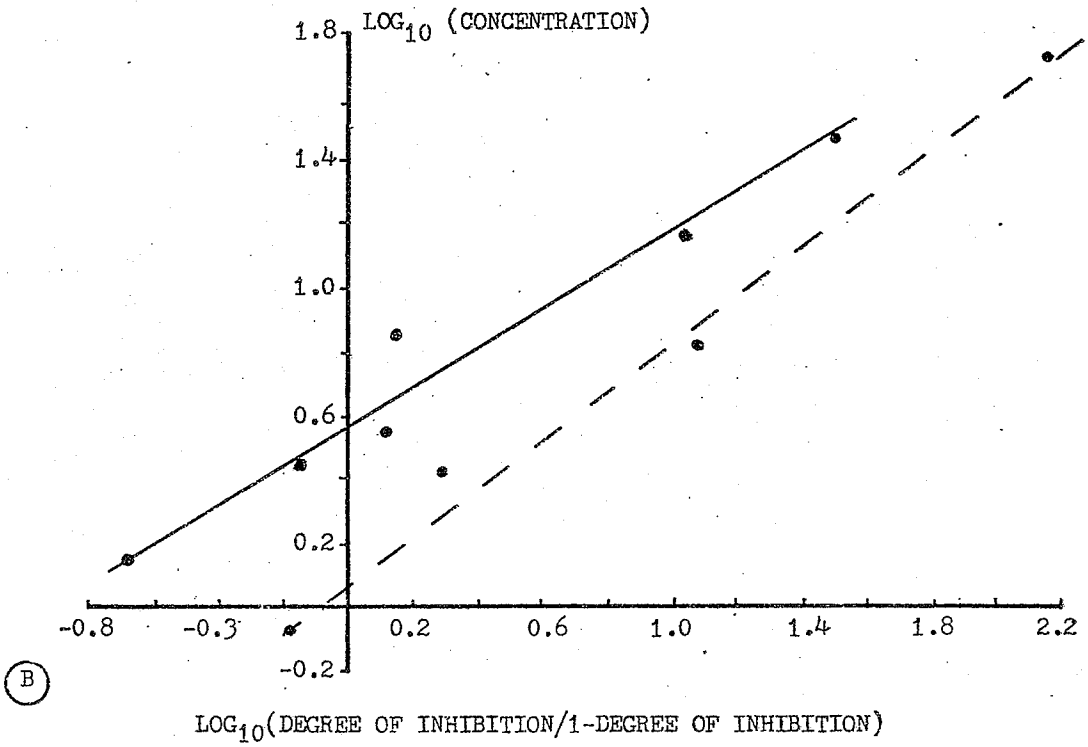


FIGURE 1.) DEGREE OF INHIBITION AT DIFFERENT CONCENTRATIONS OF INHIBITOR FOR TWO PARTIALLY PURIFIED EXTRACTS. ●— —●, MEMBRANE FILTERED; ●——●, GEL FILTERED.

Discussion

The correlation coefficient obtained for the 51 sets of lesion numbers was significant at the 99 percent level. This coefficient indicated a strong uniform dependence of the standard errors on the magnitude of the means as indicated by Kleczkowski (1955). Kleczkowski also found the slope of the regression line in most experiments to be approximately 0.5. The value obtained for the Pinto bean-TMV system was 0.46. These results showed that the lesion numbers for this system were compatible with the transformation. Since the value of C did not have to be determined with any great accuracy, a value of 20 was chosen for simplicity of calculation.

As can be seen form plot A (Figure 1) these curves do not lend themselves to an easy graphical determination of the 50 percent level of inhibition. No doubt with access to computor facilities a good non-linear least squares fit could be applied but for most laboratories only simple calculators are available. The shape of these curves are similar to the curve obtained for complement titration by immune hemolysis (Campbell et al., 1964). As the degree of hemolysis approaches 1 (100 percent), large changes in the concentration of complement results in only small changes in the degree of hemolysis. This is also true for the inhibitor curve. Similarly the straight line relationship between concentration and degree of hemolysis using a logarithmic transformation is also true for the inhibitor curves. Plot B gives good straight lines as evidenced by correlation coefficients being greater than 98 percent. Only

simple calculators are needed to fit least square straight lines.

The slopes of the two lines in plot B are slightly different. This difference may have been due to random errors or to the presence of extra inhibitors in the membrane filtered fraction. An estimate of the 50 percent inhibition amount can be calculated from a single experiment using an average value for the slope. This value would only be a rough estimate since single experiments show large deviations but it would be useful in deciding how much dilution is required to bring the inhibition level down to the region of 50 percent inhibition.

The 50 percent inhibition amounts for gel filtered and membrane filtered materials were 3.64 ug and 1.18 ug respectively. The activity of the gel filtered material was 275 units/mg protein and the activity of the membrane filtered material was 847 units/mg protein.

General Discussion

The activity in the high molecular weight fraction isolated from pepper juice has been shown not to be a result of protein bound polyphenols. While in contact with the proteins, oxidation of the polyphenols was prevented by ascorbate in the extraction buffer. Complete separation of the polyphenols and proteins was achieved by Polyclar AT treatment and gel filtration. The absence of polyphenols in the hydrolysate of the high molecular weight fraction is proof that these precautions were effective.

The heat-labile material in two of the fractions eluted from the Sephadex G-200 column confirm the supositions put forth by Apablaza and Bernier (1972). They suggested that there may be other inhibitors present in pepper that are heat-labile. They also suggested that these inhibitors may be responsible in part for the discrepency between their work on pepper and McKeen's work on pepper. However, the heat-labile inhibitors reported in this work may not be the inhibitor detected by McKeen. It remains to be shown that the inhibitors active in the Pinto bean-TMV bioassay are the same as the inhibitors active in the cowpea CMV system and if they are the same, to what extent their activity varies from bioassay to bioassay. These questions can be answered using the purified fractions and the graphical method of activity analysis described in this thesis.

The material isolated in a pure form by calcium phosphate chromatography is most likely the heat-stable high molecular weight described by Apablaza and Bernier (1972) and Apablaza (1968). There is strong evidence that this material is a glycoprotein.

All active fractions and the purified inhibitor contained carbohydrate and protein. Furthermore the stability of the active material towards strong ion-exchange resins indicates a covalent rather than an ionic complex.

The purified glycoprotein had an amino acid composition similar to glycoproteins from other sources and to a proteinaceous plant virus inhibitor from pokeweed. The sugars present in the pepper glycoprotein are also found in the other glycoproteins and in a polysaccharide from Trichothecium which inhibits plant virus infection. These findings suggest that the activity of the inhibitor could be either in the protein moiety or the carbohydrate moiety or possibly both. Previous work by Apablaza (1968) indicated that the intact molecule may be required for activity. No attempt was made to confirm this point in this study. This is an important point and should be examined carefully with a view towards establishing the identity of active agent in each bioassay method. The moiety that is responsible for activity in the mixed inoculum assay may not be the same as that responsible for activity when applied to the underleaf surface.

Apablaza and Bernier (1972) reported that their high molecular weight fraction was systemically active when applied to the lower leaf surface before inoculation of the pepper surface. Whether this was true for the purified glycoprotein was not tested. However, the high molecular weight gel filtered material from which the glycoprotein was purified was tested and was found to be systemically active. This should reside in one (or all) of the fractions from the column.

It is important to bioassay the inhibitor from the acrylamide gels. Without the assay it would be difficult to say that the material

visualized on the gels represents the active substances contained in a sample. A polysaccharide could be responsible for activity in the sample and, as a non-charged molecule, would not migrate into the gel. In this case, to say that the activity of the sample is due to the proteins present in the stained gel, would be false. The same would be true of a highly basic protein which would migrate in the opposite direction even at the high pH of the electrophoresis system. In this research it was shown that activity resides in the gel. This does not eliminate the possibility that part of the activity of the sample may be due to other components as mentioned above. To do this it would have to be shown that all the activity applied to the gel was present in the excised band. In this research it was felt that demonstrating that the sample and excised band contained the same level of heat-stable activity was sufficient evidence to say that material present in the excised band was responsible for the activity in the sample.

The graphical method of activity analysis was only applied to two partially purified pepper materials. The bioassay, in general, suffers from the fact that relatively large amounts of inhibitory material are needed. The necessity of having to do a dilution type analysis compounds the problem. The problem can be reduced by using an average value of the slopes already determined for the two partially purified fractions. Thus when analyzing pepper fractions on the Pinto bean-TMV system it will be possible to calculate an approximate concentration value for 50 percent inhibition from a single experiment. As a result the number of dilutions and the amount of material required will be reduced.

Application of Kleczkowski's equations (Kleczkowski, 1955) to the Pinto bean-TMV virus-host system has not only provided a good statistical basis for evaluating local lesion data but also provided a better estimate of the average number of local lesions per leaf. The number of local lesions produced by different leaves inoculated with the same inoculum does not follow a normal distribution (Kleczkowski, 1955) and as a result the simple arithmetic mean does not represent the most frequently observed value (mode). In this case the chances of observing a value equal to the arithmetic mean in a single experiment are less than for the mode value. Consequently the mode value is a better representation of the average number of local lesions per leaf. Normally the mode value is difficult to estimate but with the Kleczkowski transformation the mode value of the untransformed data corresponds to the arithmetic mean value of the transformed data. Reversing the transformation will give the mode value from the mean value. All the levels of inhibition given in this work were calculated using average lesion values obtained in this manner.

Summary

Pepper extract was separated into several active fractions by gel filtration and ion-exchange chromatography. Two heat-labile and one heat-stable fractions were found. These fractions may contain inhibitors reported by other workers.

An active material which gave one band on disc electrophoresis was purified by gel filtration and calcium phosphate chromatography. The material was low in aromatic amino acid and high in lysine and methionine. The sugars present in this material were galactose and arabinose.

The local lesion assay was found to be suitable for statistical analysis by the method of Kleczkowski. A plot was found which allows for the determination of specific inhibitory activity and the detection of different inhibitors.

Suggestions for Further Work

Further work into the exact nature of the pepper inhibitor is needed. The inhibitor appears to be a carbohydrate-protein complex but further confirmation is needed.

It remains to be conclusively shown that the intact molecule is required for inhibition. Because of its resemblance to other glycoproteins, it would be interesting to test the inhibitor for phytohemaggtuinin activity.

Further work could also be done on purifying the other inhibitors present in pepper and determining their 50 percent inhibition level of activity.

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