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Multiple antiviral components from an extract of Datura stramonium

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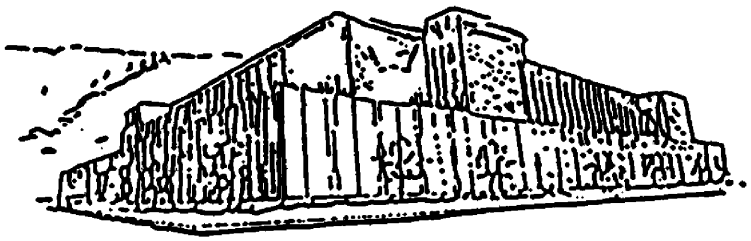

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Multiple Antiviral Components from an Extract of *Datura stramonium*

by

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Multiple Antiviral Components from an Extract of *Datura stramonium*.

Committee Chairman: Dan C. DeBorde

The objective of this project was to further characterize the compound(s) from *Datura stramonium* leaves responsible for antiviral activity. A variety of protein purification techniques were explored in the isolation of the active material. The purification steps finally established as useful were (in this order): 60-80% ammonium sulfate precipitation, dialysis, heat-treatment at 60°C, and chromatography on DEAE-cellulose followed by Sephadex G-200. The first peak eluted (following the void volume peak) from the G-200 column contained the majority of the antiviral activity. This peak was further characterized by SDS-PAGE, tested for RNase activity, and digested with trypsin. All antiviral fractions contained a protein of ~17,000 MW and the antiviral activity was destroyed by trypsin. The initial fractions from this peak also contained RNase activity, while the remainder of the fractions were only antiviral. The heat-treated extract was also fractionated using centrifugal filters having 100K, 30K, and 10K molecular weight cut-offs. These fractions were characterized by SDS-PAGE, and tested for antiviral and RNase activity. The +10,000 (10,000-30,000 MW), +30,000 (30,000-100,000 MW), and +100,000 (above 100,000 MW) molecular weight fractions were antiviral, while only the +30,000 and +100,000 fractions showed RNase activity. Data suggest the antiviral material is present as a ~87,000 MW complex which is not precipitated by heat. The data suggest the antiviral complex is composed of proteins of ~17,000 MW and ~12,000 MW, an antiviral protein of unknown size, and an RNase-active protein. Future studies of the material present in the complex should help to elucidate the nature of the components present.

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INTRODUCTION

The use of plants to treat disease has been a common practice since the beginning of history. Natural medicinals have been used to treat diseases from cancer to warts with varying degrees of success. Some of these (such as quinine, opium, and digitalis) have found their place in modern medicine. The **CRC Handbook of Medicinal Herbs** lists 365 plants of medicinal use world-wide and the **Medicinal Plants of the Mountain West** contains references to 120 medicinal plants found in our geographic area. This number of plants represents only a small fraction of the 265,000 different species of plants which are available for study and testing.

It stands to reason that of these many plants a percentage of them would have antiviral (viricidal or virustatic) components. Upon testing this turns out to be the case (1,8,22,25).

The discovery of Pokeweed Antiviral Protein (PAP) in 1925 signalled the beginning of a new area of research, plant antivirals. Sap from pokeweed reduced the infectivity of TMV when mixed together and inoculated onto a TMV-susceptible host. This sap was further purified and a protein was isolated which would inhibit TMV and a variety of other viruses from plants and animals (8). The discovery of PAP led to research into other plants, and eventually this led to the discovery of many other antiviral compounds from plants. Further testing would show that these

antivirals functioned by a number of different mechanisms.

Plant compounds function in a wide variety of ways to achieve their antiviral effect. Photosensitizers function in concert with ultraviolet light to cause damage to viral nucleic acids. This damage is accomplished by one or more of the following: DNA/protein cross-linking, guanine oxidation, or singlet oxygen production (8). Terpenoids function as antivirals at the level of the membrane, either by interference with the adsorption/penetration of the virus or at the time of assembly and/or release (8). Lignans affect viruses at some early step of their infection, while phenolic compounds appear to function at various key steps during the virus life cycle (8). Proteins and peptides have activities ranging from interference with early steps of the virus infection to exerting their effects at the level of protein synthesis (8).

Antiviral Proteins from Plants

Induced Antiviral Proteins

The induced antiviral proteins (IAVPs) are produced as a result of a viral infection, and therefore are not always present (3-7). These proteins can exert their effect at a distance from the site of infection, and upon viral challenge show marked inhibition. Examples of these proteins are antiviral factor (AVF) extracted from tobacco mosaic virus infected leaves of *Nicotiana glutinosa*, inhibitor of virus replication (IVR) found in the culture

supernatant of TMV-infected tobacco protoplasts, and antiviral substance (AVS) which is also found in the culture medium of infected tobacco protoplasts (7,8,25).

Sela and Applebaum were the first to describe the production of a substance in virus-infected plants which when isolated would protect new plants from infection (26). Sap from infected leaves was found to inhibit local lesion production when mixed with virus and inoculated onto healthy leaves. The sap from uninfected leaves was found to be non-inhibitory. They postulated that the inhibitory substance was being produced as a result of virus infection and suggested that its activity might be similar to the action of interferon observed in animal tissues (26). Further work showed that AVF was present in infected tissue, and could be isolated using methods similar to those used for the isolation of interferon from human leukocytes (25). Further work by Sela demonstrated that AVF was present in minute quantities in uninfected tissues, and that virus infection was directly responsible for the production of AVF in larger quantities (25).

Gera et al. showed that infection of protoplasts from a hypersensitive strain of tobacco with TMV caused the release of an inhibitory substance into the protoplast culture medium (5,25). This substance, inhibitor of virus replication (IVR), was found to inhibit virus replication in protoplasts isolated from both hypersensitive and systemic

responding varieties of Samsun tobacco. IVR was found to be inhibitory when applied up to 18 hours after infection with TMV (5,7).

Mitra isolated two antiviral substances (AVS) from the tissue culture medium of TMV infected protoplasts (AVS-30 and AVS-65) (25). AVS-30 was found in the medium of both infected and uninfected protoplasts and was believed to be present as an endogenous inhibitor, whereas AVS-65 was produced as a response to TMV infection. AVS-65 was able to transfer resistance to TMV infection to protoplasts in a manner reminiscent of interferon action (25).

Endogenous Antiviral Proteins

Endogenous antiviral proteins (EAVPs), in contrast, are always present within the plant. These proteins did not appear to exert their antiviral activity within the source plant from which they were extracted, because no obvious protection was observed. However, these proteins were antiviral in other plant species upon external or mechanical inoculation (8-11) under laboratory conditions, and were termed 'altruistic'. Further research with Pokeweed antiviral protein (PAP) has shown that this 'altruistic' effect may not be the case (25).

The EAVPs can be classified into three general categories based upon their mode of action: proteins that inhibit or inactivate eukaryotic ribosomes, proteins that

induce an antiviral response, and proteins that prevent the establishment of a viral infection by some mechanism other than those just described.

Pokeweed antiviral protein (*Phytolacca americana*), Gelonin (*Gelonium multiflorum*), and Dianthin (*Dianthus caryophyllus*)are included in the category of ribosomal inhibitory proteins (RIPs). RIPs are capable of inactivating the large 60S subunit of eukaryotic ribosomes and the antiviral effect(s) may be dependent upon this inactivation (8,9,13,14). The mechanism of inhibition exhibited by PAP has been shown to be catalytic in nature, implying that virus replication may be prevented as a result of interference with the host ribosomes ability to bind elongation factors (14). The inhibitor, entering the cell during mechanical inoculation along with infective viral RNA, inactivates the 60S subunit of the ribosome and consequently stops viral multiplication. This results in an aborted viral infection (14). When PAP was tested against herpes simplex and poliovirus *in vitro*, it was found that the activity was dependent upon the interaction of the virus with the cellular membrane. This interaction facilitated the entry of PAP into the cell with subsequent inactivation of the host cell ribosomes and cessation of protein synthesis (8,15).

The mechanism by which ribosomes are inactivated *in vivo* is thought to be due to the *N*-glycosidase activity of

RIPs. This activity breaks the *N*-glycosidic bond at adenine^{4324} in the 28S rRNA which allows cleavage of the RNA by aniline *in vitro*. This cleavage is responsible for the inability of ribosomes to bind elongation factors (EF1 and EF2) and the resultant inhibition of protein synthesis (14).

The second mode of action is by the induction of a systemic antiviral response (systemic resistance). The treatment of one portion of the plant results in either the entire plant mounting an antiviral response *(Boerhaavia diffusa)*(16,17), or a response which is restricted locally to the treated leaf (*Chenopodium ambrosoides*)(18). The action of the inhibitor may be attributed to the blockage of the host cell receptor or to interference with virus synthesis within the host cell. The actual mechanism by which the inhibition occurs has not been elucidated.

Proteins which prevent the establishment of a viral infection fall into the last category. The antiviral effect is of a localized nature, causing the inhibition of virus in treated areas only. Extracts of *Datura* have been shown to cause this type of resistance (11) as well as extracts from other solanaceous plants (21) and various plant lectins (8).

It was previously thought that *Datura stramonium* extract inhibited the establishment of virus in a localized reaction by competing for available receptors (10,11,19,20). However, other studies found no evidence of virus-specific receptors on the plasma membrane surface (31). This

evidence suggests that interference with the virus may occur at a different stage in the infection.

It is possible that this interference is due to degradation of the infective nucleic acids. Research by Saxena and colleagues shows that ricin (antiviral protein from *Ricinus communis*) will abolish protein synthesis in *Xenopus* oocytes when either injected or added to the oocyte incubation medium. The RNA glycosylase activity of ricin removes a single base adjacent to the alpha sarcin site from the 28 S RNA in *Xenopus* oocytes (28). Therefore, it is possible that the antiviral activity observed with other plant antiviral proteins could be a result of RNase-like activity.

Datura stramonium has been used for centuries throughout the world for the treatment of cancers, tumors and indurations. In China it is used as a treatment for flatulence, hyperacidity, and tuberculosis sweats; in Mexico for birthing pains; in Costa Rica for tumors and ulcers; and in Africa (by the Arabs) for the treatment of influenza and asthma (1). In the United States, this plant (leaves) was used for the treatment of asthma, as an analgesic and anti-inflammatory, and also as a hallucinogen in Native American religious ceremonies (2). The widespread use of this plant as a medicinal suggests that this plant may contain compounds which could be used therapeutically, some of which may prove to be antiviral.

Previous studies have shown that extracts of *Datura* contain an endogenous inhibitor that when mixed with virus and inoculated onto a permissive host exerts an antiviral effect (9,10,11). Apablaza and Bernier showed that an extract from *Datura* leaves was inhibitory against TMV when tested in a pinto bean local lesion assay. This activity was heat-labile (100°C for 10 minutes) and was retained by a 50,000 MW cutoff filter (10). Grasso and Shepherd found that upon purification by ion-exchange chromatography, an inhibitor from *Datura* gave an intense double band of 17,000 MW when visualized by SDS-PAGE (9). Using a partially purified extract of *Datura*, Zipf determined that the inhibitory activity was pH stable (pH 3-10) and was not reduced upon treatment with papain, acid phosphatase, formic acid, cysteine, or performic acid. The inhibitory activity was not affected by heating at 65°C-70°C for 10 minutes. Antiviral activity was reduced upon treatment with trypsin (10%), acetic anhydride (50%), and phenyl isocyanate (70%) (11).

Purpose of study

The purpose of this study was to further characterize the antiviral compound(s) from *Datura stramonium* leaves. The purification was accomplished by ammonium sulfate precipitation of leaf homogenate and chromatography on a DEAE-Cellulose column followed by a Sephadex G-200 column. This material was characterized by agarose and acrylamide

gel electrophoresis, and molecular weight fractionation. The antiviral activity was evaluated with a Pinto bean/Tobacco Mosaic Virus inhibition assay.

MATERIALS and METHODS

Buffers: The buffer used throughout these experiments was a 0.067M sodium phosphate dibasic/potassium phosphate monobasic buffer at pH 6.0, pH 7.0, or pH 7.9. Buffer species were made as 10X solutions and stored at room temperature (18-22˚C). For use, buffer species were diluted 10-fold with H_2O and mixed together until the desired pH was reached.

Growth of plants: The pinto bean plants (*Phaseolus vulgaris* var. Pinto) were grown in Peat Lite mix (Grace Sierra, Ca.) in a temperature-controlled growth room in a greenhouse. Two seeds were planted per 4" plastic pot and watered when the soil became dry (approximately every 2 days). *Datura* seeds were planted in plastic flats (24"x12"x2") and grown to the 2-leaf stage after which they were transplanted to individual pots. All plants were grown in a 16 hr light/8 hr dark photoperiod at 21˚C to 27˚C (70˚F to 80˚F). Pinto beans were used at 9-11 days (primary leaf stage), and the *Datura* leaves were harvested at maturity and stored at -20˚C until needed.

Protein content: The protein content of samples was determined by the use of the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, Il.) by either the tube or microplate

method as described in the literature supplied with the kit. Readings were taken at A_{565} for the tube method and at A_{650} for the microplate method. Where specified, the Coomassie Brilliant Blue Protein Assay Reagent Kit (Pierce, Rockford, Il.) was used to assay for protein content. In all cases a standard curve was generated by diluting BSA (2 mg/ml) to known concentrations and assaying under the conditions dictated by the assay method. By comparing the absorbance of each fraction with the standard curve, a protein concentration for each unknown could be determined.

Purification and Titration of TMV: A stock solution of Tobacco Mosaic Virus (TMV) was prepared as follows: Frozen (-20°C) tobacco leaves infected with TMV were homogenized 1:1 in pH 7.0 buffer. This material was centrifuged at 3,473 x g (3500 rpm) for 15 min at 4°C in a Beckman TY JS-4.2 rotor. The pooled supernatant was centrifuged through a 30% sucrose cushion at 111,000 x g (25,000 rpm) for 7 hours at 4°C in a Beckman SW-28 rotor. Pellets were resuspended in 2 ml of H_2O for each pellet and loaded on 10-40% sucrose gradients. These were centrifuged at 125,000 x g (26,000 rpm) for 4 hours at 4°C in a SW-28 rotor. The opaque band was harvested from the gradient, diluted with H_2O, and centrifuged at 111,000 x g (25,000 rpm) for 3 hours at 4°C in a SW-28 rotor. The supernatant was discarded and the virus pellets resuspended in 3 ml of H_2O per pellet. This

TMV suspension was stored at -20°C in 0.5 ml aliquots until needed.

The virus stock was titrated by making serial 5-fold dilutions in buffer, mixing 1:1 with additional buffer, and adding Celite abrasive (15 mg/ml). The inoculation was performed by placing 200 µl of the mixture onto the half-leaf and rubbing the half-leaf with a fingertip. The infected plants were grown for 7 days, the leaves harvested, and the lesions counted with the aid of a dissecting scope.

A lesion is defined as a necrotic spot on the leaf surface, red-brown to gray in color and circular with irregular edges. Lesions will in some cases be bounded by a light green area. The average number of lesions obtained throughout the year varied with the season. Control lesion numbers obtained in the spring and fall were usually higher than at other times of the year. This agrees with observations made by Zipf (11).

Preparation of the *Datura* extract: Frozen (-20°C) leaves of *Datura stramonium* were homogenized 1:1 in cold (4°C) pH 7.0 buffer in the presence of 0.1% ß-Mercaptoethanol with a Waring blendor. The resultant homogenate was placed in 250 ml centrifuge bottles, and centrifuged at 18,200 x g (8000 rpm) for 50 minutes at 4°C in a GSA rotor. The pellets were discarded and the supernatants pooled before precipitation with ammonium sulfate.

Protein precipitation was accomplished by a 60% to 80% ammonium sulfate 'cut'. This cut had been previously determined to contain the maximal antiviral activity from *Datura* (11). The pooled supernatant was brought to 60% of saturation by the addition of solid ammonium sulfate and the solution stirred overnight at 4°C. The fluid was placed into 250 ml centrifuge bottles and centrifuged at 18,200 x g (8000 rpm) for 1 hour at 4°C in a GSA rotor. The pellets were discarded and the supernatant brought to 80% of saturation with solid ammonium sulfate and stirred overnight at 4°C. The solution was centrifuged as above, the supernatant discarded, and the pellets resuspended in a total of 2 ml of pH 7.0 buffer per 40 ml of starting liquid. This material was dialyzed at 4°C for 17 hours against four 2 liter changes of pH 7.0 buffer, with buffer changes after 2, 4, and 12 hours (Spectra-por dialysis tubing 12-14,000 MW cutoff, Fisher). The dialyzed material was stored at -20°C in 1.2 ml aliquots until needed.

Inhibition Assay: Nine to eleven day-old Pinto bean (*Phaseolus vulgaris* var. "Pinto") plants were used as the assay host. The assay unit used throughout the experiments was the half-leaf. A half-leaf is defined as the leaf surface extending laterally from the central vein of the primary leaf to the margin.

Serial five-fold dilutions of TMV were made in buffer

to a dilution previously determined to give 100 to 200 lesions per half-leaf. The control virus dilution contained virus dilution 1:1 with buffer. Experimental samples contained virus dilution 1:1 with the sample to be tested. Celite abrasive was then added to a concentration of 15 mg per ml of mixture. Using a micropipettor, 200 µl of the well-mixed sample was placed onto the leaf surface and rubbed onto the entire half-leaf surface with a fingertip. The inoculated plants were grown in the green-house for 7 days, the leaves harvested, and the lesions counted with the aid of a dissecting microscope.

Heat Treatment of *Datura* extract: Zipf determined that the thermal inactivation point of the antiviral material from *Datura* was between 65°C and 70°C, but no protein data were included (11).

Two aliquots of *Datura* were thawed, and centrifuged at 12,500 x g (14-15,000 rpm) in the benchtop microcentrifuge for 30 seconds. The supernatants were transferred to new tubes. One tube was placed on ice and one tube heat-treated as follows: The tube was placed in the 60°C water bath for 10 minutes and then cooled on ice for 5 minutes. The material was centrifuged for 4 minutes at 12,500 x g (14-15,000 rpm) in a benchtop microcentrifuge at 4°C and the supernatant transferred to a new tube. The two aliquots were tested for antiviral activity by making serial 10-fold

dilutions and testing as outlined above for the inhibition assay.

Molecular Filtration: One ml of heat-treated *Datura* extract was loaded onto a 100Kda Centricon (Amicon) filter and centrifuged at 920 x g (2750 rpm) in an SS-34 (Sorvall) rotor at 4°C for 1 hour. The retentate was saved and the filtrate was loaded onto a 30kDa filter and centrifuged for 1 hour at 4715 x g (6250 rpm) at 4°C. The retentate was saved and the filtrate was loaded onto a 10kDa cutoff filter and centrifuged for 2 hours at 4715 x g (6250 rpm) at 4°C. All material was resuspended to starting volume (1 ml) with buffer to obtain four different samples: +100kDa (material retained by the 100K filter), +30kDa (<100K and >30K), +10kDa (<30K and >10K), and -10kDa (0-10K). All samples were stored at 4°C until use.

Column Chromatography:

DEAE-Cellulose: DEAE-cellulose (Whatman DE-52) column matrix was prepared as follows: One volume of DE-52 matrix was added to 15 volumes of 0.5M hydrochloric acid in H_2O, stirred, and allowed to settle for 30 minutes. Fine particles were removed by decanting and the matrix was repeatedly washed with H_2O until the pH was above 4.0. The matrix was soaked in 15 volumes of 0.5M sodium hydroxide in H_2O for 30 minutes and repeatedly washed in H_2O until the pH

was below 7.0. The DEAE-cellulose was then suspended in pH 7.9 buffer overnight at 4°C. The excess buffer was removed, and the resultant slurry poured into the column (Pharmacia, Upsala, Sweden) at 4°C. The column was equilibrated with 3 column volumes of buffer. The final column bed volume was 40 ml. Column dimensions: 1.5 cm diameter by 28 cm length.

CM-cellulose: CM-cellulose (Whatman CM-52) column matrix was prepared as follows: One volume of CM-52 matrix was added to 15 volumes of 0.5M sodium hydroxide in H_2O, stirred, and allowed to settle for 30 minutes. Fine particles were removed by decanting and the matrix was repeatedly washed with H_2O until the pH was below 8.0. The matrix was soaked in 15 volumes of 0.5M hydrochloric acid in H_2O for 30 minutes and repeatedly washed in H_2O until the pH was above 7.0. The CM-cellulose was then suspended in pH 6.0 buffer containing 0.01% sodium azide and aged at 4°C for 1 week before pouring. The excess buffer was removed, and the resultant slurry poured into the column at 4°C. The column was equilibrated with 3 column volumes of buffer. The final column bed volume was 69 ml. Column dimensions: 1.6 cm diameter by 35 cm length.

Sephadex G-200: Two grams of Sephadex G-200 (Pharmacia) were mixed with two column volumes of pH 7.9 buffer, autoclaved for 15 minutes (121°C and 15 atm), and allowed to

cool to 4°C. Excess buffer was removed, the slurry poured into the column, and the column equilibrated with 3 column volumes of buffer at 4°C. The final column bed volume was 60 ml. Column dimensions: 1.6 cm diameter by 35 cm length. The column void volume was determined using 0.5 mg/ml Blue Dextran (average MW of 2,000,000).

Column chromatography of extract:

DEAE-cellulose: Six aliquots of *Datura* extract were thawed and centrifuged for 1 minute at 12,500 x g (14-15,000 rpm) at 4°C in a benchtop microcentrifuge to sediment any particulate material. The supernatants were transferred to new Eppendorf tubes and heated in a 60°C water bath for 10 minutes. The tubes were removed and cooled on ice for 5 minutes, and centrifuged at 12,500 x g (14-15,000 rpm) in the benchtop microcentrifuge for 4 minutes to sediment any heat-aggregated material. The supernatants were pooled and brought up to 18.5 ml with pH 7.9 buffer. The material was loaded onto the DEAE-cellulose column and allowed to run into the bed. The walls of the column were washed with 12 ml of pH 7.9 buffer, and the washes allowed to run into the bed. The head-space was filled with buffer, the buffer reservoir attached, and the fraction collector set to collect 120 drop fractions (5 ml/tube). Buffer (pH 7.9) was allowed to wash the column by gravity feed. Eluted fractions were monitored at A_{280} until the absorbance had

returned to background. The adsorbed material was eluted with a linear 0-1.4M sodium chloride gradient. Fractions were tested for antiviral activity. In further purifications, tubes containing the unadsorbed material were pooled and concentrated using a Centriprep-10 centrifugal filter (10,000 MW cut-off, Amicon).

CM-cellulose: The concentrated wash-through peak (pH 7.9) from the DEAE-cellulose column was buffer-exchanged to pH 6.0 by the use of a Centriprep-10 centrifugal filter. This was accomplished by multiple resuspensions of the centrifugally concentrated material with buffer pH 6.0. Final volume of buffer-exchanged material was 1.2 ml. This material was resuspended to the starting volume of 5.2 ml with buffer pH 6.0, and a 0.5 ml aliquot was removed for testing. The remainder of the material was diluted to 25 ml with buffer and loaded onto the CM-cellulose column. The head-space was filled, and the column washed with buffer pH 6.0 until the A_{280} had returned to background collecting 5 ml fractions. Adsorbed material was eluted with a linear 0-1M sodium chloride gradient until the A_{280} had returned to background. All fractions were stored at 4°C for testing.

Sephadex G-200: The concentrated wash-through peak obtained from the DEAE-cellulose column was resuspended to 1% of the G-200 bed volume with pH 7.9 buffer (final volume 0.6 ml).

This material was loaded onto the G-200 column, and allowed to drain into the bed. The walls of the column were washed with 1 ml of pH 7.9 buffer, the head-space filled, and the buffer reservoir attached. The column was washed with 3 column volumes of buffer, and 120 drop fractions (5 ml) were collected. The A_{280} readings were used to generate a column elution profile. Aliquots of each fraction were stored at 4°C for testing, and the remainder was stored at -20°C until needed.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE): Electrophoresis was carried out by the method of Laemmli (27) in 12.5% polyacrylamide gels with a 4% polyacrylamide stacking gel under denaturing (SDS-PAGE) conditions.

Material which had previously proved to be too dilute to be visualized by SDS-PAGE was concentrated by TCA precipitation (31). Briefly, one volume of sample was mixed with four volumes of 5% trichloroacetic acid and incubated overnight at 4°C. The precipitated material was centrifuged out of solution in a benchtop microcentrifuge for 15 min at 12,500 x g (14-15,000 rpm) and resuspended in a small volume (50 µl) of 2X Laemmli Sample Buffer for electrophoresis.

<u>4X Tris-Glycine Electrophoresis Running Buffer</u>: 28.8 grams of glycine and 6 grams of Tris base were dissolved in 250 ml

of H_2O and diluted to a final volume of 500 ml with H_2O. For use, 5 ml of 10% SDS was added to 125 ml of the 4X buffer and diluted to 500 ml (denaturing gel). Buffer is pH 8.3 when diluted to a 1X concentration.

4X Tris-HCl Buffer pH 8.8 (1.5M Tris): 45.5 grams of Tris base was dissolved in 100 ml of H_2O, adjusted to pH 8.8 with 1N HCl, and diluted to a final volume of 250 ml with H_2O.

4X Tris-HCl Buffer pH 6.8 (0.5M Tris): 6.05 grams of Tris base was dissolved in 50 ml of H_2O, adjusted to pH 6.8 with 1N HCl, and diluted to a final volume of 100 ml with H_2O.

2X Laemmli Sample Buffer: 1.52 grams of Tris base, 20 ml of glycerol, 2 grams of Sodium Dodecyl Sulfate (SDS), 2 ml of ß-Mercaptoethanol, and 1 mg of Bromphenol Blue were dissolved in 40 ml of H_2O, adjusted to pH 6.8 with 1N HCl, and diluted to a final volume of 100 ml with H_2O.

30% Acrylamide/0.8% Bisacrylamide Solution: 30 grams of acrylamide and 0.8 grams of N,N'-methylene-bisacrylamide were dissolved in 40 ml of H_2O and diluted to a final volume of 100 ml with H_2O. Stored in the dark at 4°C.

Preparation of an Electrophoresis Gel (SDS-PAGE): The glass plates and 1.5 mm spacers were cleaned with 95% ethanol

before assembly. The assembled plates were placed in a bag and clamped vertically to a plexiglas stand for pouring. The analytical gel was prepared as follows: 13 ml of H_2O, 10 ml of 4X Tris-HCl pH 8.8 buffer, and 16.8 ml of the acrylamide solution were mixed and degassed with a vacuum pump. To this mixture, 400 µl of 10% SDS, 100 µl of 10% ammonium persulfate, and 20 µl of TEMED were added, mixed well, and poured between the glass plates using a 10 ml syringe fitted with a 18 gauge needle. This mixture was then overlaid with 2-3 ml of H_2O to exclude air and allowed to polymerize for 30 minutes. The stacking gel was prepared as follows: 11 ml of H_2O, 5 ml of 4X Tris-HCl pH 6.8, and 2.6 ml of the acrylamide solution were mixed and degassed. To this mixture, 200 µl of 10% SDS, 60 µl of 10% ammonium persulfate, and 20 µl of TEMED were added. The water overlay on the analytical gel was removed and the well-mixed stacking gel solution was poured as above. A comb was inserted and the stacking gel allowed to polymerize for 30 minutes. The gel sandwich was then removed from the bag, clamped in the electrophoresis apparatus, and the upper and lower buffer chambers filled with running buffer pH 8.3 containing SDS. The comb was removed and the wells rinsed with running buffer before the samples were loaded.

SDS-PAGE: All samples were mixed 1:1 with 2x Laemmli Sample Buffer, boiled for 5 minutes, centrifuged at 12,500 x g (14-

15,000 rpm) for 10 seconds, and loaded onto the gels. The gels were electrophoresed at 120 V until the marker dye was through the stacking gel, and then turned up to 200 V and electrophoresed until the marker dye was 0.5 cm from the bottom of the gel. The dye front was marked with india ink before staining as desired.

Molecular weight markers used: Phosphorylase B (~97,400 MW), Bovine Serum Albumin (~68,000 MW), Ovalubumin (~46,000 MW), Carbonic Anhydrase (~30,000 MW), and Cytochrome C (~12,300 MW).

Staining:

Coomassie Brilliant Blue Stain for Proteins: Coomassie Brilliant Blue staining was accomplished by staining the gel in Coomassie stain (50% methanol/0.05% Coomassie Brilliant Blue R /10% glacial acetic acid/40% H_2O) for 1.5 to 2 hours. The stain solution was removed and the gel destained (5% methanol/7% glacial acetic acid/88% H_2O) until the blue bands were visible against a clear background (27).

Silver Staining for Proteins: Silver staining of the gels was accomplished by the method of Wray (28). Each gel was fixed in 50% methanol for 30 min and then washed in 2 changes of dH_2O for 15 min each. The silver stain was prepared as follows: 2.3 ml of a 3.6% solution of sodium hydroxide was mixed with 20.3 ml of H_2O and 1.4 ml of 14.8M

ammonium hydroxide was added. To this mixture, 4 ml of a 20% silver nitrate solution was added dropwise with constant stirring. An additional 72 ml of H_2O was added to this solution. The gel was soaked in the stain solution for 15 minutes, the stain removed, and the gel washed with 3 changes of H_2O for 5 min each. The gels were developed by soaking in developer solution prepared as follows: 2.5 ml of 1% citric acid and 0.25 ml of 36-37% formaldehyde were brought up to 500 ml with H_2O. The gels were soaked in this solution until the bands were of the desired intensity, and the developer removed. The developing was stopped by replacing the developer with 50% methanol. Protein bands are visible as gray-brown to black bands in a clear to yellow background.

Molecular weight determination:

By SDS-PAGE: Fractions of interest and marker proteins were subjected to SDS-PAGE to determine the denatured molecular weight of the protein(s) present in the fractions (see above). After staining with silver, the migration of the marker proteins relative to the dye front was used to generate a calibration curve for the gel. The relative migration of unknown bands was compared to the calibration curve and the molecular weight calculated (29).

By Sephadex G-200: The Sephadex G-200 column was calibrated

by the use of the MW-GF-200 kit (Sigma, St. Louis, MO.) This was accomplished by elution of proteins of a known molecular weight from the column. The marker proteins used were: ß-Amylase (~200,000 MW), Alcohol Dehydrogenase (~150,000 MW), Bovine Serum Albumin (~66,000 MW), Carbonic Anhydrase (~29,000 MW), and Cytochrome C (~12,400 MW). Blue Dextran (~2,000,000 MW) was used to determine the void volume of the column. The elution volumes of these marker proteins were used to generate a calibration curve for the column. The elution volumes of the extract column fractions were compared to this curve and approximate native molecular weights were calculated for each fraction.

RNase Activity in Agarose Gels: The presence of RNase activity was monitored by the following procedure: 25 µl of the sample was mixed with 25 µl of whole TMV and 48 µl of H_2O, and incubated on ice for 30 minutes. To each tube, 2 µl of 10% SDS was added to disrupt the virus. Twenty µl of loading dye (50% Sucrose/0.5% SDS/ 0.25% Bromphenol blue/H_2O) was added to each 100 µl reaction mix. Forty µl to 60 µl of each sample was loaded onto a 1% agarose gel (1% agarose/1x TAE/1%SDS) and electrophoresed at 100 V to completion. The gels were washed in H_2O for 1 hour to remove the SDS, stained with ethidium bromide (20 µl of a 1 mg/ml ethidium bromide solution per 100 ml of H_2O) for 1 hour, and destained with H_2O. The gel was viewed with the

aid of a UV transilluminator. RNase activity was indicated by the degradation of the TMV RNA band to fragments of a lesser size and a concomitant loss of fluorescence in the degraded RNA as compared to the control.

50X TAE stock: 24.2 grams of Tris base, 5.71 ml of glacial acetic acid, and 10 ml of 0.5M EDTA pH 8.0 were added to 40 ml of H_2O and diluted to 100 ml with H_2O.

Running Buffer: 10 ml of 50X TAE and 5 ml of 10% SDS were diluted to 500 ml with H_2O.

1% Agarose gel: 0.5 grams of electrophoretic grade agarose was added to 50 ml of H_2O, and microwaved on high until the agarose was dissolved (45-60 seconds). One ml of 50X TAE and 0.5 ml of 10% SDS were added, swirled to mix, and poured. The gel was allowed to cool and solidify before use.

Inhibition of RNase Activity: Twenty-five μl of each column fraction (4-11) were treated by the addition of 2 μl of either RNasin or 0.5M EDTA. After 30 minutes on ice, the virus was added and incubated for an additional 30 minutes. Two μl of 10% SDS and 20 μl loading dye were added to each. The samples were loaded onto a 1% agarose gel, and electrophoresed as above. If RNase was inhibited, the band

corresponding to the virus RNA would not be degraded to smaller fragments.

Protease Treatment of Column Fractions: Two 600 µl aliquots of each G-200 column fraction were thawed for use in this assay. Twenty mg of trypsin conjugated to acrylic beads (Sigma, St. Louis, MO) was added to each tube of one set of G-200 column fractions, and the second set of tubes were not treated. Buffer pH 7.9 was also treated with enzyme to serve as a enzyme control, or untreated to serve as a temperature control. All samples were mixed well and incubated at 37°C for 24 hours with shaking. The tubes were removed from the water bath, and centrifuged at 12,500 x g (14-15,000 rpm) to pellet the beads or any heat-precipitated material. The supernatants were removed to new tubes and tested for activity in the inhibitor assay. If the antiviral activity was sensitive to protease treatment, a reduction in antiviral activity would be observed by an increase in lesion number compared to untreated column fraction controls.

RESULTS

Titration of TMV

Serial 5-fold dilutions of the purified virus were made in buffer ranging from 1:5 to 1:78,125. The dilutions of 1:125 to 1:78,125 were inoculated onto pinto bean half-leaves as previously described. The dilution of 1:3125 gave 236.6 ± 53 lesions per half-leaf (Table 1). For our assay system, an average of 100-200 lesions per half-leaf is optimal. This dilution falls on the linear portion of the titration curve and was used as the standard virus dilution in subsequent experiments.

Heat treatment of *Datura* extract

Serial 10-fold dilutions of the heated and unheated *Datura* extract were made to 10^{-5}, and the dilutions of 10^{-2} to 10^{-5} were tested for activity by the inhibition assay. This experiment was performed in duplicate on two separate days. These data showed that the heated extract was slightly more inhibitory than the unheated material in both experiments. The average IC_{50} was $10^{-2.6}$ for the unheated material with an average of $10^{-2.88}$ for the heated material. However, the specific activity of the heated material was 3.5-fold higher than the specific activity of the unheated material (Table 2).

The heat treatment of our extract at 60°C is a valid addition to our purification scheme for two reasons:

Table 1: Titration of Tobacco Mosaic Virus (TMV)

TMV purified from infected tobacco leaves by density gradient centrifugation was diluted in buffer pH 7.0 in a serial 5-fold dilution scheme ranging from 1:5 to 1:78,125. Each dilution was mixed 1:1 with buffer pH 7.0, abrasive added, and inoculated at 200 μl per half-leaf. The inoculated plants were grown for an additional 7 days, the leaves harvested, and the lesions counted with the aid of a dissecting microscope. The dilution of 1:3125 was the dilution found to give a countable number of lesions per half-leaf and was used as the standard virus dilution in subsequent experiments.

Dilution of Virus	Mean[1]	95% CL[2]
1:125	TNTC*	-
1:625	541.3	426 to 656
*1:3125	236.6	183 to 290
1:15625	49.9	38 to 61
1:78125	8.9	5 to 13

Table 1: Titration of TMV

[1]The number shown is an average of the lesion counts obtained from 8 half-leaves.

[2]95% confidence limits for each sample were calculated from the individual lesion counts for each half-leaf.

*Too Numerous To Count

Table 2: Heat Treatment of *Datura* Extract

Datura **extract was heated to 60°C for 10 minutes, cooled on ice for 5 minutes, and centrifuged at 12,500 x g (14-15,000 rpm) to remove any heat-aggregated material. The samples were tested for antiviral activity by mixing serial 10-fold dilutions (10^{-1} to 10^{-4}) of the heated and unheated material with the standard virus dilution and inoculating onto pinto bean half-leaves as previously described. In both of these replicates there is a 3.5-fold increase in the specific activity with heat treatment with a ~56% recovery of protein.**

Sample*	IC_{50}[1]	U/ml[2]	mg/ml of protein[3]	Specific activity[4]
Expm I D6	$10^{-2.45}$	281.8	0.37	759.5U/mg
HD6	$10^{-2.75}$	562.3	0.21	2699.2U/mg
Expm II D6	$10^{-2.7}$	501.2	0.37	1350.6U/mg
HD6	10^{-3}	1000.0	0.21	4800.0U/mg

Table 2: Heat Treatment of *Datura* Extract

[1]IC_{50} unit is defined as the dilution of the extract which inhibits 50% of lesion formation as compared to control.

[2]U/ml is the IC_{50} units contained per ml of extract.

[3]mg/ml of protein was determined by Coomassie Brilliant Blue Protein assay.

[4]Specific activity is calculated by dividing the U/ml by mg/ml to determine the IC_{50} units present per mg of total protein.

*Abbreviations: D6: Unheated *Datura*, Preparation #6
HD6: Heated *Datura*, Preparation #6

1) About 50% of the protein present was precipitated, and 2) The specific activity of the material increased considerably. The increase in specific activity may be due to heat-inactivation or precipitation of interfering compounds, or dissociation of these interfering substances from the active material. This final option may suggest a method in which the material is kept in its inactive form *in vivo* until conditions are met which result in the unmasking of the active material.

Molecular Filtration

Several filters with different nominal molecular weight cutoffs (100K, 30K, and 10K) were used to fractionate the heat-treated extract. This was done to determine the size (range of MW) of the active material in its native form. The highest antiviral activity was observed in the +30K and +100K fractions (Table 3).

Apablaza and Bernier used molecular filters to separate components in an extract of *Datura* (10). They found that the antiviral activity was confined to the high molecular weight (+50,000 MW) fraction only. The data obtained in this study indicate that the activity was present in the +100,000, +30,000, and +10,000 molecular weight fractions. These results are consistent with Zipf's statement that there may be more than one inhibitory protein

Table 3: Antiviral Activity of Molecular Filtration Fractions

The fractionation of heated *Datura* extract through molecular weight cut-off filters generated four fractions. These fractions were tested for antiviral activity in the inhibition assay as previously described. The +100K fraction contains material which was retained by the 100Kda cut-off filter, the +30K fraction contains material retained by the 30Kda filter but not the 100Kda filter, the +10K fraction contains the material retained by the 10Kda filter but not the 30Kda filter, and the -10K fraction contains the material which passed through the 10Kda filter. The +100K, +30K, and +10K fractions are all antiviral, while the -10K fraction is not.

Sample	Mean[1]	95% CL[2]	% Inhibition
Control	237.3	155 to 319	NA*
+100K	4.0	1 to 7	98.3%
+30K	0.0	0	100.0%
+10K	125.7	51 to 201	47.0%
-10K	254.0	204 to 304	-7.0%

Table 3: Antiviral Activity of Molecular Filtration Fractions

[1]The number shown is an average lesion count of 4 half-leaves for experimental samples and 8 half-leaves for the control.

[2]95% confidence limits for each sample were calculated from the individual lesion counts for each half-leaf.

*Not applicable

present in an extract from *Datura* (11). The presence of more than one active fraction has been observed for the extracts of geranium (*Pelargonium hortorum* B.) and pepper (*Capsicum frutescens* L.) where both high and low molecular weight components are active (10).

SDS-PAGE of Molecular Filtration Fractions

SDS-PAGE of these fractions show that the +100K, +30K, and -10K fractions have many proteins in common. The +10K fraction shows no protein bands in our gel. The only major difference observed between the +100K, +30K, and -10K fractions is the absence of the 17,000 MW in the -10K fraction (Figure 1). This -10K fraction also has no inhibitory activity.

The absence of protein bands in our +10K fraction and presence of protein (evaluated by BCA protein assay), suggest that either the protein is not staining or that the active protein is of a molecular weight less that 12.5K (smallest marker present on the gel). The latter is the most probable explanation since other proteinaceous antivirals have been shown to be of a low molecular weight, *i.e.* meliacin (5000 to 6000 MW from *Melia azedarach*) and several di- and tri-peptide carbobenzoxy derivatives of phenylalanine (8).

Figure 1: SDS-PAGE of Molecular Filtration Fractions
This is a 12.5% denaturing gel of the fractions obtained from the molecular weight fractionation of heated *Datura* extract. 100 µl of each fraction was mixed with 100 µl of 2X Laemmli Sample Buffer, boiled for 5 minutes, and electrophoresed as previously described. The gel was silver stained to visualize the protein bands.

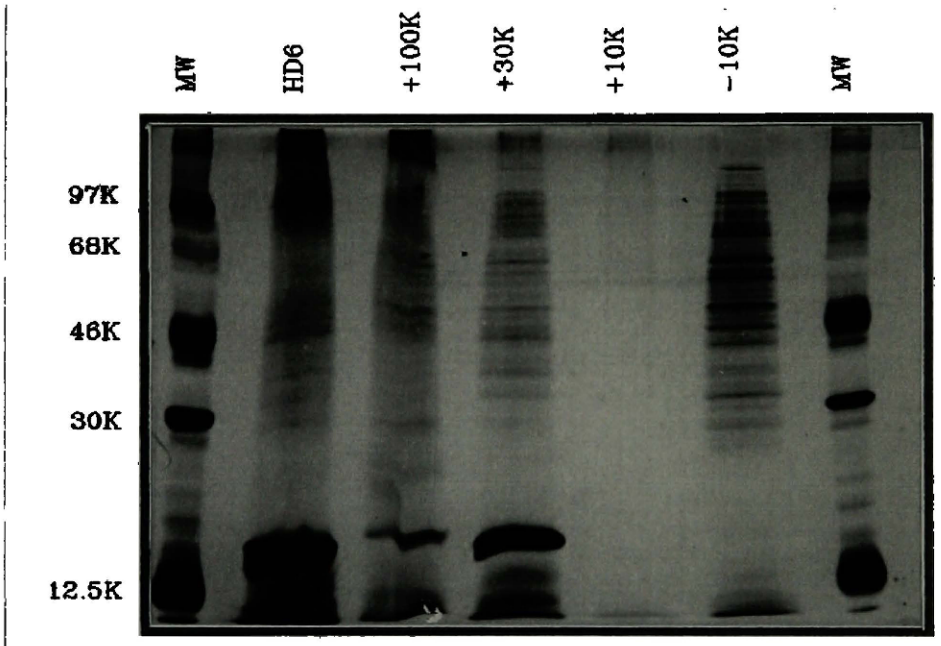

Figure 1: SDS-PAGE of Molecular Filtration Fractions

RNase Activity of Molecular Filtration Fractions

The samples obtained from the molecular filtration were tested for RNase activity as previously described. The stained gel showed that of the four fractions, the +100K and +30K had RNase activity while the +10K and the -10K fractions exhibited no RNase activity (Figure 2).

Column Chromatography:

DEAE-cellulose: Fractionation through a DEAE-cellulose column was accomplished by a modification of the method used by Grasso and Shepherd (9). The material was loaded onto the column which was then washed with buffer. Adsorbed material was eluted from the column with a linear 0-1.4M NaCl gradient. The absorbance at 280 nm of the fractions collected from the column were monitored with a spectrophotometer to generate a column elution profile (Figure 3). Fractions were pooled and tested for antiviral activity in the inhibition assay. Results showed that the antiviral activity of the extract was confined to the unadsorbed material and the gradient-eluted material was largely non-inhibitory (Table 4). Due to these data, the gradient elution step was omitted in subsequent DEAE-cellulose chromatography of the extract, and only the unadsorbed material was collected for further testing (Figure 4).

Figure 2: RNase Activity of MW Fractions

Twenty-five μl of molecular weight fractions (+100K, +30K, +10K, and -10K), heated *Datura* extract (HD6), and buffer pH 7.9 (TMV), were mixed with 25 μl of TMV and H_2O to 100 μl. These samples were incubated on ice for 30 minutes, and 2 μl of 10% SDS was added to disrupt the virus. 20 μl of loading dye was added to each sample and 40-60 μl of each was electrophoresed on a 1% agarose gel at 100V to completion. The gel was washed with H_2O, stained with ethidium bromide, and destained with H_2O. The bands were visualized with the aid of a UV transilluminator. The +100K, +30K, and heated *Datura* extract exhibit RNase activity, while the +10K and -10K fractions do not.

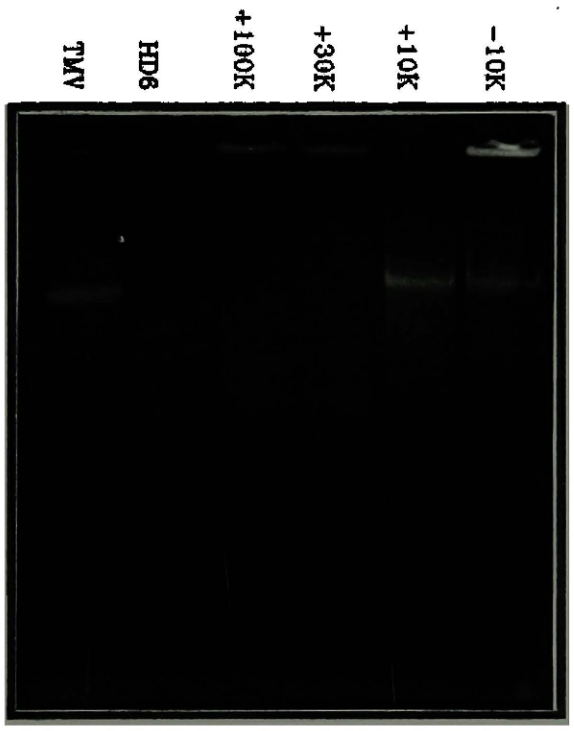

Figure 2: RNase Activity of MW Fractions

Figure 3: Elution Profile of DEAE-cellulose

Heat-treated *Datura* extract was subjected to ion-exchange chromatography on DEAE-cellulose resin. The column was washed with buffer pH 7.9 and then eluted with a linear 0-1.4M NaCl gradient to elute any adsorbed material. Absorbance at 280 nm was used to generate the column profile. Two peaks were obtained, A and C. A contains the wash-through peak and C contains the gradient-eluted material. Each fraction is ~5 ml.

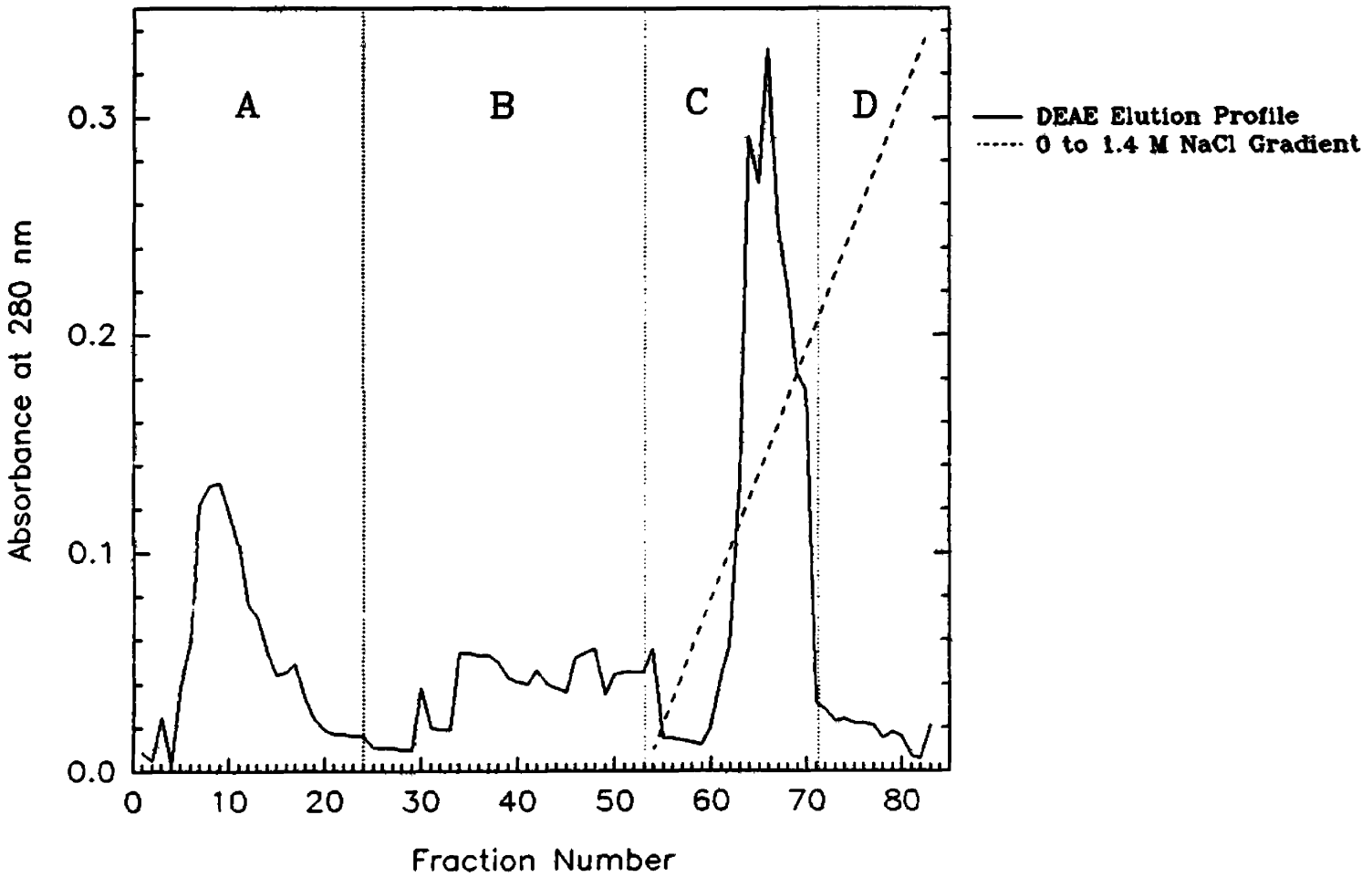

Figure 3: Elution Profile of DEAE–Cellulose

Table 4: Antiviral Activity of DEAE-cellulose Column Fractions

Aliquots (0.1 ml) of each fraction in A-D were pooled and tested for antiviral activity in the inhibition assay. Pool A encompassing fractions 1-24 was found to contain the antiviral activity, while the rest of the pools had no activity. The material in pools B-D appear to exert a stimulatory action on the virus which could indicate the presence of some compound in the extract which could function as a stimulator of virus activity.

Sample	Mean[1]	95% CL[2]	% Inhibition
Control	46.6	37 to 56	NA*
A (1-24)	24.0	13 to 34	48 %
B (25-53)	129.1	99 to 159	-177.1%
C (54-71)	78.5	35 to 122	-68.5%
D (72-83)	61.5	46 to 70	-31.9%

Table 4: Antiviral Activity of DEAE-cellulose Column Fractions

[1]The number shown is an average lesion count of 8 half-leaves for experimental samples and 16 half-leaves for the control.

[2]95% confidence limits for each sample were calculated from the individual lesion counts for each half-leaf.

*Not applicable

Figure 4: DEAE-cellulose Wash-through

In subsequent column purifications, the gradient elution was not done, and only the wash-through was collected for further testing. This figure shows the elution profile of the wash-through peak from the DEAE-cellulose column generated from the A_{280} values of the fractions.

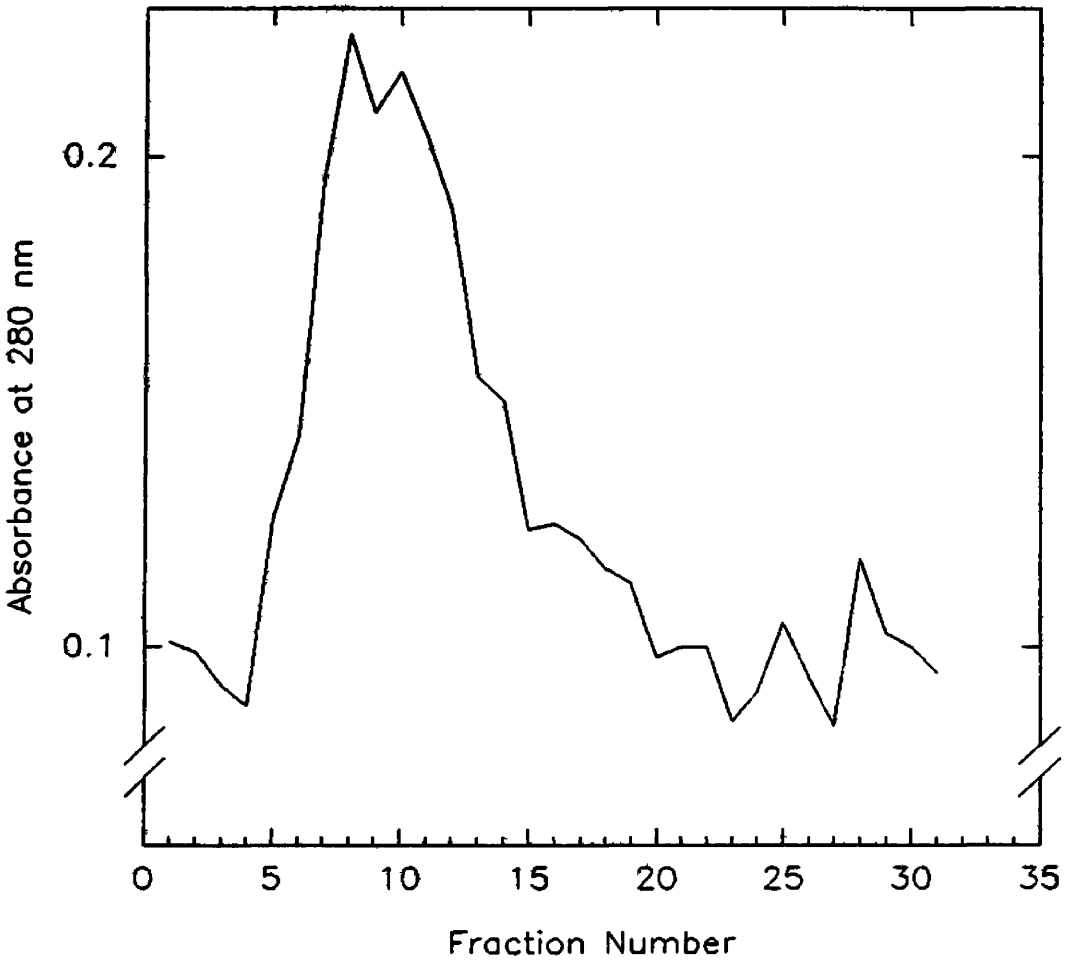

Figure 4: DEAE–Cellulose Wash–through

Fractionation through a DEAE-cellulose column allowed for the removal of about 50% of the protein present in the heat-treated extract, with one major peak of activity present in the wash-through. Since DEAE-cellulose is an anion exchanger and binds positively-charged side groups or proteins, the data led us to believe that the active material has an overall negative or neutral charge.

CM-cellulose: A CM-cellulose column was used to fractionate the concentrated, buffer-exchanged material obtained from the DEAE-cellulose column wash-through. This material was loaded onto the column, washed with buffer until the A_{280} had returned to background, and the adsorbed material eluted with a linear 0-1M NaCl gradient. The absorbance at 280 nm of each fraction was used generate a column elution profile for the CM-cellulose column (Figure 5). Seventy-six 5 ml fractions were collected.

Aliquots (0.1 ml) of each fraction were pooled to generate four distinct pools: A (fractions 1-30), B (fractions 31-45), C (fractions 46-60), and, D (fractions 61-76). These pools were tested for antiviral activity in the inhibition assay, and pools B (31-45) and D (61-76) contained the majority of the activity (Table 5). The data suggest that the use of CM-cellulose (cation exchanger, binds negatively charged material) to fractionate the

Figure 5: Elution Profile of CM-cellulose Column
The wash-through peak from the DEAE-cellulose column was concentrated and loaded onto a CM-cellulose ion-exchange column. The column was washed with buffer pH 6.0 and 5 ml fractions were collected. This elution profile was generated from the A_{280} values of the fractions.

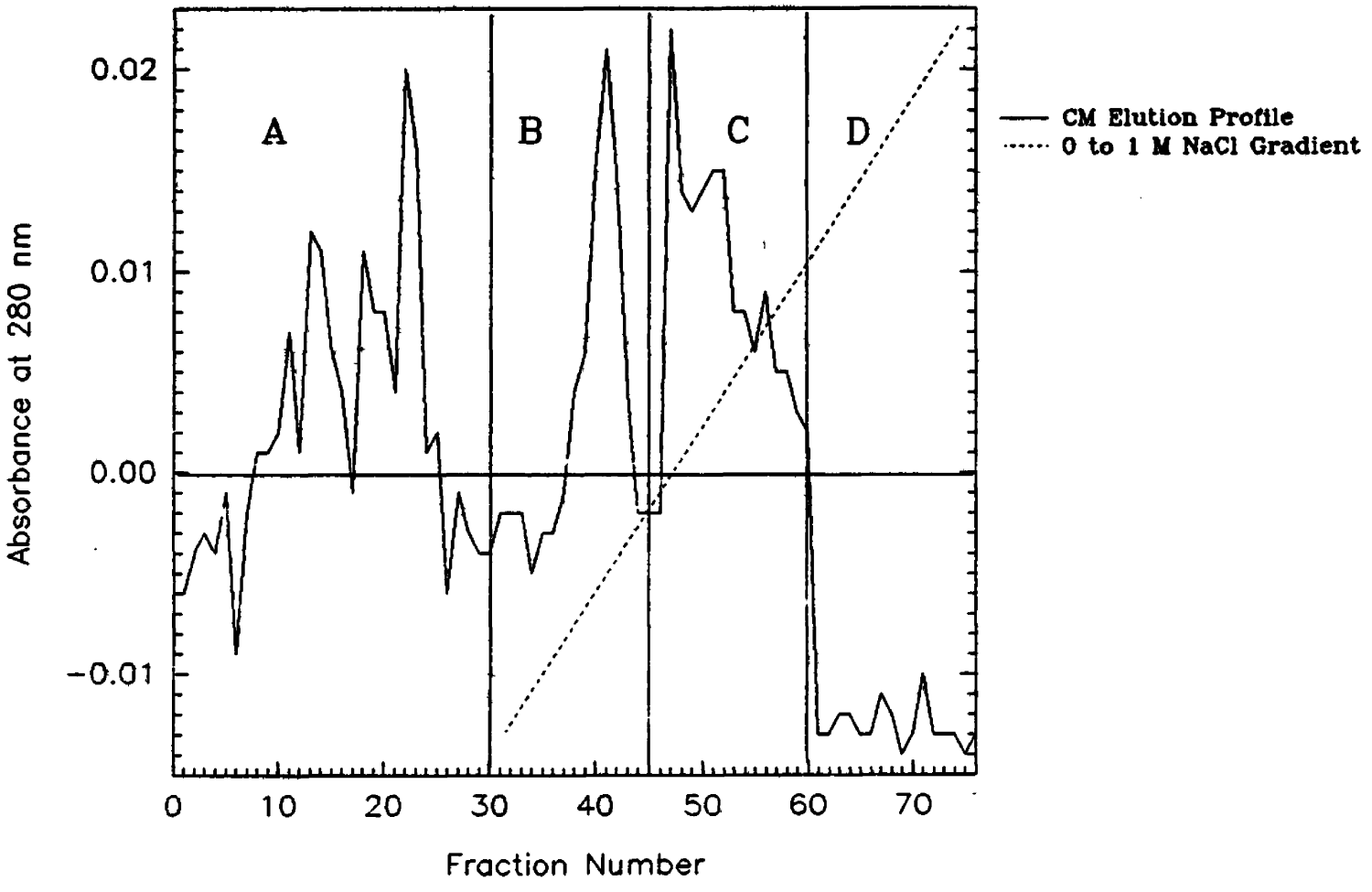

Figure 5: Elution Profile of CM-Cellulose

Table 5: Antiviral Activity of CM-cellulose Fraction Pools
The 0.1 ml aliquots of the material obtained from the CM-cellulose were pooled (Pools A-D) and tested for antiviral activity in the inhibition assay. Pools B and D were antiviral in experiment I. Pools B and D were desalted and tested for antiviral activity in experiment II, where neither was antiviral.

Sample	Mean[1]	95% CL[2]	% Inhibition
I Control	209.0	177 to 241	NA*
A (1-30)	139.5	115 to 164	33.3%
B (31-45)	88.3	62 to 115	57.8%
C (46-60)	179.5	122 to 237	14.1%
D (61-76)	20.5	14 to 27	90.2%
II Control	103.1	92 to 114	NA*
B (31-45) No Salt	161.1	128 to 194	-56.3%
D (61-76) No Salt	156.8	134 to 179	-52.1%

Table 5: Antiviral Activity of CM-cellulose Fraction Pools

[1]The number shown is an average lesion count of 8 half-leaves for experimental samples and 16 half-leaves for the control.

[2]95% confidence limits for each sample were calculated from the individual lesion counts for each half-leaf.

I and II are two different experiments. The values shown are calculated with respect to the internal control of each experiment.

*Not applicable

active material recovered from the DEAE-cellulose column resulted in the active material binding to the column.

Pools B and D were desalted and buffer-exchanged to remove the NaCl present in solution from the gradient elution. The desalted pools were tested for antiviral activity, and neither pool B or D were antiviral (Table 5). This loss of antiviral activity could be attributed to one or all of the following: 1) Proteins may undergo conformational changes in binding to ion-exchangers (32), which could cause the adsorbed protein to be stripped of cofactors necessary for its activity. 2) The salt concentration of the fractions was sufficiently high to have caused the precipitation or denaturation of the virus present. 3) The salt concentration of the fractions was sufficiently high to have caused the precipitation or denaturation of the active material. 4) The salt concentration of the fractions was sufficiently high to interfere with the pinto beans (buffer containing 0.5M NaCl mixed with the standard virus dilution and inoculated onto pinto beans caused the leaves to die). 5) The active material may be tightly bound to the column matrix and was not eluted under the conditions used.

Sephadex G-200: Due to this loss of activity, fractionation by a G-200 Sephadex column was substituted for the CM-cellulose column. G-200 Sephadex fractionates proteins on

the basis of size, with little chance that necessary cofactors would be stripped from the active material.

The unadsorbed material from the DEAE-cellulose column was concentrated and loaded onto the Sephadex column. The column was washed with three column-volumes of buffer and the absorbance of the collected fractions was used to generate a column elution profile (Figure 6). Aliquots of the eluted fractions were pooled for testing in the inhibition assay (pools A-F). Antiviral activity was present in pools A and B which corresponded to the void volume (tubes 1-5) and the major peak (tubes 6-14) of the column respectively (Table 6). Each of the individual fractions encompassing pools A and B were then tested in the inhibitor assay, and fractions 4-11 contained the majority of the antiviral activity (Table 7 and Figure 7).

SDS-PAGE of Column Fractions

TCA-precipitated G-200 column fractions 4-11 were electrophoresed on a 12.5% SDS-PAGE gel. Upon silver staining, all fractions were found to contain a band of ~17,000 MW with fractions 4 and 7-11 containing the majority of this material. Grasso and Shepherd also found a protein of ~17,000 which they believed was the active material present in their extract from Datura (9). Fractions 8-11 also contained a band of ~12,000 MW (Figure 8).

Using G-75 Sephadex for his separation, Zipf

Figure 6: Sephadex G-200 Elution profile

The wash-through peak from the DEAE-cellulose column was concentrated and loaded onto a Sephadex G-200 size-exclusion column. The column was washed with buffer pH 7.9 and 5 ml fractions were collected. This elution profile was generated from the A_{280} values of the fractions.

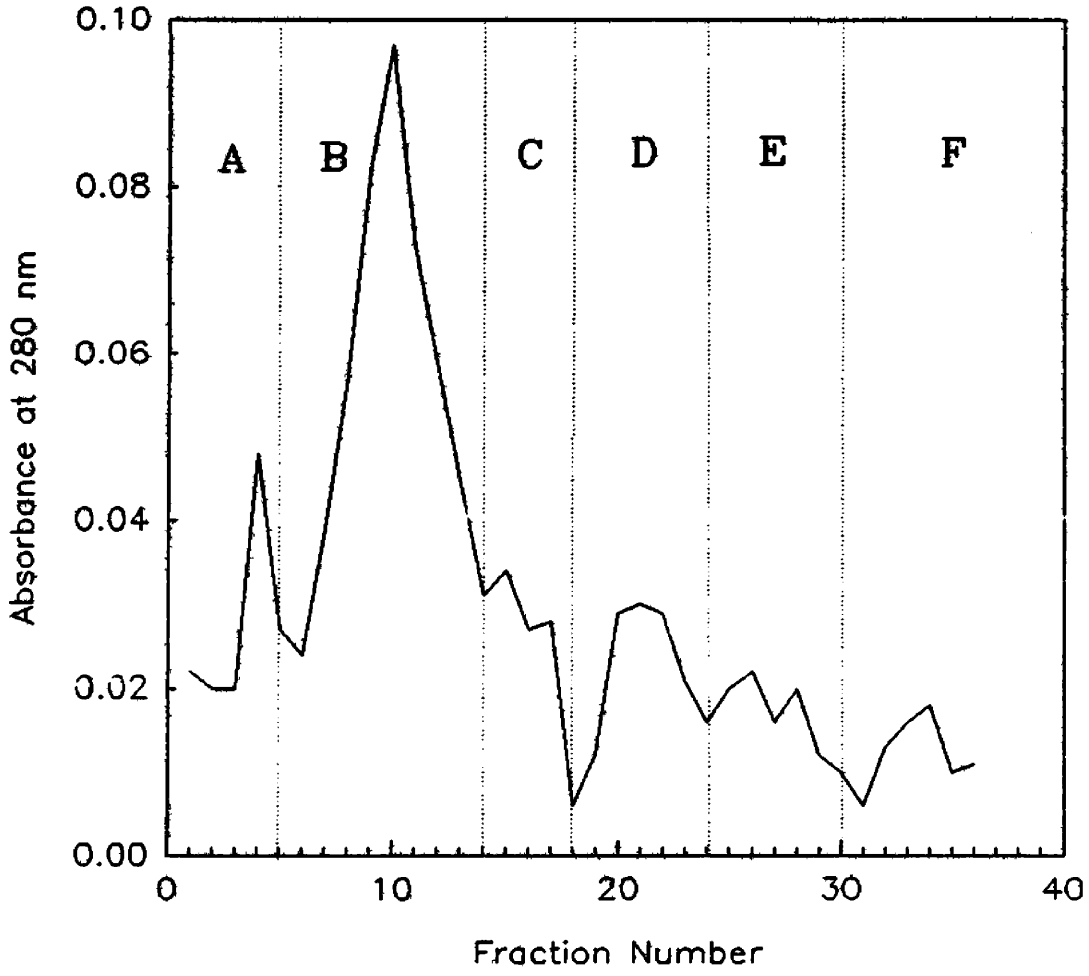

Figure 6: Sephadex G-200 Elution Profile

Table 6: Antiviral Activity of G-200 Sephadex Fractions (Pooled)

The 0.1 ml aliquots of the material obtained from the G-200 Sephadex column were pooled (Pools A-F) and tested for antiviral activity in the inhibition assay. Pools A and B were found to contain the antiviral activity, while pools C-F showed stimulatory activity

Sample	Mean[1]	95% CL[2]	% Inhibition
Control	80.3	58 to 102	NA*
A (1-5)	29.8	19 to 40	62.9%
B (6-14)	1.9	1 to 3	97.6%
C (15-18)	126.3	107 to 145	-57.2%
D (19-24)	132.0	102 to 162	-64.4%
E (25-30)	147.1	113 to 181	-83.2%
F (31-36)	189.9	156 to 224	-136.5%

Table 6: Antiviral Activity of G-200 Sephadex Fractions (Pooled)

[1]The number shown is an average lesion count of 8 half-leaves for experimental samples and 16 half-leaves for the control.

[2]95% confidence limits for each sample were calculated from the individual lesion counts for each half-leaf.

*Not applicable

Table 7: Antiviral Activity of Individual G-200 Sephadex Column Fractions

Each individual fraction encompassing pool A and B was individually tested for antiviral activity in the inhibition assay. Fractions 1-14 were tested, and fractions 4-11 were found to contain the majority of the antiviral activity observed. The peak of antiviral activity was observed at fraction 8, while the absorbance peak was in fraction 10.

Sample	Mean[1]	95% CL[2]	% Inhibition
Control	78.0	53 to 103	NA*
Fraction 1	213.4	141 to 285	-173.6%
Fraction 2	129.0	101 to 157	-65.4%
Fraction 3	137.3	64 to 211	-76.0%
Fraction 4	23.3	20 to 27	70.2%
Fraction 5	11.0	1 to 21	85.9%
Fraction 6	7.75	5 to 11	90.1%
Fraction 7	4.5	2 to 8	94.2%
Fraction 8	0.8	0 to 2	98.9%
Fraction 9	1.0	0 to 2	98.7%
Fraction 10	2.25	0 to 5	97.1%
Fraction 11	13.0	2 to 24	83.3%
Fraction 12	48.8	23 to 75	37.5%
Fraction 13	36.8	21 to 53	52.9%
Fraction 14	127.3	85 to 170	-63.1%

Table 7: Antiviral Activity of Individual G-200 Sephadex Column Fractions

[1]The number shown is an average lesion count of 4 half-leaves for experimental samples and 8 half-leaves for the control.

[2]95% confidence limits for each sample were calculated from the individual lesion counts for each half-leaf.

*Not applicable

Figure 7: Antiviral Activity of Column Fractions 4-11
The inhibition data obtained by testing the individual G-200 fractions is shown superimposed on the elution profile of the G-200 Sephadex column. Fraction 8 contains the maximal antiviral activity, while the absorbance peak is seen in fraction 10 which is slightly less active.

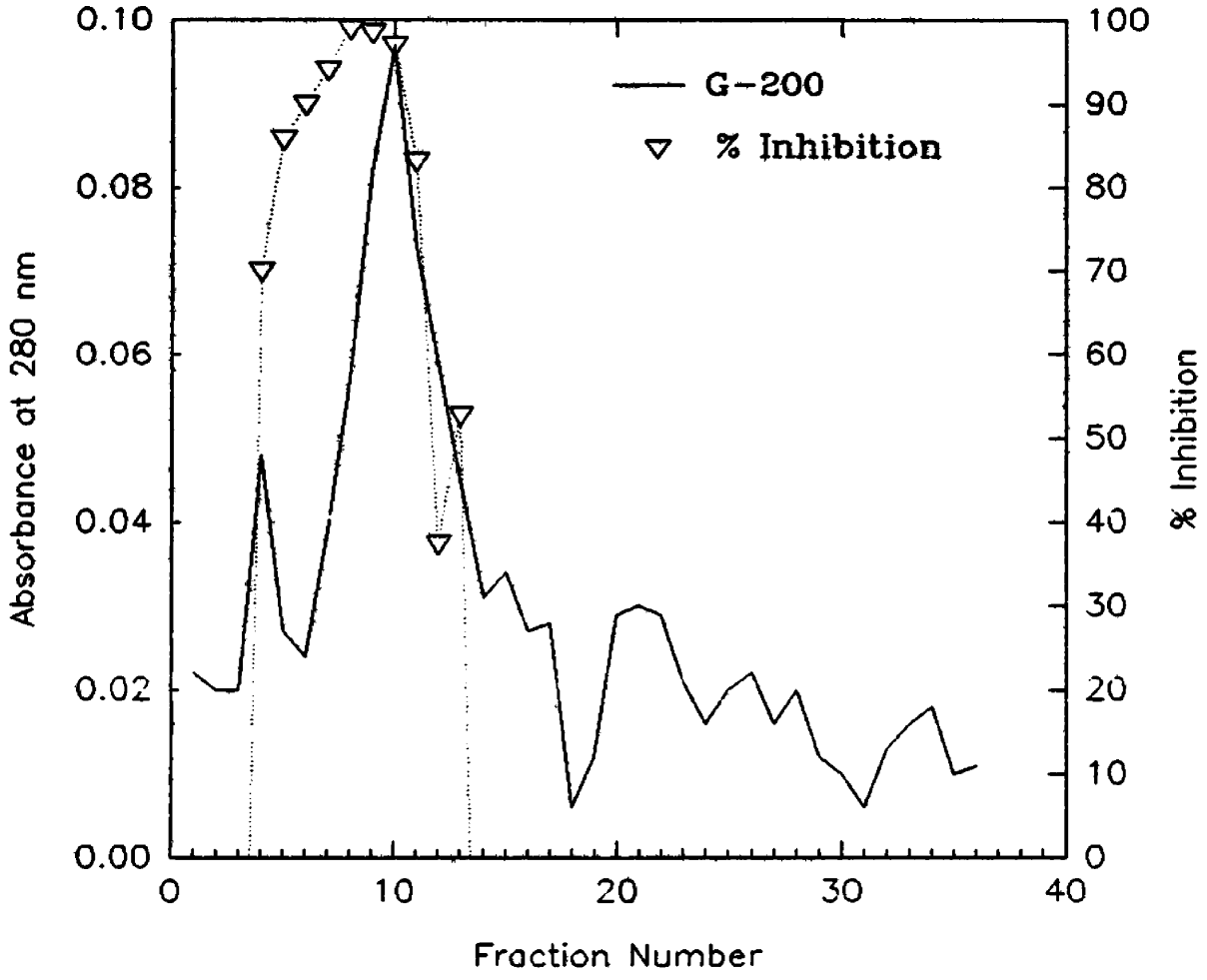

Figure 7: Antiviral Activity of Column Fractions 4–11

Figure 8: SDS-PAGE of G-200 Column Fractions
One-half ml of each G-200 column fraction was TCA-precipitated and resuspended in 50 μl of 2X Laemmli Sample Buffer for electrophoresis on a 12.5% SDS-PAGE gel. The resultant gel was silver stained to visualize the bands. All samples contain a ~17,000 MW band in common which is faintly visible in fractions 5 and 6, and is easily seen in all other fractions.

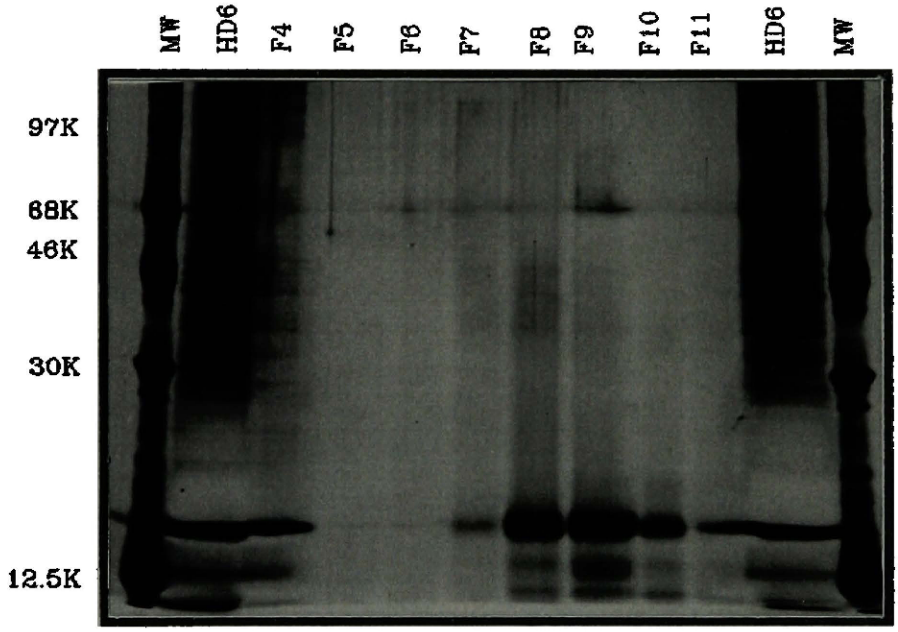

Figure 8: SDS-PAGE of G-200 Column Fractions

fractionated his extract and upon electrophoresis of this material found no evidence of a 17,000 MW protein (11). This may be due to the fact that Zipf's gels were stained with coomassie blue instead of silver. Gels which I stained with coomassie blue showed very little of this protein, but by using the silver stain this protein was readily visible. The large volumes of eluate obtained by Zipf's G-75 fractionation of the extract may have diluted the material present to a point too dilute to visualize without concentration.

RNase activity in Agarose Gels

TMV was incubated with the G-200 column fractions 4-11 to determine whether the antiviral activity of the column fractions was associated with RNase activity. Upon gel electrophoresis, Fractions 4-8 were found to degrade the TMV RNA, with the major activity observed in fractions 4-7. Fractions 9-11 had little or no RNase activity (Figure 9).

Inhibition of RNase Activity

The column fractions were treated with EDTA or RNasin before mixing them with TMV to see if the RNase activity could be inhibited. After incubation, the samples were electrophoresed on an agarose gel and stained with ethidium bromide. Treatment with RNasin (Figure 10) or EDTA (Figure 11) had no effect on RNase activity.

Figure 9: RNase Activity of Column Fractions

Twenty-five μl of column fractions 4-11 (F4-F11), heated *Datura* extract (HD6), and buffer pH 7.9 (TMV), were mixed with 25 μl of TMV and H_2O to 100 μl. These samples were incubated on ice for 30 minutes, and 2 μl of 10% SDS was added to disrupt the virus. Twenty μl of loading dye was added to each sample and 40-60 μl of each was electrophoresed on a 1% agarose gel at 100V to completion. The gel was washed with H_2O, stained with ethidium bromide, and destained with H_2O. The bands were visualized with the aid of a UV transilluminator. Fractions 4-7 and *Datura* extract degraded TMV RNA, while fractions 9-11 showed little or no RNase activity. Fraction 8 exhibits some RNase activity.

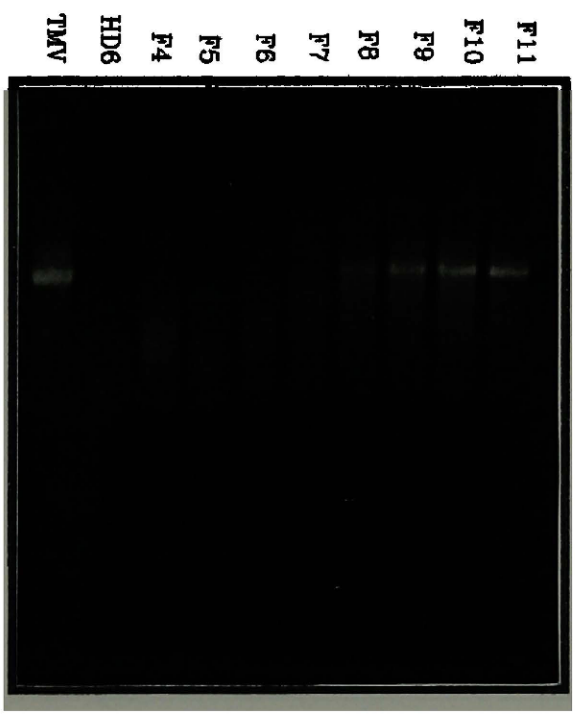

Figure 9: RNase Activity of Column Fractions

Figure 10: RNasin Treatment of Column Fractions

Twenty-five μl of column fractions 4-11 (F4-F11) and heated *Datura* extract (HD6) were treated with 2 μl of RNasin and incubated on ice for 30 minutes. Twenty-five μl of TMV and H_2O to 100 μl were added and incubated on ice for an additional 30 minutes. Two μl of 10% SDS was added to disrupt the virus. Twenty μl of loading dye was added to each sample and 40-60 μl of each was electrophoresed on a 1% agarose gel at 100V to completion. The gel was washed with H_2O, stained with ethidium bromide, and destained with H_2O. The bands were visualized with the aid of a UV transilluminator. Treatment with the RNasin had little or no effect on the RNase activity of the fractions.

Figure 10: RNasin Treatment of Column Fractions

Figure 11: EDTA Treatment of Column Fractions

Twenty-five μl of column fractions 4-11 (F4-F11) and heated *Datura* extract (HD6) were treated with 2 μl of 0.5M EDTA and incubated on ice for 30 minutes. Twenty-five μl of TMV and H_2O to 100 μl were added and incubated on ice for an additional 30 minutes. Two μl of 10% SDS was added to disrupt the virus. Twenty μl of loading dye was added to each sample and 40-60 μl of each was electrophoresed on a 1% agarose gel at 100V to completion. The gel was washed with H_2O, stained with ethidium bromide, and destained with H_2O. The bands were visualized with the aid of a UV transilluminator. Treatment with the EDTA had little or no effect on the RNase activity of the fractions.

Figure 11: EDTA Treatment of Column Fractions

The fact that RNase is not inhibited by the presence of EDTA suggests that this RNase activity is not dependent on the presence of divalent cations, a result which agrees with results obtained by Zipf (11). RNasin treatment of the fractions also showed no inhibition of activity. RNasin functions by binding to the active site of some RNases and blocking their activity (personal communication).

Protease Treatment of Column Fractions

The antiviral activity of the undigested G-200 fractions was unaffected by incubating for 24 hours at 37°C, with the exception of fraction 7. Treatment of the column fractions with trypsin conjugated to acrylic beads destroyed the antiviral activity of the fractions as compared to the controls (Table 8).

Native Molecular Weight

Markers of known molecular weights were eluted from the G-200 column and a calibration curve was generated by plotting the Ve/Vo (elution volume of the marker/elution volume of the blue dextran) of each marker against its' molecular weight (Figure 12). Comparison of the Ve/Vo for column fractions 4-11 to the calibration curve gave a molecular weight range for the proteins present in the column fractions (Table 9). Since the material eluted from the column was present as one peak, an assumption could be

Table 8: Trypsin Treatment of Column Fractions

Two 600 µl aliquots of each fraction were thawed and one aliquot of each was treated with 20 mg of trypsin conjugated to acrylic beads. Two 600 µl aliquots of buffer were also prepared, one aliquot treated with 20 mg of enzyme and the other untreated. All samples were incubated at 37°C for 24 hours with shaking. The samples were centrifuged at 12,500 x g for 1 minute to precipitate the beads or any heat-aggregated material, and the supernatants collected for testing in the inhibition assay as previously described.

Sample	Mean[1]	95% CL[2]	%Inhibition[3]
Control	46.1	36 to 56	NA*
Buffer	61.3	9 to 113	NA*
Buffer+T	152.0	0 to 324	NA*
Fraction 7	133.0	74 to 192	-177.1%
Fraction 8	18.3	15 to 21	70.2%
Fraction 9	14.3	11 to 17	76.7%
Fraction 10	7.8	0 to 16	87.4%
Fraction 11	11.3	0 to 31	75.6%
Fraction 12	112.8	68 to 158	-84.1%
Fraction 7T	56.3	31 to 81	62.9%
Fraction 8T	136.0	51 to 221	10.5%
Fraction 9T	177.3	128 to 226	-16.6%
Fraction 10T	168.8	111 to 226	-11.0%
Fraction 11T	164.5	114 to 215	-8.2%
Fraction 12T	185.3	63 to 308	-21.9%

Table 8: Trypsin Treatment of Column Fractions

[1]The value given is an average lesion count of 8 half-leaves for the control and 4 half-leaves for each experimental.

[2]95% confidence limits for each sample were calculated from the individual lesion counts for each half-leaf.95% CL[2]

[3]The % inhibition for the experimental samples have been determined by comparison with internal controls. The samples not treated with the enzyme are calculated with respect to the buffer sample. The enzyme-treated samples are calculated with respect to the enzyme-treated buffer sample.

Note: The T designation denotes treatment with trypsin for 24 hours at 37°C with shaking.

Figure 12: G-200 Calibration Curve

Markers of a known molecular weight were loaded onto the G-200 Sephadex column and eluted with buffer pH 7.9, collecting 1 ml fractions. The calibration curve was generated by plotting the molecular weight of each marker against its' V_e/V_0 (marker elution volume/elution volume of Blue Dextran). The molecular weight of each unknown fraction was determined by calculating the V_e/V_0 for each and using this value to extrapolate the approximate molecular weight from this curve.

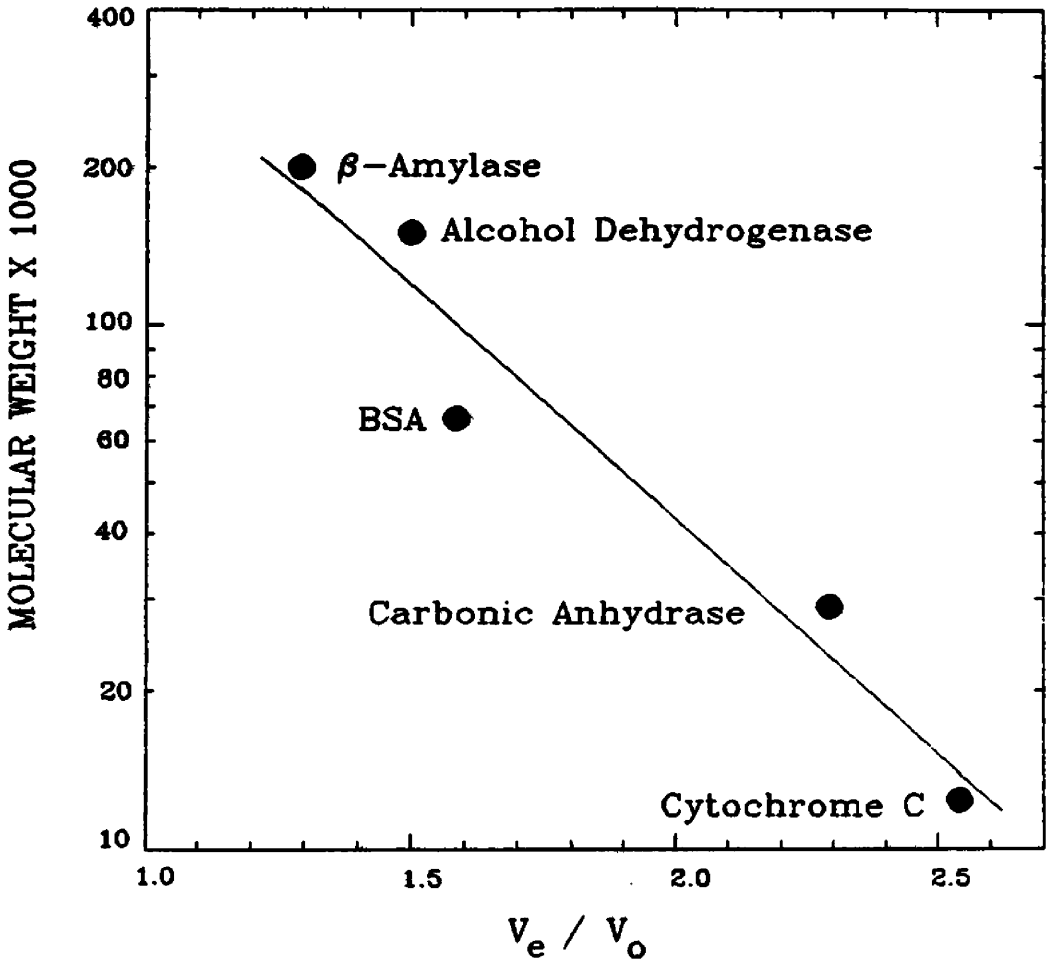

Figure 12: G−200 Calibration Curve

Table 9: Calibration of the Sephadex G-200 Column and Determination of Native Molecular Weight

The known molecular weight and the elution volume of each marker was used to generate Figure 12. The V_e/V_0 calculated for G-200 fractions 4-11 were used to extrapolate to a range of approximate molecular weights possible for the protein complex present in each fraction.

Sample	Ve[1]	Ve/Vo[2]	Approx. MW[3]
Blue Dextran	19.44 ml (VOID VOLUME)	1.00	2,000,000
ß-Amylase	25.11 ml	1.29	200,000
Alcohol Dehydrogenase	29.16 ml	1.50	150,000
BSA	30.78 ml	1.58	66,000
Carbonic Anhydrase	44.55 ml	2.29	29,000
Cytochrome C	49.41 ml	2.54	12,400
Fraction 4 (VOID VOLUME)	20-25 ml	1.02-1.29	+320,000
Fraction 5	25-30 ml	1.29-1.54	320,000-180,000
Fraction 6	30-35 ml	1.54-1.80	180,000-120,000
Fraction 7	35-40 ml	1.80-2.05	120,000-65,000
Fraction 8	40-45 ml	2.05-2.31	65,000-42,000
Fraction 9	45-50 ml	2.31-2.57	42,000-23,000
Fraction 10	50-55 ml	2.57-2.83	23,000-13,000
Fraction 11	55-60 ml	2.83-3.08	13,000-7,000

Table 9: Calibration of the Sephadex G-200 Column and Determination of Native Molecular Weight

[1]Values given are the elution volumes of each marker or fraction.

[2]Values given are Ve divided by the Ve of Blue Dextran (Vo for the column)

[3]All molecular weights given are approximate. The MW of the markers are given in the GF-3 bulletin supplied in the MW-GF-200 kit from Sigma. The molecular weights of the column fractions are extrapolated from the calibration curve.

made that the material eluted is present as a complex. This would imply that the native molecular weight of the complex is an average of the values obtained, or ~87,000 MW.

DISCUSSION

At the start of this project, the main goal was to isolate and purify to molecular homogeneity the antiviral protein from our extract. Upon obtaining a protein which was homogenous, the second goal of the project was to characterize this protein. This project was based partly on work done previously by Zipf (11). Data obtained in the study by Zipf suggested that there was probably only one antiviral factor present in the extract of *Datura*. This antiviral factor was proteinaceous in nature, but was resistant to many treatments known to inactivate proteins.

The initial step in the purification of the antiviral from a 60-80% ammonium sulfate precipitated fraction of the extract was to subject this fraction to heat. Previous work (11) had shown that the activity of the extract was not affected by temperatures up to 65°C for 10 minutes. Our extract was heated to 60°C for 10 minutes, and we found that this heating did indeed remove about half of the protein content from solution. As a result, the specific activity of our heat-treated extract was 3-4 times that of the unheated material.

Fractionation of the heat-treated extract by the use of centrifugal filters and subsequent testing in the inhibition assay indicated that native molecular weight of the active material was greater than 30,000. These data suggest that the active components may be present as a complex containing

both low and high molecular weight components, or that there is more than one antiviral factor present. This has been seen previously in other antiviral extracts (10).

Electrophoresis under denaturing conditions shows that these fractions are composed of proteins ranging from 10,000 to 100,000 mw which are present as either a complex or aggregate large enough to be retained by these filters. The sample containing proteins between 30,000 and 10,000 shows no visible bands but does possess antiviral activity. This indicates that there is either some compound which is not visible under our electrophoresis/staining, or the material is less than the resolution limit of our gel system (less than 10,000 mw). The latter is possible, as other compounds such as small proteins, peptides, and enzymes have been found to be antiviral (8).

An assay testing these samples for RNase activity showed that the +100,000 MW and +30,000 MW samples had RNase activity, while the samples less than 30,000 MW did not. This indicates that the antiviral activity observed in the greater than 30,000 MW fractions may be due in part to the activity of an RNase.

Fractionation of the heat-treated extract through the DEAE-cellulose and the Sephadex G-200 allowed us to further purify our active material. The one major peak obtained from the G-200 column was antiviral. Upon SDS-PAGE of the individual fractions obtained from this column, all the

antiviral fractions contained a protein with a molecular weight of ~17,000. This agrees with previous work done with *Datura*, which showed a intense double band at ~17,000 MW on SDS-PAGE after being purified by ion-exchange chromatography (9). This disagrees with more recent data (11), however, the purification conditions in the study were much less stringent.

In the peak of antiviral activity from Sephadex G-200, there are two main areas of activity. The first ~45% of the peak contains RNase activity and is also antiviral, while the remainder of the peak has only antiviral activity. The entire peak contains the ~17,000 MW protein indicating that this protein is probably responsible for a portion of the antiviral activity. The RNase activity is confined to the fractions containing larger molecules which is supported by the molecular weight fractionation data.

The elution volumes of the G-200 column fractions were used to determine the approximate native molecular weight of the material present. Knowing that the material was eluted in a single peak, the molecular weight values of each fraction were averaged to obtain ~87,000 MW as the probable molecular weight for the active complex.

Protease digestion of the Sephadex G-200 column fractions shows that the antiviral material is susceptible to trypsin, indicating that this material is proteinaceous in nature. This disagrees with previous data, which

indicated the antiviral activity was not susceptible to trypsin (11).

SDS-PAGE data in conjunction with results obtained in the inhibition assay and RNase activity assay suggest that there may be at least three compounds in the extract which are inhibitory. Of these proteins, one or two are strictly antiviral (no RNase activity) and the other is inhibitory by virtue of its RNase activity. All these data suggest that the active material is present as a complex of ~87,000 MW. This active complex contains proteins of ~17,000 MW and ~12,000 MW, an RNase active protein, and an antiviral protein of unknown size (probably between 10,000 and 30,000 MW).

FUTURE STUDIES

Due to the complex nature of this extract further work needs to be done to identify and characterize the individual compounds responsible for the observed activities.

Initially, the material present in the Sephadex G-200 peak needs to separated into individual components. This could be accomplished by continuous electrophoretic elution or HPLC. This would allow each component to be tested for both antiviral and RNase activity, which would serve to identify the component(s) responsible for these actions.

It is possible that the material needs to be present as a complex to be active. The components necessary for

activity could be determined by adding together the isolated components until the activity is restored. This should also indicate the stoichiometry of the components in the complex.

The RNase active molecule needs to be characterized in its activity and specificity (34), and an inhibitor of its activity found. This would allow the RNase-active portion to be inactivated to unmask any other activities this molecule may have.

The possibility that this extract could be a ribosomal inhibitory protein needs to be investigated. By using a cell-free system assay system (such as wheat-germ or rabbit reticulocyte lysate) and exogenously added mRNA or TMV RNA as the template, the activity of extract components could be further characterized.

The virus-stimulating activity observed in some of the fractions should be further investigated. Isolating and characterizing the compound responsible for this activity may provide insight into virus/inhibitor interaction.

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