PURIFICATION AND PARTIAL CHARACTERIZATION OF POKEWEED ANTIVIRAL PROTEIN IMMUNOREACTIVE SPECIES

THESIS

Presented to the Graduate Council of
Southwest Texas State University
in Partial Fulfillment of the Requirements

For the Degree

Master of Science

By

Staci S. Thomas, B.S.

San Marcos, Texas
May, 2001

TABLE OF CONTENTS

ILLUSTRATIONS

FIGURE

LIST OF TABLES

ACKNOWLEDGEMENTS

The author wishes to recognize those who provided assistance during the completion of this project. Special thanks go to the following colleagues and friends without whom this work would not have been possible.

DR. JAMES D. IRVIN for guidance and assistance for this research project.

DR. LINETTE M. WATKINS and DR. JAKOB M. KIRCHNER for their counsel in the review of this work.

LEON OEHLERS and SHEILA HEATER for their guidance in the laboratory and support.

My husband BRIAN THOMAS for his patience and support during endeavor.

CHAPTER I

INTRODUCTION

Ribosome Inactivating Proteins

Ribosome inactivating proteins (RIPs), found mainly in plants, are enzymes that selectively inhibit protein synthesis by inactivating ribosomes. Ribosomes are inactivated by catalytic removal of a specific adenine at the N-glycoside bond from the large subunit of the ribosome [1].

Ribosome specific N-glycosidases are highly sequence specific. The specific site at which a RIP depurinates rRNA is A_{4324} in rat liver 28S rRNA. The adenine is contained in the center of a conserved 14-nucleotide sequence with a tetra loop structure called the α-sarcin/ricin (SR) loop. Removal of this adenine by a RIP results in an inhibition of the translocation process in the ribosome consequently inactivating the ribosome.

There are two types of RIPs, type 1 containing a single chain enzymatically active protein and type 2 containing two proteins, an A chain and a B chain. The A chain possesses the enzymatic activity, the B chain has cell binding activity due to its affinity for specific cell surface

carbohydrates [2]. A disulfide bond holds the two chains together. As the B chain facilitates transport across the cell membrane, the A chain is released in the cytoplasm and inactivates the ribosome. For this reason type 2 RIPs are very toxic. Ricin, a protein derived from castor beans, is a notable example of a type 2 RIP having both the carbohydrate binding B chain and the ribosome specific N-glycosidase enzyme A chain [3]. Type I RIPs contain only the A chain portion and are considerably less cytotoxic. In order to inactivate the ribosome, the A chain must have a means of entering the cell. Conjugation to a cell membrane binding molecule greatly enhances cytotoxicity [2]. There are possible useful applications for RIPs such as combining them with antibodies to produce immunotoxins that can be used as chemotherapeutic treatments and by inserting RIP genes into the genome to facilitate disease resistance.

Pokeweed Antiviral Protein

In 1925, Duggar and Armstrong conducted a series of experiments to determined if tobacco plants could be infected by the tobacco mosaic virus (TMV) in the presence of sap from *Phytolacca americana* (commonly known as pokeweed) [4]. They found that TMV was unable to infect tobacco even when the pokeweed sap was diluted several fold. Other plant

extracts were tested and did not protect the plants from infection. The antiviral activity was later found to be due to a type 1, single chained RIP termed *Phytolacca americana* protein also known as pokeweed antiviral protein (PAP) [3].

PAP was first isolated from extracts of leaves and is also expressed in the leaves, stems, and roots of pokeweed [5]. In 1973 PAP was found to inhibit protein synthesis in eukaryotic cell free extracts [3]. PAP was the first single chain RIP isolated and characterized [6].

The M_r of PAP was determined to be 29,000 by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The crystal structure shows similarities to the ricin A chain [7]. Twenty-eight basic amino acids make up about 10% of the total amino acids, making the protein basic with an isoelectric point (pI) greater than 9. Four cysteines form two disulfide bonds [3]. PAP has been observed by electron microscopy to be located in the cell wall matrix of pokeweed cells [8]. It has been suggested that the first 22 amino acid residues of the primary transcript, which are not found in the mature protein, are a leader sequence that aids the protein in translocation to the cell wall [9].

Ribosome Inactivation

Depurination of ribosomes by PAP results in the inhibition of the elongation cycle of protein synthesis on the ribosome. Two enzymes, elongation factors 1 and 2 (EF 1 and EF 2), facilitate the elongation cycle. Initially, EF 1 binds aminoacyl-tRNA to the acceptor site on the ribosome. This is followed by the formation of the peptide bond by which peptidyl transferase transfers the growing peptide chain to the aminoacyl-tRNA at the acceptor site. EF 2 then mediates the translocation event, which is the movement of the peptidyl-tRNA from the acceptor site to the donor site. Depurination of rRNA by PAP results in a decreased ribosomal affinity for the elongation factors thus blocking the elongation of the chain [3].

PAP acts as a highly specific N-glycosidase that recognizes the sarcin/ricin (SR) loop of the 28S rRNA and hydrolyzes the N-glycoside bond at A_{4324} in mammalian 28S rRNA by an irreversible depurination [6]. The specific adenine is in the center of a 14 nucleotide sequence (5'-AGUACGAGAGGAAC-3') which is the most highly conserved structural feature of the large ribosomal subunit [10].

The proposed mechanism for PAP's enzymatic action involves arginine 179 forming an ionic bond with both the 3' and 5' phosphates of the RNA backbone, while glutamate 177 stabilizes the oxycarbonium ion

formed from the ribose after the adenine leaves. It appears that the leaving group adenine is protonated by arginine 179 [6].

Antiviral Activity

PAP has been shown to be a broad-spectrum antiviral agent; it is effective against many plant viruses. Plant viruses such as southern bean mosaic virus and cucumber mosaic virus combined with PAP did not infect when rubbed with an abrasive onto leaves [3].

The potent antiviral activity of PAP may be in part due to the unique ability of PAP to extensively depurinate viral RNA, including HIV-1 RNA. Recent evidence has shown that PAP efficiently depurinates adenine-containing polynucleotides, suggesting that the activity of PAP is not restricted to the SR loop of rRNA. PAP displays high activity against HIV, and the anti-HIV activity of PAP cannot be explained by RIP inactivation of ribosomes due to the fact that the ricin A chain does not show any detectable anti-HIV activity under identical experimental technique [11].

Immunotoxins

PAP's ribosome inactivating ability can be harnessed to cure certain diseases by preparing immunotoxins targeted against diseased cells. The

cell killing activity of PAP is greatly enhanced by conjugation to monoclonal antibodies. The antibodies are targeted to cell surface proteins so that they are capable of being internalized. Acute lymphoblastic leukemia (ALL), which affects 3500 adults and children each year, can be treated with PAP conjugated to B43 (an IgG1) anti CD19 monoclonal antibody. The immunotoxin kills leukemic progenitor B cells displaying CD19, which is not present on normal cells but is displayed on every ALL cell. In addition B43 – PAP does not harm pluripotent stem cells [6]. At the Parker Hughes Institute in Minnesota, more than 100 patients have been treated with B43-PAP since 1999 and show minimal side effects in phase III clinical trials [12].

Immunoreactive Species

Bonness *et al.* demonstrated that PAP is effective against pokeweed ribosomes [13]. PAP's sensitivity toward its own ribosomes implies that the protein should be either spatially separated in the cell or temporarily inhibited until it reaches the cell wall.

A protein species that exhibited PAP immunoreactivity with an apparent molecular mass of 50 kDa was found in leaf extracts [14]. This species has a pI lower than that of PAP as shown by its retention on

diethylaminoethyl (DEAE) cellulose anion exchanger resin. Desvoyes *et al.* proposed that this species is a complex of PAP associated with another species. It was showed that the enzymatic activity of PAP is strongly reduced in complexed PAP and that its thermal denaturation induced a release of enzymatically active PAP [15].

Preliminary studies involving isoelectric focusing of the fraction retained on the DEAE resin showed the separation of a species with a pI of 5.2. Western blotting of this species showed an immunoreactive band with a molecular weight of 50 kDa [16].

This research proposes to isolate this species using chromatographic methods as well as analyze the relative amount of PAP present, RIP activity, and other properties.

Cancer Research Significance

The identification of the chemical nature and specific structural features of cellular components that strongly interact with PAP may allow medical researchers to indirectly identify analogous proteins or other molecules found in humans. These proteins or molecules may be impossible to identify or isolate in humans, but with an understanding of the fundamental binding characteristics of PAP, strong correlations can be

made. For example, identification of a protein component followed by sequence determination would allow a search of human genome for proteins of similar sequence and presumably similar interactions with PAP. An understanding of the types of molecules PAP interacts with will be useful when considering the dose limiting toxic side effects, such as vascular leak syndrome, associated with the use of PAP immunotoxins in therapy [17]. Such studies should provide valuable clues relating to the nature of the unexplained activities of PAP against the HIV virus that do not appear to be due to ribosome inactivation [11].

CHAPTER II

MATERIALS

Diethylaminoethyl (DEAE) cellulose anion exchange resin was purchased from Whatman® International Ltd., Maidstone, England.

S-Sepharose® cation exchange resin, Q Sepharose®Fast Flow anion exchange resin, Superdex™ 75 prep grade gel filtration media, and the HiTrap™ NHS-activated affinity column was purchased from Amersham Pharmacia Biotech Inc., Piscataway, New Jersey. Affinity purified rabbit anti-PAP antibody was provided by Dr. James D. Irvin.

High-performance liquid chromatography (HPLC) was performed using the following equipment: two Gilson Model 302 mobile phase pumps, a Gilson Model 802B monometric module, a Gilson Model 811 dynamic mixer, a Gilson Model HM Holochrome UV detector, and a Rheodyne Model 7125 injector.

Novex NuPAGE™ pre-cast 10% Bis-Tris polyacrylamide electrophoresis gels (No. NP0301), Novex electrophoresis reagents, Novex

Mark 12™ wide range molecular weight protein standards, and Novex SeeBlue™ pre-stained molecular weight standards were purchased from Novel Experimental Technology, San Diego, California.

A Novex XCell-II™ electrophoresis module and a Novex XCell-II™ blotting module were used for all polyacrylamide gel electrophoresis and Western Blot analyses.

The Novex Mark 12™ molecular weight standards contain: myosin (200 kDa), β-galactosidase (116.3 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.3 kDa), glutamic dehydrogenase (55.4 kDa), lactate dehydrogenase (36.5 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.0 kDa).

The SeeBlue™ molecular weight standards contain: myosin (188 kDa), bovine serum albumin (62.0 kDa), glutamic dehydrogenase (49.0 kDa), alcohol dehydrogenase (38.0 kDa), carbonic anhydrase (28.0 kDa), myoglobin (18.0 kDa), lysozyme (14.0 kDa), aprotinin (6.0 kDa), and insulin.

Pierce GelCode® Blue Stain Reagent (No. 24592) and Pierce GelCode® SilverSNAP™ Stain Kit (No. 24602) were purchased from Pierce, Rockford, Illinois. Goat anti-rabbit IgG-alkaline phosphatase conjugate (No. 170-6518) and the Alkaline Phosphatase Conjugate Substrate

Kit (No.170-6432) was purchased from Bio-Rad Laboratories, Hercules, California. Goat anti-rabbit horseradish peroxidase was purchased from Sigma®, St. Louis, Missouri.

Protein bands were analyzed with a Kodak Digital Science™ Image Station 440CF, Eastman Kodak Company, Rochester, New York, using Kodak Digital Science™ ID Image Analysis Software, version 3.0.

Centricon® and Microcon® YM-10 centrifugal filter devices were purchased from Millipore Corporation, Bedford, Massachusetts.

Slide-A-Lyzer mini dialysis units (molecular weight cut off 10,000) were purchased from Pierce, Rockford, Illinois.

All other reagents were at least reagent-grade or better and were purchased from Sigma® or Fisher.

CHAPTER III

METHODS

The purification method for the anti-PAP immunoreactive protein is outlined in a flowchart in Figure 1 at the end of the chapter.

Purification of Proteins Immunoreactive with Anti-PAP

Immunoreactive proteins were purified from *Phytolacca americana* leaves harvested from the wild in Hays, Caldwell, Bastrop, and Wilson Counties, Texas between the months of April and August. Pokeweed extracts were obtained based on methods described by Irvin [18]. The PAP immunoreactive species was isolated using the procedure developed below

Pokeweed Extracts

Ten kilograms of leaves were juiced with extraction buffer [10 mM Tris-HCl pH 8, 200 mM NaCl, 5 mM β-mercaptoethanol] in an Acme Model 5001 juicer. The crude extract was fractionated with 40%-90% saturation ammonium sulfate, centrifuged, and the pellet was dialyzed

against DE buffer [10 mM Tris-HCl pH 8, 5mM β-mercaptoethanol] for 48 hours with one buffer change. The dialysate was passed through a DEAE-cellulose (Whatman DE-52) anion exchange column

Ion Exchange Chromatography

A salt gradient (0-500 mM NaCl, 10 mM Tris-HCl pH 8) was applied to the DEAE cellulose to determine at what salt concentration most of the bound protein eluted from the resin. A bulk elution of the retained fraction was performed with 500 mM NaCl, 10 mM Tris-HCl pH 8. The salt was removed through dialysis against 8 L of 10 mM Tris-HCl pH 8 for 48 hours (with one change of buffer). After the sample was frozen at -20°C and thawed, the sample was centrifuged at 10,000 rpm for 15 minutes and passed through a glass membrane filter (Millipore) and 5.0 micron cellulose acetate filter (MSI) to remove precipitant.

The sample was loaded onto an S-Sepharose® (Pharmacia) cation-exchange column (18.5 x 4.9 cm) equilibrated with 20 mM potassium phosphate pH 6.5. After the unbound material was collected, the column was eluted with 500 mM NaCl, 20 mM potassium phosphate pH 6.5. The unbound material was loaded onto a Q-Sepharose® (Pharmacia) anion exchange column (16 x 4.9 cm) equilibrated with 20 mM Tris-HCl pH 8 and

eluted with a salt gradient (0-750 mM NaCl). Fractions were collected and analyzed by Enyme Linked Immunosorbant Assay (ELISA). Fractions with high ELISA response were carried on to affinity purification.

Affinity Purification

A HiTrap™ NHS-activated affinity column (Pharmacia) was coupled with affinity purified IgG from rabbit anti-PAP serum using the coupling procedure outlined by Pharmacia. The Q-Sepharose® fraction was loaded onto a 5-mL affinity column equilibrated with 20 mM HEPES pH 7.0, 100 mM NaCl and eluted with 3.5 M $MgCl_2$, 20 mM HEPES pH 7.0. Bound protein was eluted with 100 mM glycine pH 3.5, and the fractions were concentrated using Centricon® (Millipore) centrifugal devices.

Gel Filtration-HPLC of Affinity Fraction

HPLC analysis

A 300 mm x 7.5 mm SigmaChrom™ GFC-100 gel filtration HPLC column was equilibrated with 20 mM potassium phosphate pH 6.5, 150 mM NaCl or 20 mM glycine pH 3.5, 150 mM NaCl. A 100-µL affinity sample centrifuged for ten minutes at 12,000 *g* and the supernatant was injected onto

the equilibrated column. The column was eluted at a flow rate of 1.0 mL/minute while monitoring the eluate absorbance at 230 nm.

Dissociation/Reassociation Studies

Affinity purified fractions were dialyzed using Pierce Slide-A-Lyzer® Mini Dialysis Units against 20 mM glycine-HCl pH 3.5, 150 mM NaCl. Samples were dialyzed for 10-15 minutes at 4°C and the dialysate was analyzed on the gel filtration HPLC columns with 20 mM glycine-HCl pH 3.5, 150 mM NaCl as the equilibration buffer. Samples equilibrated at pH 3.5 were redialyzed against 20 mM glycine, 150 mM NaCl for 10-15 minutes and analyzed on the gel filtration HPLC column equilibrated with 20 mM potassium phosphate pH 6.5, 150 mM NaCl.

Protein Measurements

Total Protein Amounts

Total protein concentrations of the DEAE, S-Sepharose®, S-Sepharose® retained, Q-Sepharose®, and Affinity fractions were measured using the method described by Kalb and Bernlohr [19]. Absorbances at 230 nm and 260 nm were measured with the use of Molecular Devices Spectra Max 190 plate reader. Protein concentration was

estimated using the following equation: μg/ml total protein = $183A_{230} - 75.8A_{260}$ [19].

Quantitation of PAP

The amount of PAP present in each purification sample was determined with the use of PAP standard curves generated from Western blotting (below).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electophoresis (SDS-PAGE)

The SDS-PAGE procedure described below is based on the method of Laemmli [20]. Samples were prepared in reduced sample buffer [1.17 M sucrose, 563 mM Tris-Base, 423 mM Tris-HCl, 278 mM SDS, 4% DTT, 2mM EDTA, 0.88 mM Serva Blue G250, 0.70 Phenol Red]. Samples were both heated (ten minutes at 90°C) and unheated (overnight at 25°C). The prepared samples and molecular weight markers (Novex) were electrophoresed at constant 200 volts through NuPAGE™ pre-cast 10% Bis-Tris polyacrylamide gels using MES-SDS pH 7.3 running buffer [50 mM 2-(N-morpholino) ethane sulfonic acid, 50 mM Tris-Base, 3.5 mM SDS, 1 mM EDTA]. The protein bands were detected with the use of Pierce

GelCode® Blue Stain Reagent or Pierce GelCode® SilverSNAP™ stain kit. The molecular weight values were determined by Kodak Digital Science™ Image Station 440CF and the included software.

SDS-PAGE/Western Blotting

The Western Blot procedure described below is based on modifications of protocols provided by Bio-Rad Laboratories.

Samples and molecular weight markers (Novex) were electrophoresed through 10% polyacrylamide gels as described above. The protein bands were electrophoretically transfered onto a Bio-Rad Trans-Blot® Transfer Medium nitrocellulose membrane for one hour at 20 volts (constant) using Bicine/Bis-Tris pH 7.2 transfer buffer [25 mM bicine, 25 mM bis-tris, 1 mM EDTA, 20% methanol]. The nitrocellulose membrane was rinsed with TBS [20 mM Tris-HCl pH 8, 500 mM NaCl] and blocked using 3% gelatin in TBS for one hour at room temperature. The membrane was washed in TTBS [20 mM Tris-HCl pH 8, 500 mM NaCl, 0.05% Tween-20] and incubated in 50 mL of 1:4000 rabbit anti-PAP serum in 1% gelatin in TTBS for one hour at room temperature. The membrane was rinsed and incubated in 50 mL of 1:3000 goat anti-rabbit IgG alkaline phosphatase (Bio-Rad) conjugate for 30 minutes at room temperature. The membrane was rinsed

with TTBS followed by a final rinse in TBS. The membrane was developed using Bio-Rad Alkaline Phosphatase Conjugate Substrate Kit. The molecular weight and band intensities were determined using Kodak Digital Science Image Station 440CF.

Enzyme Linked Immunosorbant Assay (ELISA)

Samples were diluted in PBS [100 mM sodium phosphate pH 7, 100 mM NaCl] to final volumes of 100 µL in the wells of a Costar® 96 well assay plate and incubated for 15 minutes at 37°C with shaking in a Bio-Tek Instruments Model CERES UV900C. The wells were washed three times with PBS-T [100 mM sodium phosphate pH 7, 100 mM NaCl, 0.05% Triton X-100], and the plate was blocked with PBS-Milk [2.5% non-fat dry milk in PBS] for 15 minutes at 37°C with shaking. The wells were washed, as above, and to each well was added 50 µL 1:3000 rabbit anti-PAP serum in PBS-Milk followed by incubation for 15 minutes at 37°C. The wells were washed and incubated for 15 minutes at 37°C with 50 µL 1:4000 goat anti-rabbit horseradish peroxidase conjugate (Sigma®). The wells were washed and the plate was developed using 75 µL of substrate mix [0.7 mg/mL 2,2'-azinodiethylbenzothiazoline-6-sulfonic acid (ABTS), 0.03% H_2O_2, 100 mM sodium citrate pH 4.5]. Absorbances at 405 nm were read using a Molecular

Devices Spectra Max 190 microplate reader and analyzed with the use of SOFTmax® PRO. For quantitative determination of amount of PAP in each purification fraction, incubation times were increased to 50 minutes.

Ribosome Inactivating Protein (RIP) Activity Assay

The affinity purified fraction and the S-Sepharose® bump fractions were assayed for inhibition of *in vitro* protein synthesis. The assay mix included 1.2 µL 1 mM amino acids minus leucine, 2.2 µL 1 µM [^{14}C]Leucine (specific activity 20 mCi/mmol), 16.6 µL rabbit reticulocyte lysate (Ambion), added to 5 µL RNAse-free water (control) or a series of diluted samples. The translation mixes were incubated in bullet tubes for 60 minutes at 30°C. The reactions were stopped by transferring the samples to tubes containing water. Decolorizer solution [1 M NaOH, 1.5% H_2O_2, 1 mM leucine] was added to the tubes and incubated for 10-20 minutes at 25°C. The translation products were precipitated by adding 5 mL of 25% trichloroacetic acid (TCA) and incubating for 30 minutes on ice. The precipitates were collected on Whatman glass-fiber filter discs. The tubes were rinsed twice with 10% TCA followed by one rinse with 95% ethanol. The filters were dried at 105°C for about 15 minutes. The dried filters were transferred to scintillation vials containing 0.5% 2,5-diphenyloxazol in

toluene, and the ^{14}C emissions were counted for 10 minutes in a Beckman Model LS 100C liquid scintillation counter.

CHAPTER IV

RESULTS AND DISCUSSION

The purpose of this research was to attempt to isolate a pokeweed antiviral protein (PAP) complex based on immunodetection. A species with a molecular weight of 40 kDa was isolated using a variety of chromatographic methods. The data did not give clear evidence that this species is a complex.

A protein with a molecular weight of 36 kDa and possessing PAP immunoreactivity was found retained on the S-Sepharose® column and characterized be SDS-PAGE, Western blotting, and inhibition of *in vitro* protein synthesis assays.

The following results were obtained by the use of the protocols as outlined in the Methods section. A purification table displaying relative amounts of total protein is presented in Table 1 at the end of the chapter.

Ion Exchange Chromatography

DEAE-Cellulose

A protein fraction exhibiting PAP immunoreactivity was found to bind to DEAE cellulose anion exchange resin [14]. A salt gradient was applied to the DEAE resin to determine what salt concentration eluted a majority of the bound protein. It was determined that 500 mM NaCl eluted all of the protein from the resin. The bulk elution with 500 mM NaCl was subsequently used to recover the sample. After a freeze/thaw step, an unwanted precipitant was observed and removed by centrifugation and filtration. The filtrate was labeled **DEAE** fraction and further purified.

SDS-PAGE shows numerous proteins present in the DEAE fraction (Figure 2). Western Blotting and ELISA (Figure 3) confirm that PAP is present in this sample (15 mg).

S-Sepharose®

The DEAE fraction was loaded on an S-Sepharose® cation exchanger as described in the methods section, and the immunoreactive fraction did not bind to the cation exchanger. This fraction was labeled **S-Seph** and was used in the subsequent purification steps. Total protein decreased by thirty

percent (Table 1); however, SDS-PAGE shows essentially all the protein bands were still present in the sample (Figure 2).

Q-Sepharose®.

The quaternary amino functional group is a stronger anion exchanger than diethylaminoethyl; therefore, the Q-Sepharose® ion exchange column was used to chromatographically separate the negatively charged complex.

S-Seph fraction was loaded onto an equilibrated Q-Sepharose® column. Protein concentration was monitored at 280 nm and the fraction was screened for anti-PAP activity using an ELISA procedure. The Q-Sepharose® elution profile (Figure 4) shows two significant peaks: a major protein peak with a high absorbance at 280 nm and a major ELISA peak at lower protein concentration. The smaller ELISA response is possibly due to high background from large protein concentrations at these fractions. A major ELISA peak away from the majority of the protein indicates that these fractions represent the PAP immunoreactive species and not a background response.

Fractions exhibiting the highest ELISA responses were pooled and labeled **Q-Seph** fraction and stored at -20°C. Total protein amount present is reported in Table 1. SDS-PAGE shows a significant decrease in the

number of proteins present between the S-Sepharose® and Q- Sepharose® steps. The separation of an immunoreactive complex from the majority of the protein in the S-Sepharose® sample demonstrates purification performed at this step.

Immunoaffinity Chromatography

The purpose of this research is to isolate a PAP complex. Therefore the target fraction should be immunoreactive with anti-PAP, and it should bind to immobilized anti-PAP. To eliminate any nonspecific antibody binding, the column was coupled with affinity purified anti-PAP from rabbit serum IgG. Several attempts at affinity chromatography were made before the best procedure was determined. Two different elution methods (Scheme I and Scheme II) produced different results for the eluted sample. Elution by these two methods brought about speculation of a possible interaction between PAP and the isolated species.

Scheme I: Acidic Elution Method

The Q-Seph fraction was loaded onto the affinity column equilibrated with phosphate buffered saline (PBS), and a sample containing 0.72 mg of PAP was eluted from the column with 100 mM glycine pH 3.5. The

fractions were neutralized with Tris-HCl pH 8. HPLC gel filtration data of the affinity eluate showed peaks at 10.38 (peak area: 1324100), 13.32 (peak area: 901050), and 20.79 (peak area: 3523000) minutes (Figure 5). A PAP standard had a retention time around 13-14 minutes, so it was determined that the affinity purified sample included PAP, a substance with a higher apparent molecular weight than PAP, and one with a lower apparent molecular weight than PAP. It appeared possible that the affinity purified sample was a complex containing PAP which may have dissociated into its parts somewhere through the purification.

To examine what conditions may cause dissociation, the affinity purified sample was subjected to acid and heat denaturing conditions. In both the acid and heat treatments, the gel filtration peak around 10 minutes disappeared or relatively decreased. It was concluded that the elution buffer in the immunoaffinity step in the purification method was too harsh. A gentler method would have to be determined in order to keep the complex intact for further characterization.

Scheme II: Gentle Elution Method

The acidic elution buffer was replaced with 3.0 M $MgCl_2$, 20 mM HEPES pH 7.0. Because magnesium chloride is incompatible with

phosphate buffers, the equilibration buffer was changed to 20 mM HEPES pH 7.0.

When this sample was loaded onto the HPLC gel filtration column, a peak with area counts of 2617800 at a 10.58 minute retention time was detected along with a relatively smaller peak (peak area 473370) at 13.42 minutes, and a peak (peak area 1001300) at 19.23 minutes (Figure 6). The species with the 10-minute retention time remained more intact with the gentle elution method of the affinity column than the acidic elution method based on a 48% decrease in area counts of the peak around 13 minute retention time (PAP). Gentle elution of an immunoaffinity column was adapted into the purification procedure.

Secondary Elution of Affinity Column

After the affinity column was eluted with magnesium chloride, a more acidic buffer (100 mM glycine pH 3.5) was passed through the column to ensure that all of the material had eluted. The collected sample had absorbances at 230 nm, 260 nm, and 280 nm of 0.81, 1.32, and 0.63 respectively. Nucleic acids absorb light in the 260 nm wavelength region. The high 260/280 nm ratio (2:1) led to suspicion of the presence of a nucleic acid.

RNA aptamers have been shown to inhibit the ribosome inactivation activity of ricin A-chain [21]. PAP, which has RNA as a substrate, could be inhibited by a segment of RNA; therefore, it may be possible that the 40 kDa immunoreactive species is a complex of PAP and a small molecule of RNA. Analysis of the preliminary data indicates that nucleic acid does not appear to be present in the isolated species. More careful and intensive studies could be performed to determine the nature of this sample.

Fractions were analyzed for possible RNA compounds as follows. SDS-PAGE was performed on samples of the affinity purified fraction and the acidic elution of the affinity column. Ethidium bromide is frequently used to detect nucleic acids, therefore the gel was stained in a 0.5 μg/mL solution of ethidium bromide, and fluorescence was measured. Although a set of RNA standards fluoresced, no fluorescence was detected in the fractions.

Affinity Purification

The amount of total protein in the affinity fraction is reported in Table 1. Western Blot data verify that PAP is present in the fraction (Figure 4). SDS-PAGE analysis shows that most of the protein migrates as a heavy band with a molecular weight of 40 kDa (Figure 3). This molecular weight

is lower than that determined in previous studies which used isoelectric focusing to isolate a 47 kDa protein [16]. A band with the same molecular weight as the PAP standard (30 kDa) was also present in the fraction, but its intensity was significantly less than the calculated intensity of the higher molecular weight band.

S-Sepharose® Retained Fraction

Because PAP is a positively charged protein, it binds to a S-Sepharose® cation exchange column. The purpose of the cation exchange step was to eliminate any free PAP that may be present in the sample. When the column was eluted with 500 mM NaCl, a protein with a molecular weight of 36 kDa was found retained on the S-Sepharose®. All PAP isozymes bind to cation exchangers; hence, this protein may be related to PAP.

The eluate was labeled **S-Seph retained**, and its molecular weight was determined by SDS-PAGE (Figure 2). Western Blot analysis shows that 3.6 mg of PAP is present in this species (Figure 3). This may indicate that PAP has a weak affinity for this protein. The sample was stored at -20°C and analyzed for PAP activity.

Western Blotting of Unheated Samples

It was previously shown that the components of PAP complexes could be dissociated with heat [15]. Western blots were preformed on unheated samples to indicate if an immunoreactive species with a molecular weight higher than that of PAP was in each of the purification fractions (Figure 7). A protein band with a greater molecular weight than that of PAP was detected in each of the purification fractions with Western blotting using antibodies against PAP. These bands were not detected when the samples were heated (Figure 3).

RIP Activity Assay

A possible explanation of the purified species' presence in pokeweed is that it is an inhibitor complex of PAP for the purpose of protection of pokeweed ribosomes. The affinity purified fraction and the S-Seph retained fraction were assayed for inhibition of *in vitro* protein synthesis and compared to PAP.

PAP inhibits protein synthesis by enzymatically inactivating ribosomes [3]. A PAP standard assayed under the conditions outlined in the Methods section shows a decrease in the amount of protein synthesis (determined by a percent of control) as the concentration of PAP increases.

Both the affinity purified fraction and the S-Seph retained fraction displayed the same trend of inhibition of protein synthesis (Figure 8). The dose-response at 50% of control for the inhibition of protein synthesis by pure PAP is around 1 pg; however, the inhibition for the S-Seph retained fraction and the affinity purified fraction was found to be 0.5 ng and 12 ng, respectively. Thus, the S-Seph retained fraction exhibited 0.2% of PAP activity and the affinity purified fraction exhibited 0.01% of PAP activity.

Two assumptions might be made form this data. One is that both fractions are inhibitor complexes of PAP. If this is true, the complex found in the affinity purified fraction inhibits PAP more than the complex found in the S-Seph retained fraction due to the difference in PAP activity. The other assumption is that there is no complex, and that the PAP activity may be due to a small amount of free PAP in the sample. Assaying for PAP activity under conditions that would possibly dissociate a PAP complex should be further studied and would determine if an inhibitor complex is responsible for the low PAP activity.

Dissociation/Reassociation of Complex

To test if the affinity purified sample is a complex of PAP, the sample was subjected to conditions that would dissociate the complex. The goal

was to determine if the complex could be broken up and then recombined. Dialysis was carried out to gradually equilibrate the complex to the desired pH.

The affinity purified fraction was dialyzed against 20 mM glycine pH 3.5, 150 mM NaCl, and a sample was loaded onto a gel filtration HPLC column equilibrated with 20 mM glycine pH 3.5, 150 mM NaCl. The peak with a 10-minute retention time was no longer present, however, the peaks around 13 and 19 minutes changed little.

If the sample is a complexed form of PAP, high hydrogen ion concentration should disrupt the affinity of the components in the complex. The disappearance of the large peak around 10 minutes when the sample is dialyzed against a lower pH could be due to the components in the complex dissociating from each other. This dissociation should show increased amounts of free PAP appearing in the fraction; this was not seen. The protein precipitating out of solution may be the more reasonable explanation for the disappearance of the peak because of the lack of evidence of a subsequential increase in PAP (retention time of 13 minutes) with disappearance of the 10 minute peak. This data seems to say that the affinity purified species cannot be considered a complex of PAP.

Summary

This research initially attempted to isolate a pokeweed antiviral protein (PAP) complex from pokeweed leaf extracts. Data indicates that a protein (molecular weight 40 kDa) was isolated by affinity purification. However, the amount of PAP present (determined by Western blot intensities) relative to total amount of protein demonstrates a failure to enrich the amount of PAP in the sample (Table 1). Although the protein was isolated based on immunoreactivity with PAP, the amount of PAP changes little as the higher molecular weight protein is enhanced. The fault here may be in the detection method. The purification method to isolate the 40 kDa protein was based upon screening for PAP immunoreactivity. The PAP that is detected may be a small contaminant of free PAP in the sample. Gel filtration/HPLC data did not give clear evidence of a complex but did display a small amount of PAP even in untreated samples. Because PAP is not quantifiably enriched in the purification, as it would if included in a complex, it can be assumed that the 40 kDa protein is not a PAP complex.

Although evidence of a complex and a purification of PAP do not exist, a 40 kDa protein was isolated (Figure 2). The protein is not detected in appreciable amounts in anti-PAP Western blots (Figure 3) but follows a very small amount of a presumably heavier complex (Figure 7). Somehow

the 40 kDa protein is retained on the anti-PAP affinity column possibly by either associating with PAP or nonspecifically binding to the antibody or to the column material. Future studies to determine the reason for the retention of the 40 kDa protein by the anti-PAP column may include changes in the affinity column such as the use of monoclonal antibodies.

A protein (molecular weight 36 kDa) retained on the S-Sepharose® column was isolated as well. The protein was initially studied because all of the PAP isozymes bind to the cation exchanger. The possibility that it was a PAP related protein was considered. However, this protein also did not show appreciable interaction with anti-PAP (Figure 3).

The determined purification method did not purify a complex. However, it did isolate a 40 kDa protein as well as a 36 kDa protein. Further purification using this method and concentration of both proteins should be carried out. The amino-terminal sequence of the protein should subsequently be determined for characterization. Amino-terminal sequence data may shed light on why the proteins possess PAP immunoreactivity. For the 36 kDa protein, an amino-terminal sequence determination may determine if this protein is PAP related.

Figure 1

Flowchart for Purification of Immunoreactive Proteins

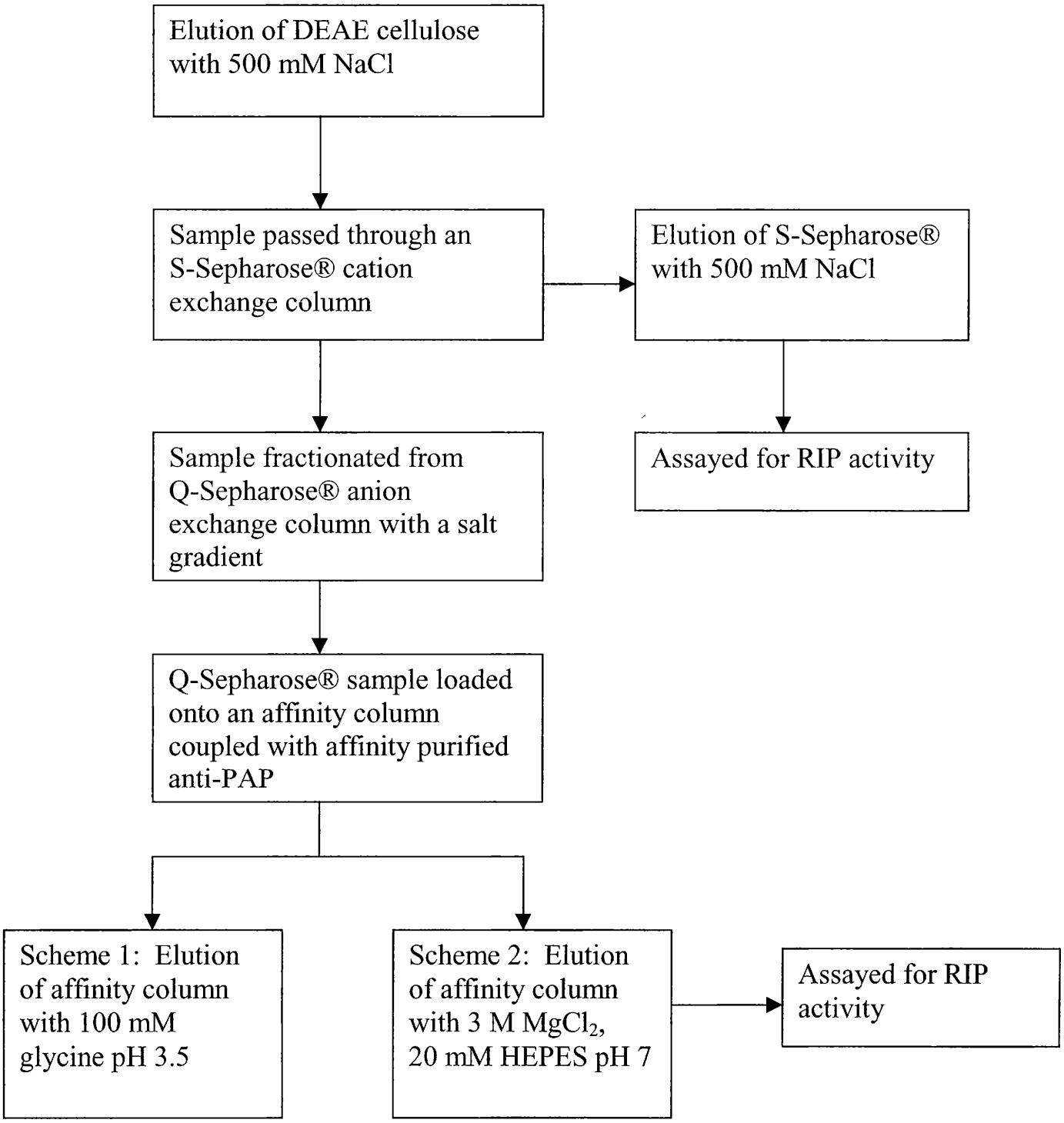


Table 1

Purification of Immunoreactive Protein

Purification Fraction	Volume (mL)	Total Protein (mg)[a]	Amount PAP (mg)[b]
DE Bump	400	3980	15
S-Seph	400	2750	15
Q-Seph	100	381	0.45
Affinity	1.5	32.6	0.39

[a]Determined by Kalb and Bernlor method (mg/mL = $183A_{230}$ - $75.8A_{260}$)
[b]Determined by Western Blot intensities

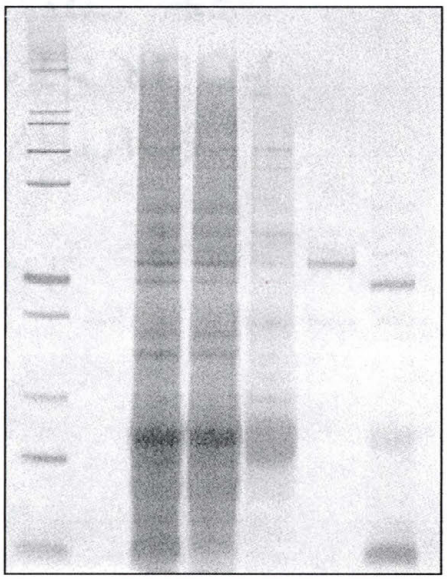


Figure 2. SDS-PAGE of purification fractions. Samples taken at each step in the purification were diluted in reduced SDS-PAGE sample buffer and incubated for five minutes at 80°C. The samples were electrophoresed in a 10% polyacrylamine gel, and the bands were developed using Pierce SilverSNAP™ stain. Lane 1, Mark 12™ protein standards (Novex); Lane 2, 54.5 µg purified PAP; Lane 3, DEAE (25 µg); Lane 4, S-Sepharose (17 µg); Lane 5, Q-Sepharose (95 µg); Lane 6, Affinity (5.4 µg); Lane 7, S-Sepharose Retained (6.5 µg).

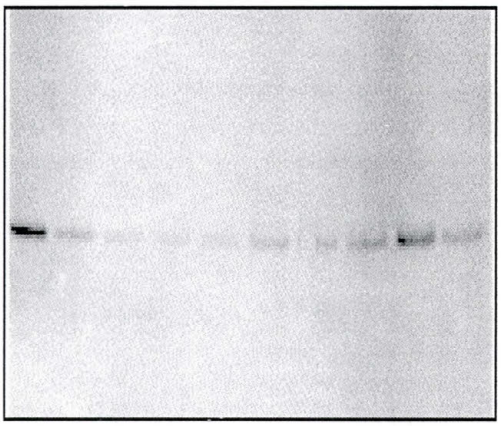


Figure 3. Western Blot of purification fractions. Samples taken at each step of the purification were diluted in reduced SDS-PAGE sample buffer and incubated for five minutes at 80°C. The samples were electrophoresed in a 10% polyacrylamide gel followed by Western blotting using anti-PAP rabbit serum. Lane 1, 25 ng purified PAP; Lane 2, 12.5 ng purified PAP; Lane 3, 6.3 ng purified PAP; Lane 4, 3.1 ng purified PAP; Lane 5, 1.6 ng purified PAP; Lane 6, DEAE (2.5 μg total protein); Lane 7, S-Sepharose (1.7 μg total protein); Lane 8, Q-Sepharose (9.5 μg total protein); Lane 9, Affinity (5.4 μg total protein); Lane 10, S-Sepharose Retained (0.65 μg total protein).

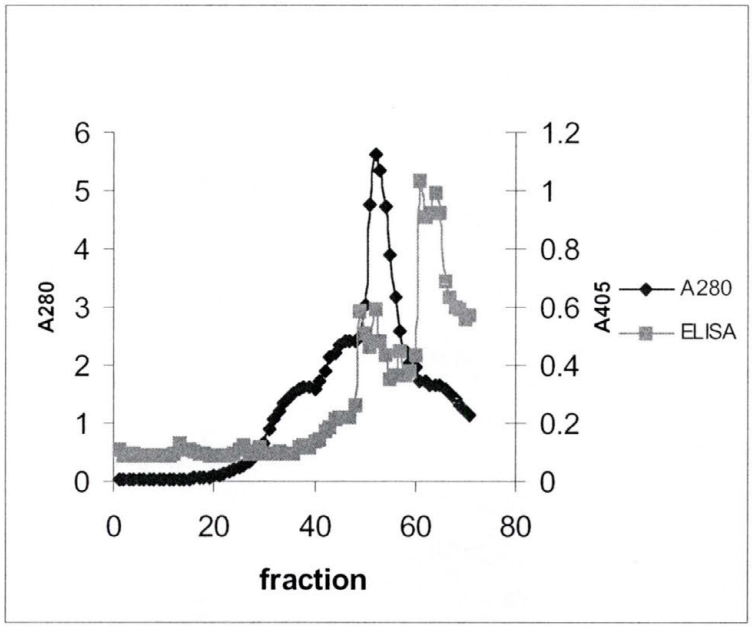


Figure 4. Q-Sepharose® Elution Profile. Fractions were eluted from a Q-Sepharose® anion exchange. The absorbance at 280 nm was determined for each fraction, and an ELISA using rabbit anti-PAP serum was performed on each fraction to assay anti-PAP activity. ELISA responses were measured as absorbances at 405 nm.

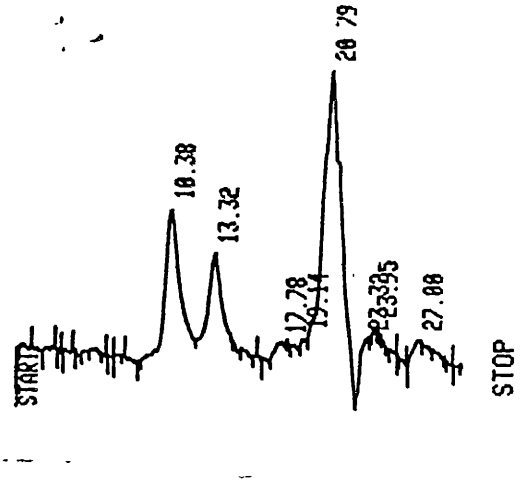


Figure 5. Gel Filtration/HPLC of Affinity Fraction Eluted with 100 mM glycine pH 3.5. A 50 μL sample of the fraction eluted from the affinity column with 100 mM glycine pH 3.5 was injected onto a SigmaChrom™ GFC-100 gel filtration/HPLC column equilibrated with 20 mM potassium phosphate pH 6.5, 150 mM NaCl. The column was eluted with a flow rate of 0.5 mL/minute and monitored at 230 nm.

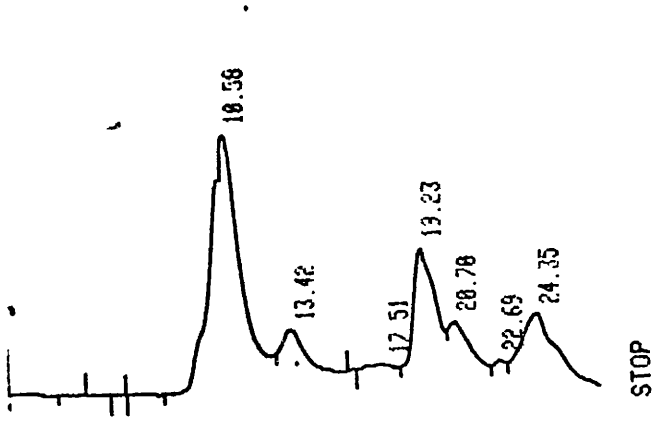


Figure 6. Gel Filtration/HPLC of Affinity Fraction Eluted with 3 M $MgCl_2$, 20 mM HEPES pH 7. A 100 µL sample of the fraction eluted from the affinity column with 3 M $MgCl_2$, 20 mM HEPES pH 7 was injected onto a SigmaChrom™ GFC-100 gel filtration/HPLC column equilibrated with 20 mM potassium phosphate pH 6.5, 150 mM NaCl. The column was eluted with a flow rate of 1 mL/minute and monitored at 230 nm

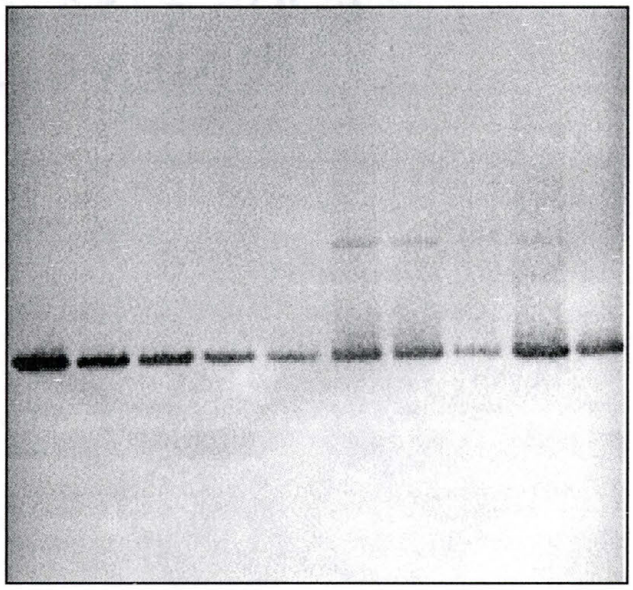
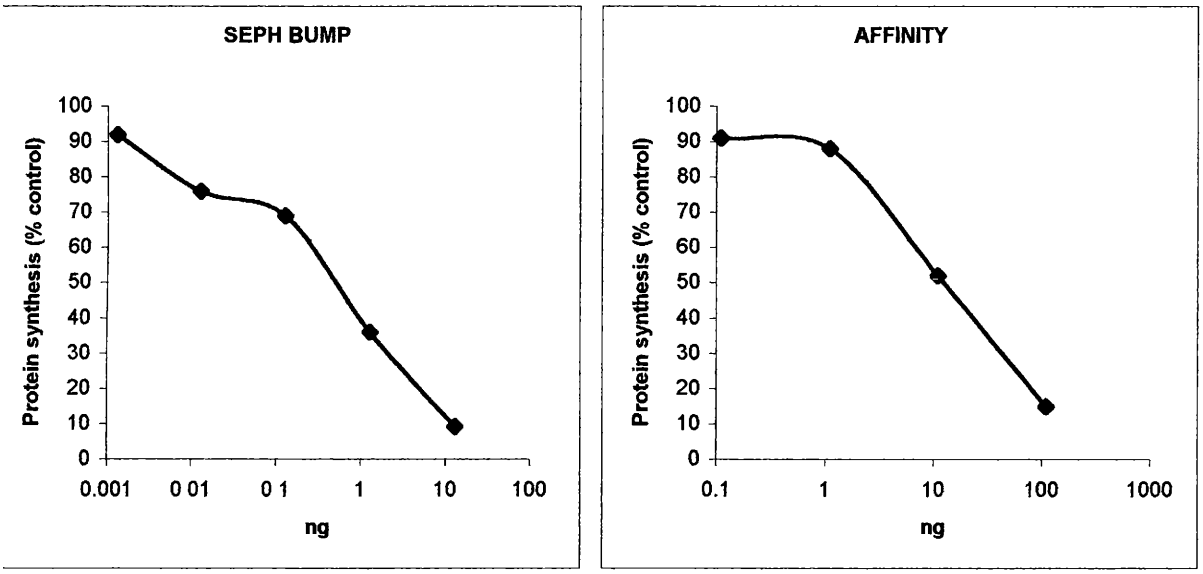


Figure 7. Western Blot of Unheated purification fractions. Samples taken at each step of the purification were diluted in reduced SDS-PAGE sample buffer and incubated overnight at 25°C. The samples were electrophoresed in a 10% polyacrylamide gel followed by Western blotting using anti-PAP rabbit serum. Lane 1, 100 ng purified PAP; Lane 2, 50 ng purified PAP; Lane 3, 25 ng purified PAP; Lane 4, 12.5 ng purified PAP; Lane 5, 6.3 ng purified PAP; Lane 6, DEAE (2.5 μg total protein); Lane 7, S-Sepharose (1.7 μg total protein); Lane 8, Q-Sepharose (9.5 μg total protein); Lane 9, Affinity (5.4 μg total protein); Lane 10, S-Sepharose Bump (0.65 μg total protein).



A **B**

Figure 8. Inhibition of *in vitro* protein synthesis. Assays were performed in 25 µL reaction volumes of rabbit reticulocyte lysate as described in the Methods section. **A)** The S-Sepharose® bump fraction, and **B)** the affinity purified fraction were assayed and a response was measured as a percent of ^{14}C incorporated into the translated product relative to uninhibited controls.

REFERENCES

1. Hartly, M. R., Chaddock, J. A., Bonness, M. A. (1996) *Trends in Plant Science,* **1**, 254.

2. Stirpe, F., Barbieri, L. (1986) *FEBS Lett.*, **195**.

3. Endo Y., Tsurugi, K. (1988) *J. Biol. Chem.*, **263**.

4. Duggar, B. M., Armstrong, J. K. (1925) *Ann. Mo. Bot. Gard.,* **12,** 359.

5. Irvin, J. (1983) *Pharmac. Ther.*, **21**, 371.

6. Hur, Y., Ching-Tack, H., Jueson, M. (1997) *J. Plant Biol.*, **40**, 53.

7. Irvin, J., Uckun, F. M. (1992) *Pharmac. Ther.*, **55**, 279.

8. Ready, M., Brown, D.T., Robertus, J.D. (1986) *Proc. Natl. Acad. Sci. USA,* **83**, 5053.

9. Ago, H., Kataoka, J., Tsuge, H., Habuka, N., Inagaki, E., Noma, M., Miyano, M. (1994) *Eur. J. Biochem.*, **225**, 369.

10. Lodge, J. K., Kaniewski, W. K., Tumer, N. E. (1993*) Proc. Natl. Acad. Sci. USA*, **90**, 7089.

11. Rajamohan, F., Taracad, V. K., Irvin, J. D., Uckun, F. M. (1999) *Biochem. and Biophys. Research Comm.*, **260**, 453.

12. Parker Hughes Institute (2000) *Discoveries*, **2**, 8.

13. Bonness, M. S., Ready, M. P., Irvin, J. D., Mabry, T. J. (1994) *The Plant Journal*, **5**, 173.

14. Oehlers, L. P. (1997) Master of Science Thesis, Southwest Texas State University.

15. Desvoyes, B., Poyet, J., Schlick, J., Adami, P., Jouvenot, M., Dulieu, P. (1997) *FEBS Lett.*, **401**, 303.

16. Heater, S., personal communication.

17. Baluna, R., Rizo, J., Gordon, B., Ghetie, V., Vitetta, E. (1999) *Proc. Natl. Acad. Sci. USA*, **96**, 3957.

18. Irvin, J. D. (1975) *Arch. Biochem. Biophys.*, **169**, 522.

19. Kalb, V. F., Bernlohr, R. W. (1977) *Analytical Biochem.*, **82**, 362.

20. Laemmli, U. K. (1970) *Nature*, **227**, 680.

21. Hesselberth, J. R., Miller, D., Robertus, J., Ellington, A. D. (2000) *J. Biol. Chem.*, **275**, 4941.