IMMUNO-DETECTION OF POKEWEED ANTIVIRAL PROTEIN

THESIS

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

For the Degree

Master of Science

By

Matthew Chien-Hung Lee

San Marcos, Texas
August, 1999

TABLE OF CONTENTS

ILLUSTRATIONS

FIGURES

TABLES

ACKNOWLEGEMENTS

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 providing guidance, preparation of this manuscript and the opportunity for this endeavor.

DR. LINETTE WATKINS and DR. FRANCIS HORNE for their guidance and counsel in the review of this work.

LEON PIERRE OEHLERS, JR. and SHEILA HEATER for their assistance and numerous useful advice in the laboratory.

STACI THOMAS for providing editorial assistance during the completion of this project.

My parents for their unconditional love and support throughout my educational pursuit.

And especially BETTY YANG, my love and inspiration.

ABSTRACT

IMMUNOLOGICAL DETECTION OF POKEWEED ANTIVIRAL PROTEINS

by

Matthew Chien-Hung Lee, B.A.
Southwest Texas State University
1999

Supervising Professor: Dr. James D. Irvin

Pokeweed antiviral protein (PAP) is a ribosome-inactivating protein isolated from the leaves of *Phytolacca americana*. It belongs to a larger family of proteins known as ribosome inactivating proteins (RIPs). These proteins enzymatically function as ribosome-specific *N*-glycosidases by removing a single adenine from the larger subunit of animal and plant ribosomes. It has been proposed that RIPs are defensive proteins which protect the host plant from invading organisms. Aside from being an biological curiosity, the enzymatic activity of PAP has found practical applications in the formulation of immunotoxins that are currently being tested for their anticancer and antiviral efficacy.

During purification, a number of different forms of PAP have been identified: the original PAP from spring leaves, PAP-II from summer leaves, and PAP-S from the seeds of *Phytolacca americana*, among which PAP-II was of particular interest to us.

Affinity-purified rabbit antibodies against PAP and PAP-II were developed and used in enzyme linked immunosorbent assay (ELISA), Western blot and slot blot detection of PAP and PAP-II in crude samples as well as samples at various stages of purification.

CHAPTER 1

INTRODUCTION

History and Background

Research on pokeweed antiviral protein can be traced back to 1925 when Duggar and Armstrong (1) discovered that sap from pokeweed (*Phytolacca americana*) was capable of reducing the infectivity of tobacco mosaic virus. They concluded from this observation that the pokeweed plant contained a substance which was capable of inactivating the virus. The actual isolation of this antiviral agent was achieved in 1948 by Kassanis and Kleczkowski. They isolated this agent from pokeweed sap by ethanol precipitation followed by adsorption to and elution from celite. This method of preparation yielded a substantial amount of carbohydrate in the product, which led Kassanis and Kleczkowski to the erroneous conclusion that the antiviral agent was a glycoprotein (2).

In 1969 the isolation of this antiviral protein was reinvestigated by Wyatt and

Shepherd using ion exchange chromatography. Wyatt and Shepherd's procedure (3) completely resolved the protein from the carbohydrate and produced a protein preparation which contained predominately a single protein with a minimum molecular weight of 13,000. This resolved protein is now identified by the acronym PAP which currently stands for pokeweed antiviral protein, although it previously stood for *Phytolacca americana* peptide or protein.

We now know that PAP belongs to a family of proteins known as ribosome-inactivating proteins (RIPs). There are two types of RIPs termed Type 2 RIPs and Type 1 RIPs. Type 2 RIPs are dimeric proteins consisting of a toxin A-chain subunit disulfide-bonded to a cell-binding B-chain. The cell-binding B-chain can bind to cell-surface receptors and facilitate the uptake of the toxic A-chain subunit into the cell. These dimeric proteins are very potent toxins; for example, ricin has a minimum lethal dosage intraperitoneal (MLD i.p.) at 48 hours in mice of 0.001 μg per gram body weight (23). Some examples of Type 2 RIPs are ricin, abrin and modecin. Type 1 RIPs, on the other hand, have only an A-chain and a much lower toxicity. Some examples of Type 1 RIPs are dodecandrin from *Phytolacca dodecandra*, dianthin from carnation leaves, tritin from wheat germ, and PAP and PAP-II from pokeweed leaves. It is interesting to note that PAP was identified as a Type 1 RIP in 1973 when it was determined that PAP has ribosomal inhibitory activity. In fact, it was the first example of Type 1 RIP to be identified (4).

In 1987, Endo and Tsurugi discovered that the A-chain subunit of ricin exhibits rRNA-specific *N*-glycosidase activity. It was recognized then that this *N*-glycosidase activity was the general mechanism of the RIPs' ribosomal inhibitory activity (5). Subsequent studies confirmed that other RIPs, including PAP and dodecandrin, all possess the same *N*-glycosidase activity.

The specific mechanism by which PAP inhibits ribosome activity is the enzymatic removal of a single adenine from a highly conserved stem-loop structure sequence (5'-AGUACGAGAGGAAC-3') at position 3017 in plant 25S rRNA and at position 4324 in mammalian 28S rRNA (6, 7). These sites are important in the interaction between ribosome and elongation factors during the peptide elongation cycle. By irreversibly depurinating the rRNA at these sites, PAP effectively shuts down protein synthesis inside any cells it may enter.

The depurination of rRNA by PAP was assayed by incubating PAP-exposed rRNA in acidic aniline followed by gel electrophoresis of the resulting RNA fragments. The removal of the adenine leaves the phosphodiester backbone chain susceptible to a base-promoted β-elimination reaction, which results in chain scission at the site of depurination (8).

Having discovered the mechanism of PAP's ribosome inactivation, scientists began to propose theories on how PAP performs its role as an antiviral agent in pokeweed. In connection with the results revealed by electron microscopy studies that PAP is only found in the cell wall matrix (9), an initial theory was proposed that PAP might function as a "suicide" agent which kills breached cells during the initial stages of viral infection (11).

In contradiction to the hypothesis, it was later observed that while PAP applied to other plants could protect them from viral infections, it failed to protect pokeweed, its originating plant, from a number of plant virus (10).

To explain this paradox, initial hypothesis proposed that PAP was ineffective against pokeweed ribosomes. It was further assumed that, in general, ribosomes are not sensitive to their conspecific RIPs (12). However, further studies showed that ribosomes isolated from pokeweed are depurinated during cell disruption and

ribosome isolation (12, 13). More detailed studies demonstrated that pokeweed ribosome are, in fact, sensitive to PAP, thus invalidating the initial assumptions (14).

A second hypothesis suggests that a cytosolic agent in pokeweed may act to prevent PAP from inactivating pokeweed ribosomes by forming an inactive complex with PAP. This hypothesis is currently under study in Dr. Irvin's lab at Southwest Texas State University (15).

The potent ability of PAP to inactivate ribosomes has found applications in the pharmaceutical arena. The use of immunotoxins in which PAP is conjugated to antibodies specific for certain cell-surface is currently an area of intense research. For example, an immunotoxin consisting of PAP conjugated to a monoclonal anti-human CD-19 antibody (designated as B43-PAP) has shown promising results as a therapeutic agent in the treatment of acute lymphoblastic leukemia. B43-PAP is currently undergoing national clinical trials established by the Hughes Institute. Other immunotoxins containing PAP and PAP-S have demonstrated potent anti-HIV activity (IC_{50}<100 pM) (16). For instance, an immunotoxin consisting of PAP conjugated to the anti-CD7 monoclonal antibody (Mab) TXU was tested on a surrogate severe combined immunodeficient (SCID) mouse model of human AIDS and was found to be a potent and nontoxic *in vivo* anti-HIV agent (17).

The Problem

During purification of PAP, a number of different forms of PAP have been identified. The purification procedure currently employed consists of extracting the soluble fraction by using a centrifugal kitchen juicer, ammonium sulfate fraction,

dialysis, exclusion from DEAE cellulose, and rapid flow cation exchange resin S Sepharose (18). PAP was recently reported to belong to a multigene family, thus it is possible that some of the different forms of PAP identified in purification may be similar isozymes (19).

A list of the different forms of PAP and their characteristics are summarized in Table 1. We are particularly interested in PAP-II which is purified from pokeweed leaves harvested during the summer months by the same procedure outlined above. PAP-II is resolved from PAP in the final ion exchange step of the PAP purification, eluting behind PAP at a higher salt concentration. It has been observed over the years that PAP-II concentrations in plants are highly variable and rarely reach the levels originally observed (20). The factors that contribute to the variability of PAP-II in pokeweed plants are not well known at this time.

Historically, the concentration of PAP had been assayed by two activity assays: protein synthesis inhibition assay and depurination assay. The protein synthesis assay, although sensitive, is a complex assay and is highly dependent on the skill of the person performing the assay. The depurination assay is also a sensitive assay but it is hard to obtain reproducible results and it requires purified ribosomes, RNA isolation, chemical β-elimination and electrophoretic analysis. Neither of these two assays is selective enough to distinguish among the various PAP isozymes, thus, we are limited in our abilities to analyze PAP isozymes.

Research Goals

The goal of this thesis is to investigate and develop alternative assays for

analyzing the concentrations of PAP isozymes. As a guideline for evaluating possible alternatives, a successful assay should meet the following criteria: the new assay should be easy to perform, it should be specific to each of the PAP isozymes and possess comparable levels of sensitivity as that of the activity assays.

Previous results in our lab suggested immunologically based techniques as promising alternatives for PAP isozymes (20). This thesis extends our previous studies by developing three immunologically based assays (ELISA, Western Blot and Slot Blot) and as well as evaluating their usefulness as alternative assays.

CHAPTER II

MATERIALS

Affinity Purification of rabbit IgG

3M Emphaze™ Biosupport Kit was purchased from Pierce, Rockford, Illinois. Purified PAP and PAP-II were obtained from S-Sepharose fractions routinely prepared in our laboratory. Rabbit anti-PAP and anti-PAP-II serum was prepared by William Kirkpatrick, San Marcos, TX .

Enzyme Linked Immunosorbent Assay (ELISA)

Nunc-Immuno™ PolySorb Surface microtier-plates were purchased from Nalge Nunc International and SANALAC instant nonfat dry milk was purchased at a supermarket. Goat anti-rabbit IgG-peroxidase conjugate and 2,2'-azino-bis(3-ethylbenz-

thiazoline-sulfonic acid) (ABTS) substrate were purchased from Sigma Biosciences, St. Louis, Missouri.

A Ceres UV900C plate reader from Bio Tek Laboratory Instruments, Winooski, Vermont was used to measure absorbance.

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

Novex NuPAGE™ pre-cast 10% Bis-Tris polyacrylamide electrophoresis gel (No. NP0301) and Novex electrophoresis reagents were purchased from Novel Experimental Technology, San Diego, California. GELCODE® Blue Stain reagent (No. 24590, Lot 98122169) was purchased from PIERCE, Rockford, Illinois.

A Novex Xcell-II™ electrophoresis module was used for polyacrylamide electrophoresis.

Western Blotting

Trans-Blot® Transfer Medium (Catalog 162-0095), EIA Grade Reagent Gelatin (Catalog 170-6537), Amplified Alkaline Phosphatase Immun-Blot™ Assay Kit (No. 170-6412) were purchased from Bio-Rad Laboratories, Hercules, California. A Novex Xcell-II™ electrophoresis module and a Novex Xcell-II™ blotting module, an UMAX Astra 1200S flatbed scanner, SigmaGel software version 1.0 from Jandel Scientific,

Chicago, Illinois and Microsoft Excel 97 from Microsoft Inc., Redmond, Washington, were used for western blot analysis.

Slot Blotting

The materials used in slot blotting were the same as in Western blotting, except for the membranes which were Trans-Blot® Transfer Medium (Catalog 162-0117) purchased from Bio-Rad Laboratories, Hercules, California.

One Bio-Dot® SF Microfiltration Apparatus (Catalog 170-6542) purchased from Bio-Rad Laboratories, Hercules, California was used for slot blot analysis.

CHAPTER III

METHODS

Purification of PAP and PAP-II from *Phytolacca americana*

Purification of pokeweed antiviral protein as performed in our lab is a five-step process based on methods described by Irvin (8, 18).

The process starts by first processing the leaves into crude juice (**Crude extract**) with extraction buffer [10 mM Tris-HCl pH 8, 200 mM NaCl, 5 mM β-mercaptoethanol] followed by ammonium sulfate salt precipitation [40–90% saturation] and centrifugation. The precipitated pellets are then re-dissolved in dialysis buffer [10 mM Tris-HCl pH 8, 5 mM β-mercaptoethanol] and dialyzed (**AMS extract**) to exclude the ammonium salt. After dialysis, the dialysate is passed through a DEAE-cellulose (Whatman DE-52) anion-exchange column (**DE-52 eluate**), followed by the final step of cation-exchange chromatographic separation with a S-Sepharose (Pharmacia) column and eluted with a linear 0-500 mM KCl gradient in 20 mM potassium phosphate pH 6 buffer.

The eluted fractions containing PAP as well as those containing the PAP-II are separately pooled and concentrated by ultrafiltration (**purified PAP**) and is stored at -20°C.

Affinity Purification of Rabbit Anti-IgG

The method described below is based on modifications of the method of Ready, Brown and Robertus (9) and suggested protocol provided by Pierce, Rockford, Illinois.

1. Affinity Column Preparation

3M Emphaze™ Biosupport Medium AB 1 (Pierce), 0.75 gram, was swollen in a 12-ml plastic column with 10 ml of coupling buffer (0.8 M sodium citrate, 0.1 M sodium bicarbonate pH 8.4) containing 4.6 mg/ml of PAP as coupling ligand. The swollen column was incubated with gentle shaking for one hour at room temperature and then washed with 100 ml of PBS [0.1 M sodium phosphate pH 7, 0.1 M NaCl]. The wash was collected to determine the coupling efficiency of the column. The coupling reaction was quenched by adding 20 ml of quenching solution (3.0 M ethanolamine pH 9.0). The coupled column was stored in PBS at 4°C.

Preparation of the PAP-II affinity column followed the same procedure with the exception of using purified PAP-II as the coupling ligand.

2. Antibody Preparation

Crude anti-PAP and anti-PAP-II sera were produced by inoculating rabbits with the appropriate antigens.

One milliliter of anti-PAP serum (73 mg) was applied to the column and washed with PBS buffer until the A_{280} of the effluent returned to baseline. The column was then eluted with 0.1 M phosphoric acid (pH 2.0). One-milliliter fractions were collected in tubes containing 1 ml of 0.5 M Tris-HCl (pH 7.0). Approximately 29% of anti-PAP activity was recovered from the affinity purification (Table 2).

The purification of anti-PAP-II followed the same procedure as outlined above with the exception of one additional step of passing the serum through the PAP-affinity column first before applying it to PAP-II-affinity column. Approximately 39% of anti-PAP-II activity was recovered from the affinity purification (Table 3).

Enzyme-Linked Immunosorbent Assay (ELISA)

All samples to be assayed were diluted in PBS [0.1 M sodium phosphate pH 7, 0.1 M NaCl] to final volumes of 50 µl in the wells of a 96-well polystyrene microtiter plate. Plating of antigens takes place at 4°C overnight followed by washing three times with PBS-T [PBS include 0.05% Triton X-100]. The antigen-plated wells were then blocked by PBS-Milk [2.5% non-fat dry milk in PBS] for 30 minutes to one hour at 37°C. The blocked wells were washed as above and then to each well was added 50 µl of first antibody [2 µg/ml of affinity purified rabbit anti-PAP IgG/20 µg/ml of affinity purified

rabbit anti-PAP-II IgG in PBS-Milk], and incubated at 37°C for 30 minutes. The first antibody-treated plate was washed and 50 µl of second antibody [1:500 diluted goat anti-rabbit IgG-peroxidase (Sigma)] added to each well and incubated at 37°C for 30 minutes. After the second antibody treatment, the plate was washed and 75 µl of substrate [0.7 mg/ml 2,2'-azino-bis(3-ethylbenzthyiazoline-6-sulfonic acid) (ABTS), 0.03% H_2O_2, 100 mM sodium citrate pH 4.5] added to each well. The ELISA response was measured as an end-point measurement at A_{405} following a 15-20 minute incubation period. A Bio-Tek instruments Model CERES UV900C microplate reader was used to collect ELISA data.

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

The SDS-polyacrylamide gel electrophoresis procedure described below is based on suggested protocols provided by Novel Experimental Technology (Novex).

Samples and standards were prepared in sample buffer (Novex Catalog NP0007) with reducing agent (Novex Catalog NP0004) and denatured by heating for 10 minutes at 80°C. Predetermined amounts of samples and standards were applied to pre-cast 10% gels and eletrophoresed at a constant voltage of 200 volts using MOPS-SDS pH 7.7 [50 mM 3-(N-morpholino)-propane sulfonic acid, 50 mM Tris-Base, 35 mM SDS, 1 mM EDTA] as running buffer.

The protein bands were detected by staining the gels with GELCODE® Blue Stain reagent for one hour and destained with water overnight. After staining the gel was destained and dried between cellophane sheets in a Hoefer Scientific Instruments Model

SE-1140 Slab Gel Dryer. The dried gel was then digitized with a UMAX Astra 1200S flatbed scanner and the image analyzed by Sigma Gel version 1.0 software.

SDS-PAGE/Western Blotting

The Western blot procedure is based on suggested protocols provided by Bio-Rad and NOVEX.

Reduced samples and standards were prepared and eletrophoresed as outlined above. Immediately following electrophoresis the protein bands were electrophoretically transferred onto a nitrocellulose membrane with bicine/bis-Tris pH 7.2 transfer buffer [25 mM bicine, 25 mM bis-Tris, 1 mM EDTA, 10% methanol] for one hour at a constant voltage of 25 volts.

The nitrocellulose membrane containing transferred proteins was washed with TTBS [20 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.05% Tween-20] and blocked for one hour with 3% gelatin-TBS [EIA grade reagent gelatin, 20 mM Tris-HCl pH 7.5, 500 mM NaCl] at room temperature. After blocking the membrane was rinsed with TTBS for 10 minutes and treated with first antibody solution for 30 minutes [1 μg/ml of affinity purified rabbit anti-PAP or 2 μg/ml of affinity purified rabbit anti-PAP-II in 1% gelatin-TTBS]. Following the treatment, the membrane was rinsed again with TTBS for 10 minutes and treated with second antibody solution [blotting grade affinity purified goat anti-rabbit IgG (H+L) alkaline phosphatase conjugate 1:3000 diluted in TTBS] for 30 minutes.

After the second antibody treatment the membrane was rinsed with TTBS for 10 minutes followed by a final rinse with TBS for 5 minutes and was developed in 0.1 M Tris-HCl pH 9.5 buffer containing 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Bio-Rad, Catalog 170-6432). The color development reaction was stopped when a clear color contrast was observed, and the developed membrane was digitized and the image analyzed with UMAX Astra 1200S flatbed scanner and Sigma Gel version 1.0 software.

Slot Blotting

The procedure outlined below is based on suggested protocol provided by Bio-Rad.

Samples were prepared in PBS and placed in the wells of a Bio-Dot® SF microfiltration apparatus. Any serial dilutions were also performed directly on the apparatus. After samples had been loaded, the samples were applied onto a nitrocellulose membrane by vacuum suction from under the membrane. Immediately following the application of the samples, the membrane was then blocked with 3% gelatine-TBS and treated with first and second antibody solutions and analyzed as outlined in the SDS/Western blotting section.

Protein Measurements

Protein concentrations were determined spectrophotometrically by the A_{230}/A_{260} method of Kalb and Bernlohr (21). A Beckman Model DU7400 spectrophotometer was used to measure the absorbances at 230 nm and 260 nm. Protein concentration was estimated using the equation: μg/mL total protein = $183A_{230} - 75.8A_{260}$.

CHAPTER IV

RESULTS

Affinity Purification of Antibodies

The successful development of immunoassays depends largely on the availability of antibodies with the desired characteristics. Specifically, the antibodies must be able to distinguish between the two analytes of interest, namely PAP and PAP-II.

To assess the ability of the antibodies in distinguishing between PAP and PAP-II (the specificity), crude sera of each antibody were assayed for activities against both antigens. Our results showed that anti-PAP serum reacted only with PAP (Figure 1a) while anti-PAP-II serum reacted with both PAP and PAP-II (Figure 2a). The cross-reactivity of anti-PAP-II occurred when the amount of serum was in the range of 10 ~ 100 μg, which coincided with its range of anti-PAP-II activity. In situations where only PAP is present in the sample, anti-PAP-II serum when used as a PAP-II detecting agent will produce false positive results.

Affinity purification was employed to eliminate the cross-reactivity from anti-PAP-II serum. After affinity purification, anti-PAP-II showed no anti-PAP activity (Figure 2b).

Although anti-PAP serum did not cross-react with PAP-II, it was subjected to affinity purification to minimize other non-specific activities. Cross-reactivity tests showed that the affinity purified anti-PAP remained specific to PAP (Figure 1b).

In addition to eliminating non-specific reactions of the sera, the affinity purification procedures also afforded the added advantage of increasing the specific activities of the antibodies approximately 10 fold. Table 2 and 3 summarizes the results of the purification procedures.

Optimization of PAP and PAP-II ELISA

One of the most critical aspects of quantitative ELISA is the preparation of standard curves that establish the sensitivity and range of the analysis. To prepare the standard curves for our ELISAs, the affinity purified antibodies were titrated against a known dilution series of PAP and PAP-II (1 ~ 1000 ng). The ideal profile of a standard curve should have a region of saturation, a region of linearity and a region of diminished response. In general, it should look like a stretched out 'S'. In the anti-PAP titration experiment, we found that 2 μg/mL of affinity purified anti-PAP produces the desired profile (Figure 3a). In the case of anti-PAP-II, 20 μg/mL of affinity purified anti-PAP-II produced the desired result (Figure 3b).

Optimization of SDS-PAGE/Western Blotting

Due to the large volume of antibody solutions required to perform the blotting experiments, lower concentrations of antibodies than those used in the ELISA experiments were employed. It was found that antibody concentration 1/10 of those used in ELISA assays produced excellent color contrast in the blots.

Titration experiments were performed to determine the range of the standard curves (Figure 4). Both PAP and PAP-II Western blots were observed to reach saturation at approximately 10 ng (Figure 5). It was also observed that once saturated, PAP blots tend to leave a trail behind the main bands while PAP-II blots, in addition to the same trailing effect, also showed a second band indicative of dimerization.

Optimization of Slot Blotting

In the slot blot titration experiment, serial dilutions starting with 200 ng of PAP produced a good standard curve (Figure 7a). In the case of PAP-II, the response seemed to fall off rapidly below 100 ng of PAP-II (Figure 7b). The linear range of the standard curves appeared to be very narrow. In addition, the curves had poor reproducibility, which could be observed even by simple eye inspection of the scanned images of the titration experiments. In Figure 6, the spot at the upper left corner of the blot contains the 200 ng of starting antigen. This starting antigen was serially diluted across the row first and then the entire first row was serially diluted down the columns. Theoretically, the

first row should be identical to the first column, the second row to second column and so forth. However, digitized results showed disagreements among the identical series. Furthermore, repeated experiments showed varied results as well.

Analysis of Samples

ELISA, Western blot and Slot blot were applied to the analysis of three sets of samples taken from routine preparations done at different periods of the season (April, May, and June). Each set of the samples consisted of two fractions, one of which (AMS) contained relatively impure sample, while the other (DE) contained relatively pure sample. The results were compared to data previously obtained by activity assays and summarized in Table 4.

It was found that ELISA over estimated the amount of PAP in all cases while under estimating PAP-II. Western blot, in most cases, produces reasonably close estimates in the DE fractions for both PAP and PAP-II, but seemed to be under estimating PAP and over estimating PAP-II in the AMS fractions. Results from Slot blot showed no consistent patterns. Furthermore, false positive result from Slot blot was observed in the analysis of PAP-II.

Both the Western and Slot blot methods as described in this thesis relied on an off-the-shelf digital scanner as a densitometer. To validate this unconventional use of equipment, SDS-PAGE analysis with GELCODE® Blue stain on all samples was performed as a control for our densitometry analysis. The scanned images of the SDS-

PAGE are shown in Figure 8. Results from the stained SDS-PAGE analysis confirmed that our choice of digital scanner was a judicious one and that it served as a capable densitometer (Table 5).

CHAPTER V

DISCUSSION

Prior to this study the methods used to quantifying the various isozymes of PAP in crude samples have been found to have a number of deficiencies. Previous assays based on protein synthesis inhibition and depurination activities are complex, non-specific assays which do not differentiate among the various PAP isozymes. In this thesis, three immuno-detection methods (ELISA, Western blot, and Slot blot) were developed specifically to quantify PAP and PAP-II. The results and suitability of each of these three methods as quantitative techniques are discussed in this section.

1. PAP and PAP-II ELISA:

Quantitative analysis of PAP by ELISA technique has been previously explored in our lab (15). In our experience, the major difficulty associated with ELISA quantitation of PAP was the nonspecific reactions of the antibodies in crude extracts. Attempts to quantify PAP in crude samples by ELISA using goat antibodies typically

failed due to weak or non-linear ELISA responses. Quantitation of PAP in purified fractions by ELISA produced higher estimates than those obtained from protein synthesis inhibition assays.

This thesis extended our previous results by using new antibodies from rabbits in place of goat antibodies. In addition to rabbit anti-PAP, rabbit anti-PAP-II was also produced to develop an ELISA assay for PAP-II.

Cross-reactivity tests of our new antibodies showed that rabbit anti-PAP had a strong affinity to PAP and no significant affinity to PAP-II. This latter observation agrees with previous results which demonstrated very low cross reactivity of anti-PAP with PAP-II (22). Anti-PAP-II, on the other hand showed a moderate cross reactivity to PAP which was later eliminated by affinity purification.

The range of linear response for the PAP ELISA using affinity purified rabbit anti-PAP was from 10 ~ 100 ng of purified PAP. In comparison to the previous ELISA using the goat antibodies which had a linear range from 2.5 ~ 15 ng of purified PAP, the new ELISA appeared to be less sensitive but with a greater dynamic range of detection.

Standard curves for PAP-II ELISA using rabbit anti-PAP-II had a similar response range as that of PAP ELISA. Its linear range was also from 10 ~ 100 ng.

When applied to samples, the PAP ELISA produced higher estimates of PAP content in AMS fractions than protein synthesis inhibition assays in all samples. However, the amount of over-estimation seemed to decrease in a chronological order. Samples taken from April were over-estimated by twice the protein inhibition estimate, whereas the amount of over-estimation steadily decreased in samples taken from May and June. This chronological decrease in over-estimation was not observed when PAP

ELISA was applied to DE fractions. In the DE fractions, all samples were over-estimated by relatively the same amount.

One might surmise that the over-estimation in ELISA was due to non-specific reactions of antibodies with other interfering immunoreactive material. This explanation fits well with the systematic over-estimation in the DE fractions, and seems to suggest that the amount of interfering material in AMS fractions decreased in later months.

PAP-II ELISA, surprisingly, under-estimates PAP-II in almost all cases and does not produce false positive results.

2. Western Blot

The Western blot method as a protein quantitation method had been studied by others with positive results (24, 25). The major advantage of the Western blot method is that the high-resolution gel electrophoresis step separates the interfering proteins from the protein of interest, which eliminates the inherent uncertainty of non-specific binding by antibodies observed in the ELISA method.

The sensitivity of Western blot was comparable to that of protein synthesis inhibition assay and depurination assay; it could detect both PAP and PAP-II in nanogram amounts. However, the advantage gained in sensitivity was offset by the fact that the range of detection was very limited (the linear response of the standard curves was from 1 ~ 10 ng). Given the limited number of wells on a gel, the number of dilutions of a given sample that could be analyzed on a single gel was severely limited, hence more experiments were required to analyze a sample.

Several other factors also contributed to the limitation of Western blot as an efficient quantitative method for analyzing PAP and PAP-II. Most notably in the titration experiments, PAP-II formed dimers and appeared as two bands with smearing in between the two bands when the amount of PAP-II was greater than 10 ng (Figure 4). PAP standards also became over-loaded when the amount was greater than 10 ng. The effect of over-loading appeared on the final blot as a 'bleeding' of color, giving the blotted spots fuzzy and smeared shapes that made quantifying the spots a difficult exercise in image processing.

The amount of time required to perform Western blot analysis was another limiting factor. On average, it required two working days by a skilled technician to complete two blots, producing 20 data points. In comparison, a skilled technician could perform 4 ELISA assays in one working day, producing 384 data points.

When applied to the samples, Western blots produced results that paralleled the activity estimates most closely on both estimates of PAP and PAP-II, albeit the consistency left much to be desired.

3. Slot Blot

Theoretically, the format of slot blotting offered an efficient alternative to both Western blotting and the ELISA technique. It required less time than the ELISA and produced far more data points per blot (48 data points per blot) than Western blotting. In practice slot blot suffered from the same interference problem encountered in ELISA assays.

The linear range of the standard curves on slot blot was from 10 ~ 100 ng for PAP and 50 ~ 100 ng for PAP-II. When applied to the samples, estimates of PAP by slot blot was significantly lower than the activity estimates. Estimates for PAP-II by slot blot, on the other hand, appeared to be higher. Furthermore, on samples taken from April where all other methods detected no PAP-II, slot blot showed significant false positive response.

In conclusion, as alternative quantitative assays for PAP and PAP-II, Western blot and ELISA each showed advantages and disadvantages. ELISA was less time consuming to perform but its results were more prone to error by non-specific interfering reactions. Western blot was more sensitive and less affected by interfering reactions, but it was also more time consuming and less reproducible. If used in conjunction, ELISA and Western blot may compliment each other and form a two-stage assay where ELISA is used for fast screening followed by Western blot to increase the accuracy of the result.

Slot blot, because of the unpredictable nature of its results, was simply unfit as a quantitative method for measuring PAP and PAP-II.

Table 1

Summary of Known PAP Isozymes and Their Properties

Name	Descriptions
PAP	PAP is purified from spring leaves of pokeweed. It is a basic protein with isoelectric point (pI) of approximately 9.5 as determined by isoelectric focusing. The pI calculated base on its amino acid sequence deduced from gene sequence is 9.2. Its molecular weight as determined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) is 29,000. The calculated molecular weight is 29,306. Reverse phase HPLC reveals multiple peaks of PAP; the two major peaks observed were isolated and found to have identical *N*-terminal sequence and mobility in SDS-PAGE as well as antiribosomal activity. None of the different forms was found to have detectable carbohydrate.
PAP-R	PAP-R is purified from the roots of pokeweed. It has same pI and *N*-terminal amino acid sequence as PAP.
PAP-C	PAP-C is purified from cultured pokeweed cells. It is most likely identical to PAP.
PAP-II	PAP-II is purified from summer leaves of pokeweed. It is resolved from PAP in the final cation exchange step of the purification procedure, elution behind PAP at a higher salt concentration. It is more basic and slightly larger than PAP. It has a molecular weight of 30,000 as determined by SDS-PAGE. It does not cross react with anti-PAP.
PAP-S	PAP-S is purified from seeds of pokeweed. It appears slightly larger than PAP on SDS-PAGE. It cross-reacts weakly with anti-PAP. Contains carbohydrate.
PAP-III	Purified from late summer leaves. Resolved from PAP and PAP-II in the same manner as in PAP-II. *N*-terminal sequence identical to PAP-II. Cross-reacts with anti-PAP-II. It has a molecular weight of 30,000 as determined by SDS-PAGE.

Table 2

Purification Table: Affinity Purification of Anti-PAP IgG

Step	mg	Activity	Specific Activity (mg^{-1})	Fold Increase	% Activity Recovery
crude anti-PAP serum	73	1.6×10^5	2.2×10^3	1 X	100%
affinity purified anti-PAP	2.2	4.7×10^4	2.1×10^4	10 X	29%

Table 3

Purification Table: affinity purification of Anti-PAP-II IgG

Step	mg*	Activity**	Specific Activity (mg^{-1})	Fold Increase	% Activity Recovery
crude anti-PAP-II serum	79	6.0×10^3	7.7×10^1	1 X	100%
affinity purified anti-PAP-II	2.3	2.4×10^3	1.0×10^3	13 X	39%

* IgG Concentration was determined by A_{280} measurement ($E^{0.1\%} = 1.4$)

** Activity of IgG was determined by ELISA, using 50% of saturated response as one activity unit.

Table 4
Summary of Results

PAP		Act*	ELISA	Western	Slot
April	AMS	700	1400	800	100
	DE	400	700	500	50
May	AMS	4000	4500	1500	450
	DE	2000	2400	1500	100
June	AMS	200	300	500	50
	DE	100	200	400	0
PAP-II & PAP-III					
April	AMS	0	0	0	500
	DE	0	0	0	50
May	AMS	1200	350	2500	4500
	DE	700	100	900	400
June	AMS	200	120	700	3400
	DE	150	200	300	200

* Activity values were based on the final yields of PAP and PAP-II estimated from historical yield data determined from the inhibition of protein synthesis (15, 22).

Note: Activity of IgG was determined by ELISA, using 50% of saturated response as one activity unit.

Table 5

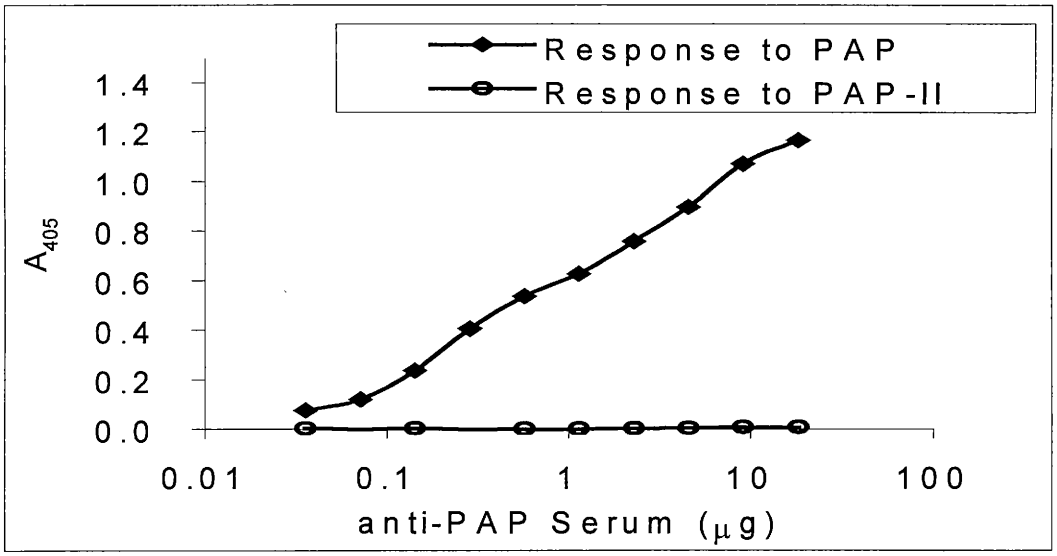
Densitometry Measurement of Stained SDS-PAGE on Three Samples

PAP	April	May	Jun
Exp 1	400	2000	150
Exp 2	350	2000	200
Average	375	2000	175
Activity Estimate*	420	2300	140

PAP-II & PAP-III	April	May	Jun
Exp 1	0	700	150
Exp 2	0	1500	300
Average	0	1100	225
Activity Estimate*	0	700	130

* Activity values are the same as in Table 4.

(a)



(b)

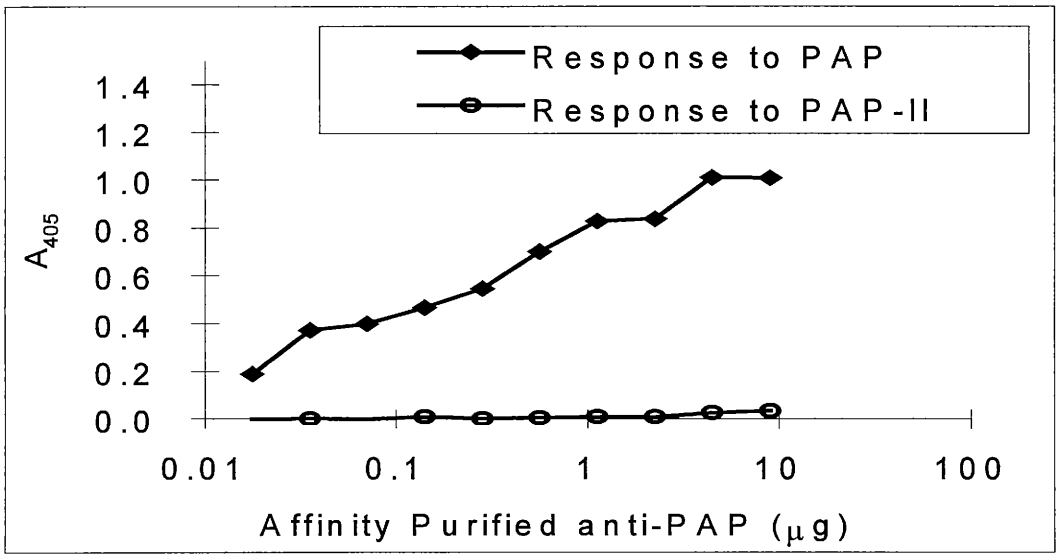
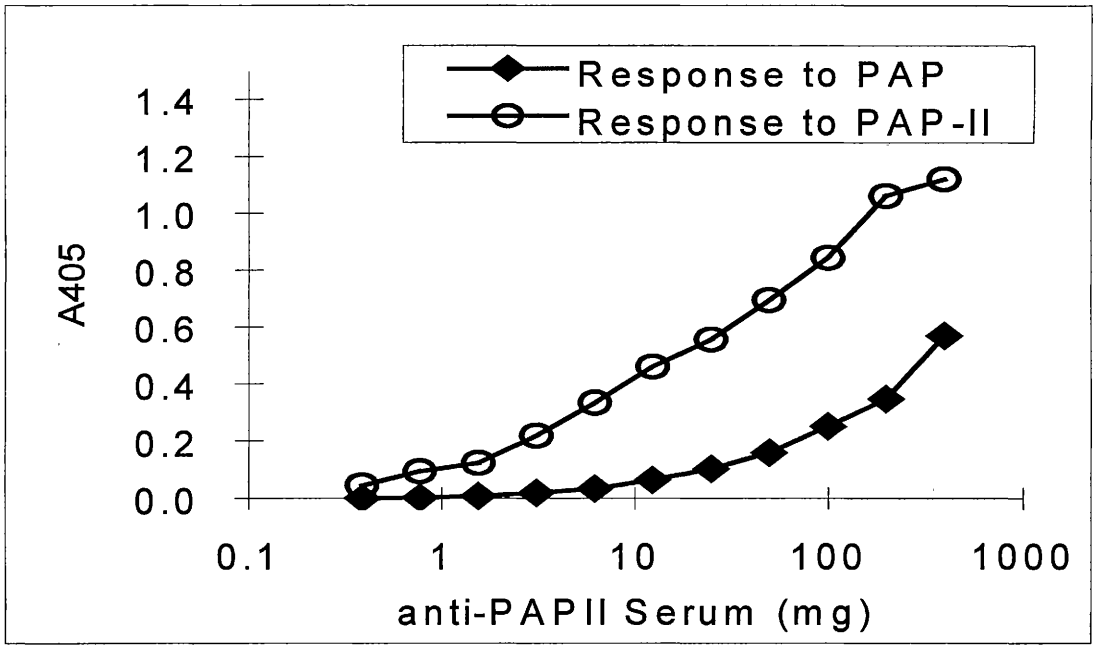


Figure 1. The reactivity of rabbit anti-PAP IgG's were assayed using ELISA. The assay was standardized on microtiter plates coated with 25 ng of antigens, 50 μl/well. **(a)** The responses of anti-PAP serum to PAP and PAP-II. **(b)** The response of the affinity purified anti-PAP to PAP and PAP-II are shown here.

(a)



(b)

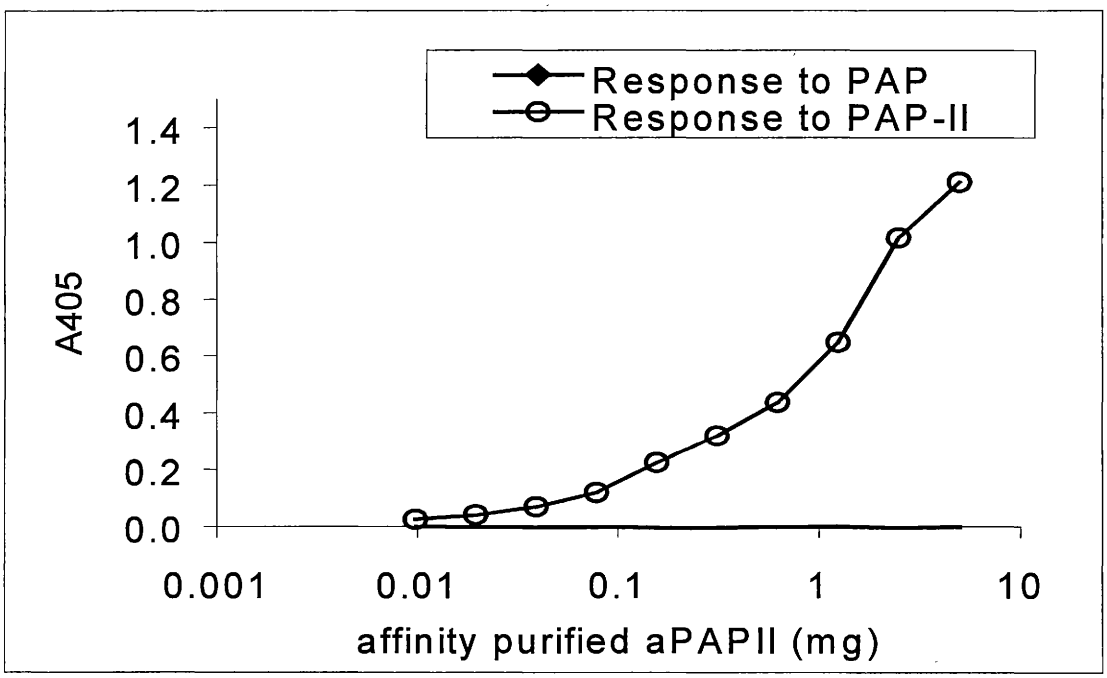
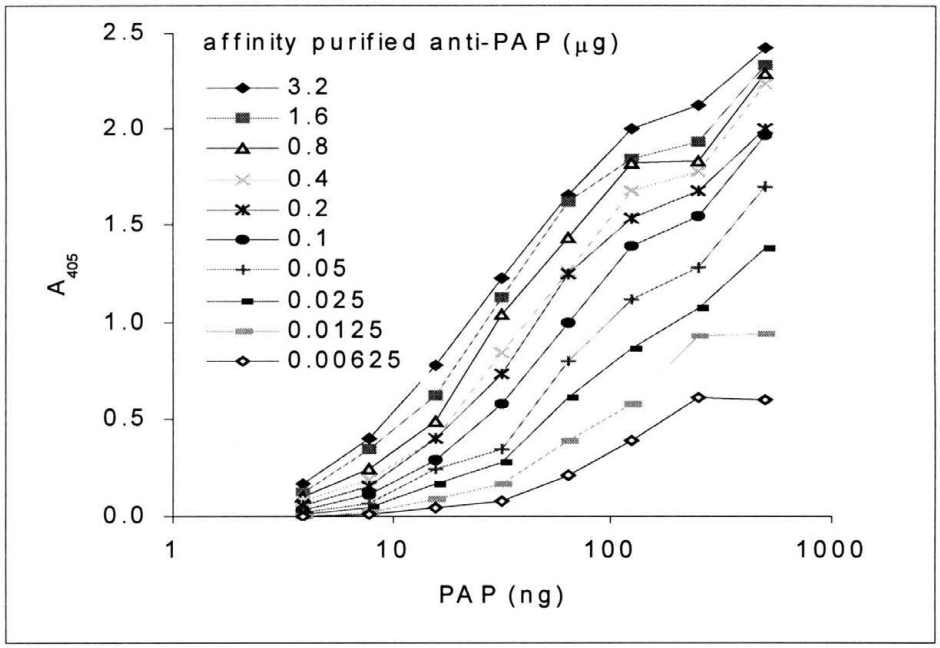


Figure 2. The reactivity of rabbit anti-PAP-II IgG's were assayed using ELISA. The assay was standardized on microtiter plates coated with 25 ng of antigens, 50 µl/well. **(a)** The responses of anti-PAP-II serum to PAP and PAP-II. **(b)** The responses of the affinity purified anti-PAP to PAP and PAP-II.

(a)



(b)

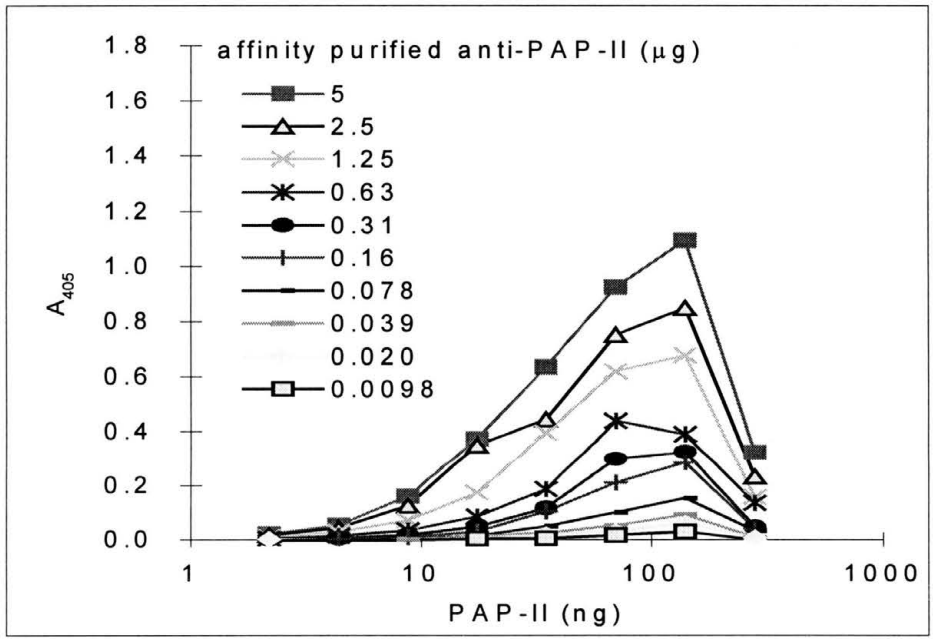
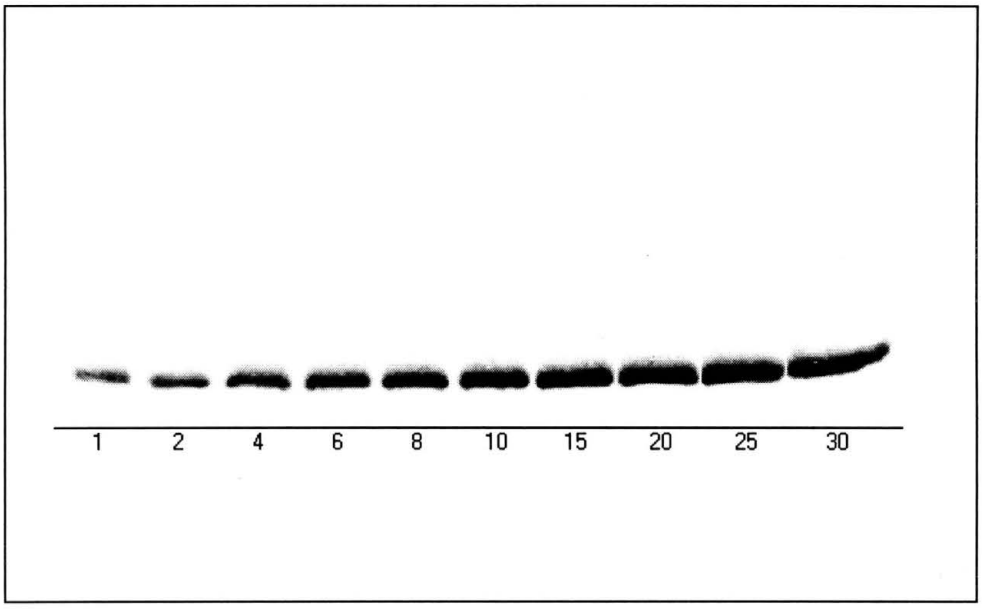


Figure 3. ELISA Titration: The affinity purified antibodies were titrated against standard antigen series to determine the optimal amount of antibodies to be used in the PAP and PAP-II ELISA. The assay was done in a 50 μL/well format. **(a)** The curves represent the responses of affinity purified anti-PAP at different concentrations. **(b)** The curves represent the responses of affinity purified anti-PAP-II at different concentrations.

Scanned Image of PAP Western Blot



Scanned Image of PAP-II Western Blot

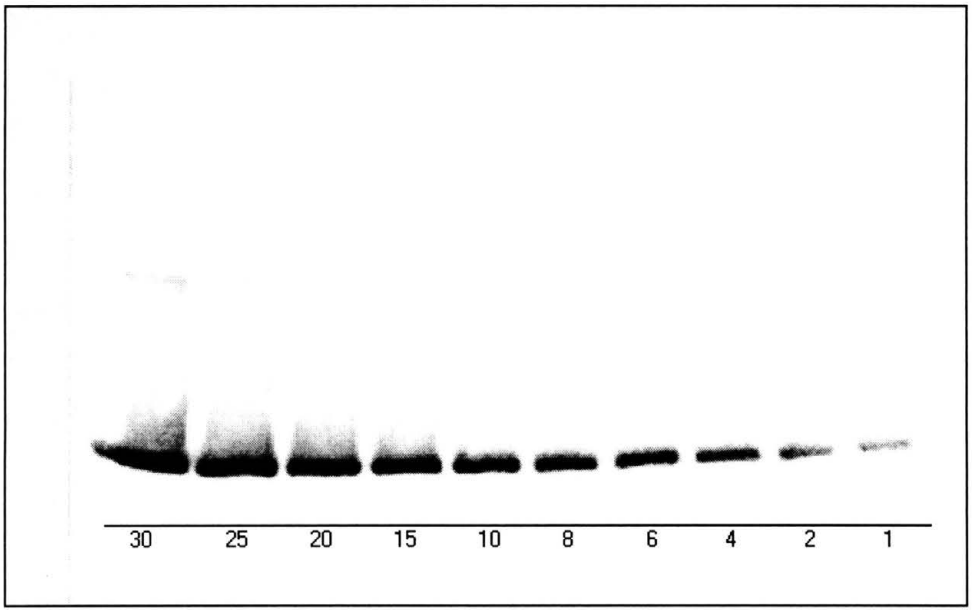
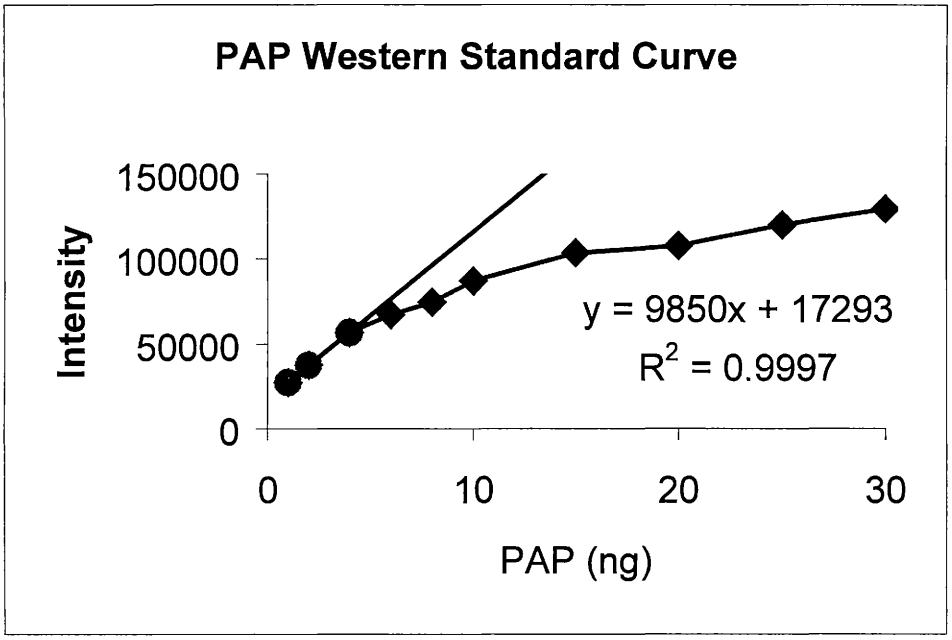


Figure 4. Western Blot Titration Image: The numbers at the bottom of the images indicate the amount of antigen of the corresponding spot in ng.

(a)



(b)

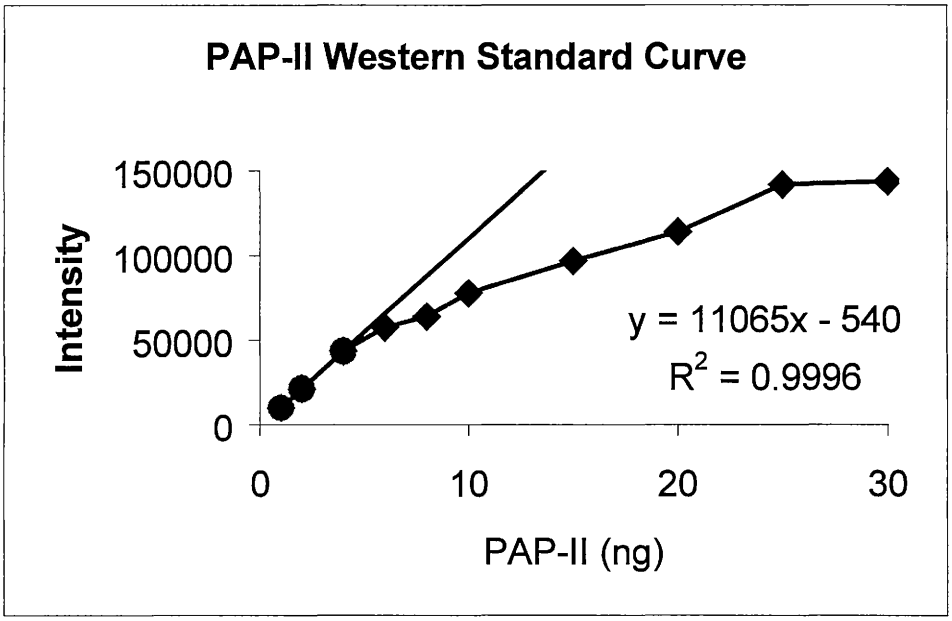
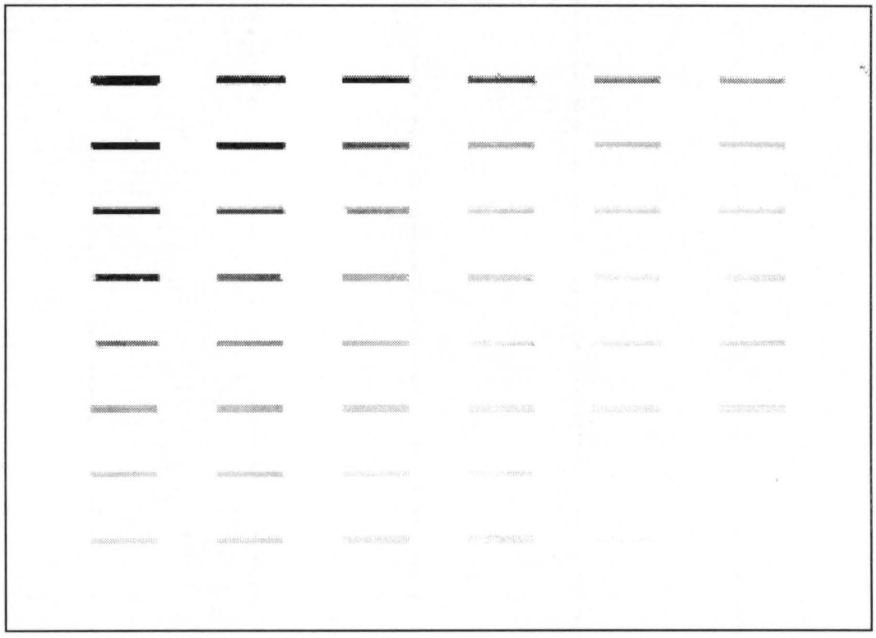


Figure 5. Western Blot Titration Curves: These two plots represent the digitized results of the images shown in **Figure 4.**

Scanned PAP Slot Blot Image



Scanned PAP-II Slot Blot Image

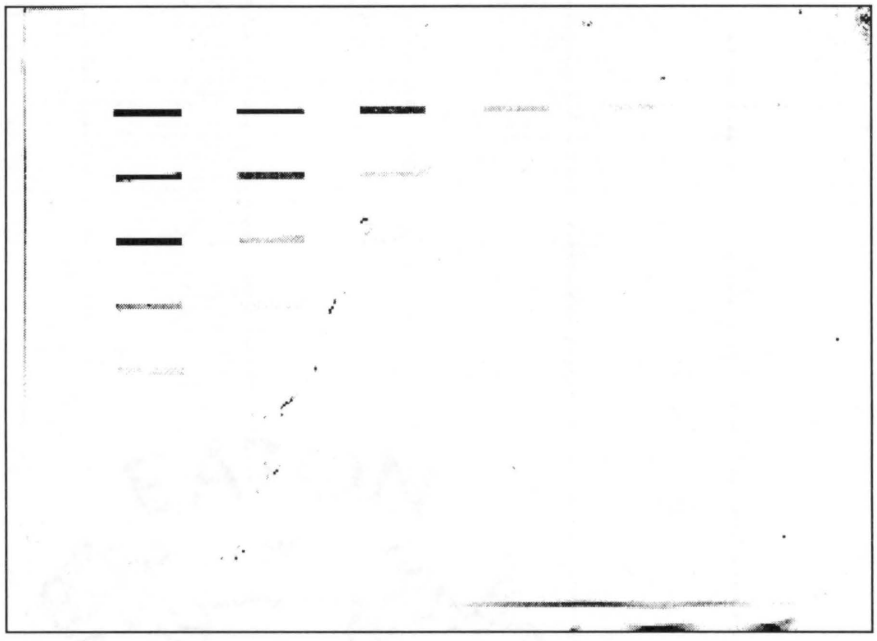
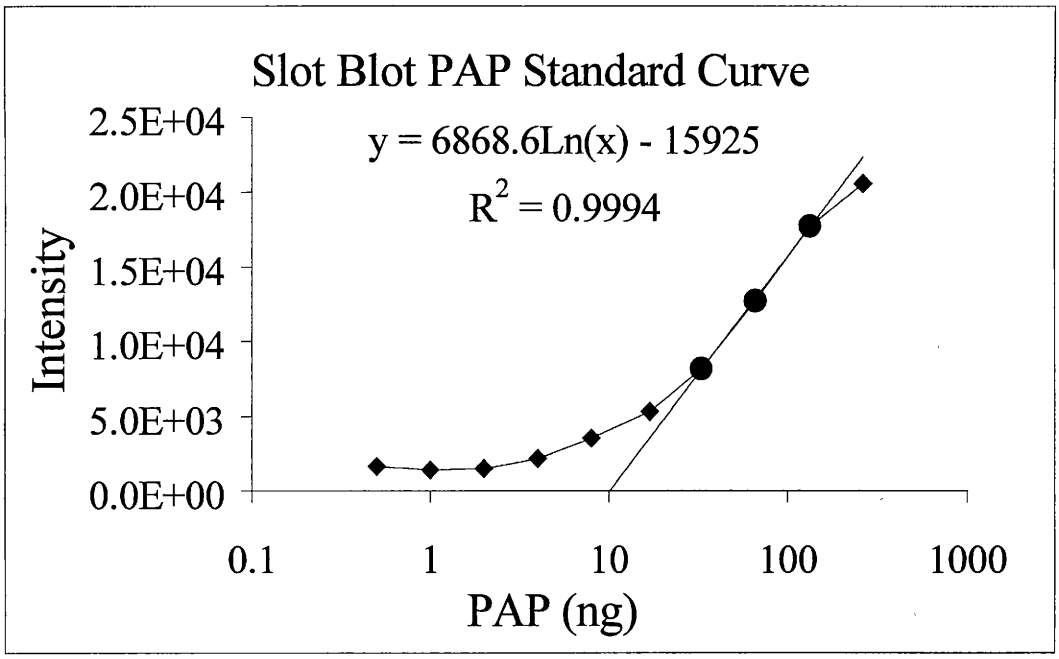


Figure 6. Slot Blot Titration Images: Titration on the slot blot started with the most concentrated antigen in the upper left corner of the blot and serial diluted first horizontally across the wells and then vertically down the wells.

(a)



(b)

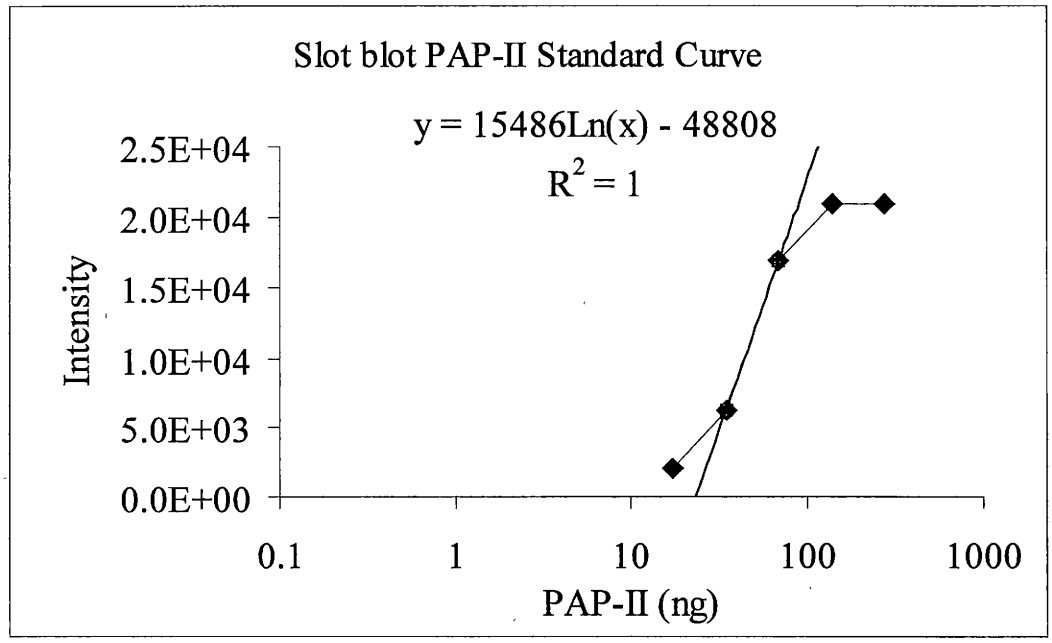
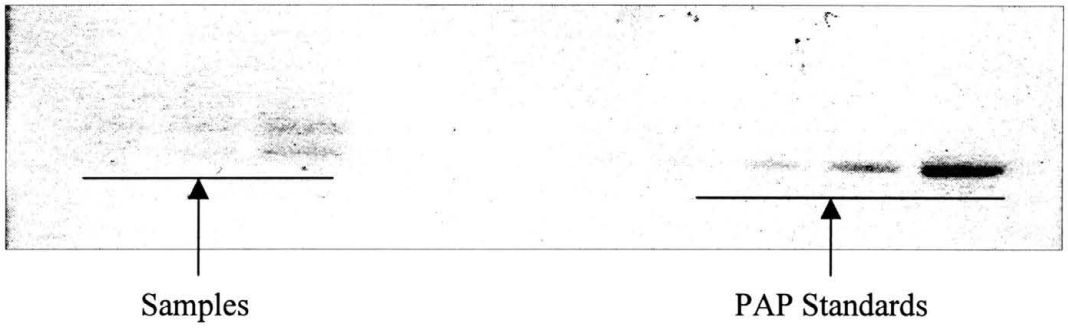


Figure 7. Slot Blot Titration Curves: (a) and **(b)** represent the standard curves as determined by densitometry analysis of the images in **Figure 6.**

(a)



(b)

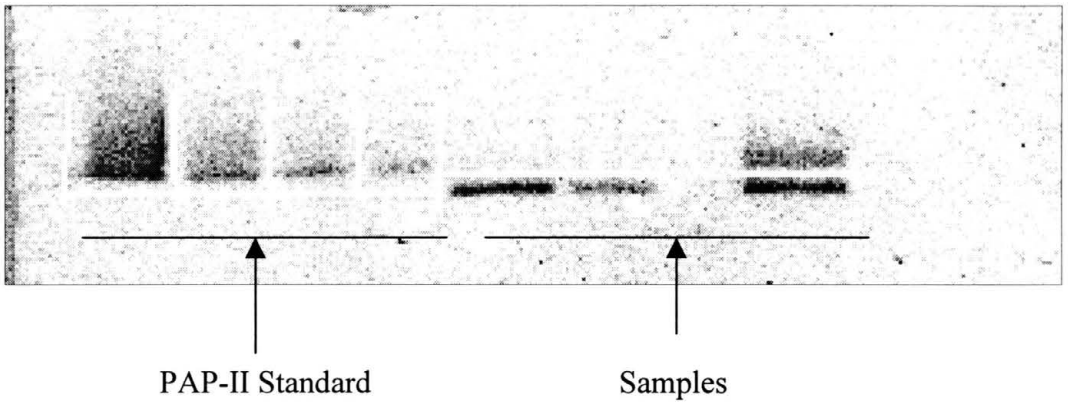


Figure 8. Scanned images of dried SDS-PAGE stained with GELCODE® Blue. **(a)** Image of gel loaded with samples from DE fraction and PAP as the standard. **(b)** Image of gel loaded with samples from DE fraction and PAP-II as the standard.

REFERENCE

1. **Duggar, B. M. and Armstrong, J. K.**, The effects of treating the virus of tobacco mosaic with the juice of various plants, *Ann. Mo. Bot. Grad*, 12, 359, 1925.

2. **Kassanis, B. and Kleczkowski, A.,** The isolation and some properties of a virus-inhibiting protein from *Phytolacca esculenta, J. Gen. Microbiol.*, 2, 143, 1948.

3. **Wyatt, S. D. and Shepherd, R. J.,** Isolation and characterization of a virus inhibitor from *Phytolacca americana, Phytopath.* 59, 1787, 1969.

4. **Obrig, T. G., Irvin, J. D. and Hardesty, B.,** The effect of an antiviral peptide on the ribosomal reactions of the peptide elongation enzymes, EF-I and EF-II, *Arch. Biochem. Biophys.,* 155, 278, 1973.

5. **Endo Y., Tsurugi, K.,** RNA *N*-glycosidase activity of ricin A-chain mechanism of action of the toxic lectin ricin on eukaryotic ribosomes, *J. Biol. Chem.,* 262, 8128. 1987.

6. **Endo, Y., Tsurugi, K., Lambert, J. M.,** The site of action of six different ribosome-inactivating proteins from plants on eukaryotic ribosomes: the RNA *N*-glycosidase activity of the proteins, *Biochem. Biophys. Res. Commun.,* **150**, 1032, 1988.

7. **Stirpe, F., Bailey, S., Miller, S. P., Bodley, J. W.,** Modification of ribosomal RNA by ribosome-inactivating proteins from plants, *Nucleic Acids Res.,* **16,** 1349, 1988.

8. **Irvin, J. D., Uckun, F. M.,** Pokeweed antiviral protein: ribosome inactivation and therapeutic applications, *Pharmac. Ther.,* **55**, 279, 1992.

9. **Ready, M., Brown, D. T., Robertus, J. D.,** Extracellular localization of pokeweed antiviral protein, *Proc. Natl. Acad. Sci. U.S.A.,* **83**, 5053, 1986.

10. **Chen, Z. C., Andoniw, J. F., White, R. F., Lin, Q.,** Effects of pokeweed antiviral protein (PAP) on the infection of plant viruses, *Plant Pathol.,* **40,** 612, 1992.

11. **Bonness, M. S.,** Pokeweed antiviral protein inactivates pokeweed ribosomes: implication for the antiviral mechanism, *Ph.D. Dissertation, The University of Texas at Austin,* 1992.

12. **Stirpe, F.,** On the action of ribosome-inactivating proteins: are plant ribosomes species-specific?, (1982) *Biochem. J.,* **202**, 279, 1982.

13. **Taylor, B. E., Irvin, J. D.,** Depurination of plant ribosomes by pokeweed antiviral protein, *FEBS Lett.,* **273** 144, 1990.

14. **Prestle, J., Schonfelder, M., Adams, G., Mundry, K. M.,** Type 1 ribosome-inactivating proteins depurinate plant 25S rRNA without species specificity, *Nucleic Acids Res.,* **20**, 3179, 1992.

15. **Oehlers, L. P. Jr.,** Characterization of crude and partially purified leaf extracts of *Phytolacca americana*, Masters Thesis, Southwest Texas State University, 1997.

16. **Irvin, J. D., Uckun, F. M.,** Pokeweed antiviral protein: ribosome inactivation and therapeutic applications, *Pharmac Ther.,* **55.** 279, 1992.

17. **Uckun, F., Chelstrom, L. M., Tuel-Ahlgren, L., Dibirdik, I., Irvin, J. D., Langlie, M., Myers, D. E.,** TXU (Anti-CD7)-Pokeweed Antiviral Protein as a Potent Inhibitor of Human Immunodeficiency Virus, *Antimicrobial Agents and Chemotherapy*, 383-388, 1998

18. **Irvin, J. D.,** Purification and partial characterization of the antiviral protein from Phytolacca americana which inhibits eukaryotic protein synthesis, *Arch. Biochem. Biophys.,* **169**, 522 1975.

19. **Lin, Q., Chen, Z. C., Antoniw, J. F., and White, R. F.,** Isolation and characterization of a cDNA clone encoding the anti-viral protein from *Phytolacca americana, Plant Mol. Biol.,* **17**, 609, 1991.

20. **Irvin, J. D.,** unpublished data, 1990.

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

22. **Irvin, J. D., Kelly, T., Robertus, J. D.,** Purification and properties of a second antiviral protein from *Phytolacca americana* which inactivates eukaryotic ribosomes, *Arch. Biochem. Biophys.,* **200,** 418, 1980.

23. **Bunclavari, S.,** *Merck Index, Merck & Co., Inc., Rahway, N.J., USA* 1989.

24. **Rudolph, C., Adam, G., Simm, A.,** Determination of Copy Number of C-Myc Protein per Cell by Quantitative Western Blotting, *Analytical Biochem.* **269**, 66-71, 1999.

25. **Bannur, S. V., Kulgod, S. V., Metkar, S. S., Mahajan, S. K., Sainis, J. K.,** Protein Determination by Proceau S Using Digital Color Image Analysis of Protein Spots on Nitrocellulose Membranes, *Analytical Biochem.* **267**, 382-389, 1999.